Transcriptional, microscopic and macroscopic investigations into monogenic and polygenic interactions of tomato and powdery mildew

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Contents

Chapter 1

General introduction

Crop production needs to be increased more than two-fold to satisfy the increasing demands of high-quality food for an increasing human population and enough feed for livestock. Besides the development of high-yielding cultivars, protecting crops from damage by weeds, animal pests and pathogens is another major sustainable way for producing enough good-quality food and feed (Oerke et al., 2004). Worldwide, crop losses due to plant diseases have steadily increased to 12-15% annually (Food and Agriculture Organization, 1993; Oerke et al., 2004). Therefore, the fight against plant diseases is among the most important issues to guarantee a sufficient global food supply.

Plant-biotrophic fungus interaction

Of infectious plant diseases, fungal diseases, which include all white and true rusts, smuts, needle casts, leaf curls, mildew, sooty molds and etc, represent the great majority, an estimated two-thirds (Holliday, 1998). The parasitic fungi can be divided into biotrophs that need a living host to complete their life cycle and necrotrophs that kill the host and absorb nutrients from the dead tissue. In order to feed on their hosts, many but not all biotrophic fungi have the ability to differentiate special interfacial structures, so-called haustoria (Schulze and Panstruga, 2003). The interaction between the plant and biotrophic fungus is compatible (susceptible, from the plant side) or incompatible (resistant, from the plant side). An incompatible interaction between plant and biotroph results in the arrest of the growth of the biotroph at different infection stages and often is associated with programmed death of host cells (hypersensitive response, HR). By contrast, during the compatible interaction certain biotrophs establish haustoria within living plant cells for nutrient uptake and reprogram the host's metabolism to favor their own without causing host cell death (Panstruga, 2002). For a long time, the majority of research on the plant-biotrophic fungus interaction has been focused on plant resistance to the pathogen. On the contrary, little attention is paid to plant susceptibility to biotrophic fungi. As for resistances, nonhost resistance is considered as one of the ideal resistance types to achieve durable resistance although little milestone progress has been made (Mysore and Ryu, 2004). Host resistances, which are among the hottest topics in plant pathology, are monogenic (dominant and recessive) or polygenic resistances, depending on the genetic control of the resistance. Below, details and comparisons between susceptibility and resistance, nonhost and host resistance, monogenic and polygenic resistance, and dominant and recessive resistance of plants to biotrophic fungi are described.

Susceptibility and resistance

Biotrophic fungi need to be successful in all the infection stages to finish their life cycle, including spore deposition, spore germination and germ tube development, finding a stoma, stoma recognition and appressorium formation, stoma penetration/cell wall

penetration, haustorium formation, colonization and sporulation (Niks and Rubiales, 2002). However, it should be mentioned that the above-described infection stages are relevant for many different plant-biotrophic fungus interactions, while individual interactions may need only some of these infection stages, for example stomata recognition, which is not necessary for powdery mildew. Plants can virtually arrest biotrophic fungal growth at any of these infection stages, however, so far the plant resistances to biotrophs, which are selected by breeders and/or studied by researchers, are mainly associated with the following infection stages: cell wall penetration and stages after haustorium formation. At the prehaustorial stage, plants can react with papilla formation, and at the posthaustorial stage, plants can initiate an HR. Resistances based on papilla formation are well exemplified by *mlo*-mediated resistance against the barley powdery mildew fungus in barley (Hückelhoven et al., 1999) and *ol2*-mediated resistance to tomato powdery mildew in tomato (Bai et al., 2005). However, to our knowledge, there are few other good examples of resistance associated with papilla formation. More frequently, HR accompanies the plant resistance to biotrophic fungi, by arresting the fungal growth at the posthaustorial stage (Parker, 2002). Most of these disease resistances fit the classic "gene-for-gene" model (Flor, 1971). *Hm1* from maize, the first *R* gene cloned through transposon tagging, is a resistance gene to the fungal pathogen *Cochiobolus carbonum* (Johal and Briggs, 1992). *Hm1* encodes a NADPH-dependent reductase unlike the later isolated *R* genes and the mechanism does not involve interaction via an *Avr* gene. Martin et al (1993) successfully isolated the tomato *Pto* gene*,* encoding a serine/threonine kinase, which renders tomato resistant to a bacterial pathogen (*Pseudomonas syringae* pv *tomato*) expressing the *Avr-Pto* gene. It is the first case of cloning an *R* gene by using map-based cloning. *Cf-9* is the first cloned *R* gene, mediating resistance to a fungal pathogen (*Cladosporium fulvum*) that fits the gene-for-gene model (Jones et al., 1994); *C. fulvum* belongs to the semi-biotrophic extra-cellular fungi without haustoria that enter the leaf via stomata. Cloned *R* genes against biotrophic intracellular fungi with haustoria include *mlo*, *Mla, Mla6, RPW8.1 and RPW8.2* (reviewed by Hammond-Kosack and Parker, 2003). So far, more than 50 *R* genes have been cloned (Coaker et al., 2005) and most of them share homologous domains, like leucine rich repeats, nucleotide binding sites, kinase domains and etc. The *R* genes cloned in the past 10 years greatly increased our knowledge on plant disease resistance.

However, we should also pay attention to the other side of the coin: "plant disease susceptibility". Screening natural populations or induced mutant libraries resulted in the identification of recessively inherited *R* genes against different biotrophic fungi, such as Barley *mlo* (Büschges et al. 1997), tomato *ol-2* (Ciccarese et al., 2000), and *Arabidopsis pmr* genes (Vogel and Somerville, 2000; Vogel et al., 2002; Nishimura et al., 2003). Because the resistances mediated by *mlo*, *pmr6* and *ol-2* are not associated with the constitutive expression of know defense markers (Panstruga, 2002; Vogel et al., 2002; This thesis), it is plausible to assume that the dominant counterparts to these genes could be the candidates of host genes required for susceptibility to the pathogen or involve some uncharacterized host defense pathways. Studies of the cloned *pmr4, pmr6* and *mlo* genes support the assumption that the host proteins MLO, PMR6 and PMR4 are located and functional at the extrahaustorial membrane, the extrahaustorial matrix and the plant

cell wall at sites of infection, respectively (Figure 1). In contrast to the hypersensitive cell death caused by the incompatible interaction, vitamin B synthesis in the haustorium suppresses cell death during the compatible interaction, which may target host bax inhibitor (reviewed by Panstruga, 2003). This cell death suppression is illustrated by the so-called “green island effect” in the compatible interaction of barley and the barley powdery mildew fungus *Blumeria graminis f. sp. hordei (Bgh*) (Schulze and Vogel, 2000). Pathogen Plasma Membrane-localized H^+-ATPase may contribute to the establishment of the compatibility as well. Despite this progress, our understanding of plant-biotroph compatibility is still limited. However, it is clear that identification of more “susceptibility” genes will not only enrich our understanding of compatibility mechanisms, but it will also provide new resistance sources for breeding. The barley *mlo* gene illustrates this point, since it has successfully been used in disease resistance breeding programs for many years. The resistances mediated by this kind of “susceptibility” genes are likely broad-spectrum to all the isolates of a fungal species, as is the case for *mlo*.

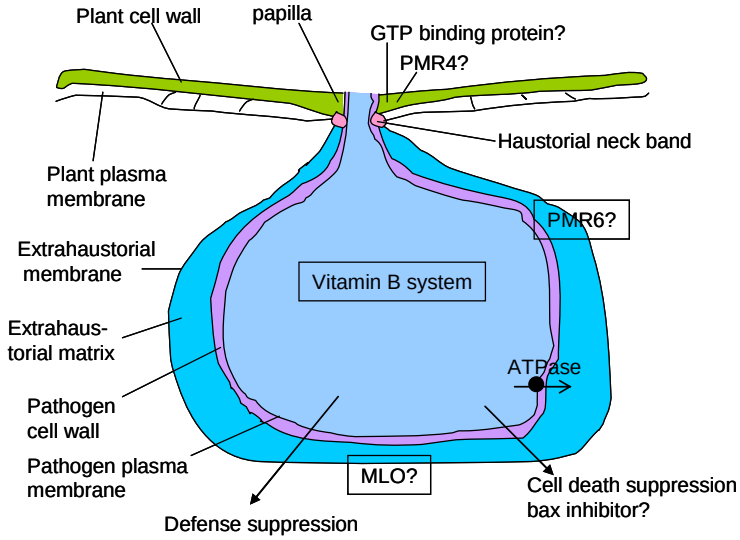


Figure 1 A central role for haustoria in compatible plant-biotroph interactions. The artificial scheme generalized from different plant-biotroph interactions represents a virtual haustorium of a fictitious phytopathogenic biotroph within a host cell.

As reviewed by Panstruga (2003), the host plasma membrane and extrahaustorial membrane are separated by the haustorial neck band and have different qualities. The haustorium absorbs nutrients (e.g. hexoses and amino acids) from the extrahaustorial matrix via proton-symport transporters. The pathogen PM-localized H^+-ATPase is crucial for establishment of a proton gradient. In the haustorium, vitamin B synthesis mediates the suppression of host defense and, cell death suppression may target host bax inhibitor. The host protein MLO, PMR6 and PMR4 might be located and function at the extrahaustorial membrane, the extrahaustorial matrix and the plant cell wall at sites of infection, respectively. A small GTP binding protein is required for the fungal penetration in barley. (Adapted from Panstruga 2003 and Schultheiss et al, 2002)

Nonhost resistance and host resistance

Although plant disease is a big issue in crop production, most plants are resistant to most plant pathogens. Disease actually is the exception rather than a commonplace

phenomenon in nature. Even if diseases occur, it is often restricted to a limited host range and specific tissues; for example, tomato powdery mildew mainly affects tissues of leaves and young stems. The terms “nonhost plant” and “nonhost pathogen” refer to the above (Thordal-Christensen, 2003). A plant species is nonhost for a specific pathogen, if all accessions tested are resistant to all strains of that pathogen. If some cultivars in a plant species are susceptible to a particular pathogen, the plant species is considered as a host of this pathogen. Thus, nonhost resistance is operating between species, whereas host resistance is active within the species, including race non-specific resistance (also named broad-spectrum resistance), race-specific resistance and basal defense. Race non-specific resistance is effective to all known isolates of the pathogen, and race-specific resistance is only effective against specific isolates of the pathogen, while basal defense differentiates levels of disease severity between susceptible plant genotypes.

Compared to host resistance, nonhost resistance is more common and durable. It can be classified into two types, Type I nonhost resistance does not induce any visible symptoms while type II nonhost resistance leads to a rapid HR (Mysore and Ryu, 2004). Thordal-Christensen (2003) listed five possible obstacles of the host during the process of disease, which the nonhost pathogen could meet. In fact, the first four obstacles, which include pathogen-differentiation-required signals from host, preformed barrier, “ancient” inducible barriers, and obstacles for nutrient uptake, can result in type-I nonhost resistance. The fifth hypothesized obstacle, which is associated with robust surveillance, mediated by multiple independent *R-/Avr*-gene recognition events, may be the cause of type-II nonhost resistance. Besides the surveillance based on a single *R-/Avr*-gene recognition event, host resistances share the preformed barrier and “ancient” inducible barriers (Thordal-Christensen, 2003). We assume that pathogen-differentiation- required signals from host and obstacles for nutrient uptake are also involved in host resistance (This thesis). There may be no clear boundary between nonhost resistance and host resistance, which explains why many studies showed that gene expression of defense responses to nonhost pathogens and host pathogens are similar (Tao et al., 2003). Several defense signaling components, such as salicylic acid, ethylene, SGT1 and heat shock proteins, were found to play roles in both host and nonhost resistance responses (reviewed by Mysore and Ryu, 2004). *NHO1* (Kang et al., 2003)*, EDS1* (Parker et al., 1996) and *PEN1* (Colins et al., 2003) are broad-spectrum disease resistance genes bridging host and nonhost resistance. Even though many particular examples showed a large extent of overlap between host and nonhost resistances, nonhost resistance is still far from being fully understood. Nevertheless, investigations on the development of the pathogen on the surfaces of artificial material, host and nonhost plants, could give help to elucidate Type I nonhost resistance. Further studies on the interactions between a particular plant and nonhost pathogens with different evolutionary distances from the host pathogen or between a particular pathogen and nonhost plants with different evolutionary distances from the host plants may decipher nonhost resistances activated at different levels.

Monogenic and polygenic resistances

Variation in host resistance, like many other traits, is qualitative or quantitative. Hence,

host resistance can be classified into qualitative resistance (usually controlled by a major gene, also called monogenic resistance) and quantitative resistance (usually contributed by many minor genes, so-called polygenic resistance). Most of the resistance exploited in breeding programs is monogenic, while polygenic resistance, although used much less, is even more abundantly available (Ribeirro do vale et al., 2001). Polygenic resistance is governed by many genes, which individually contribute with a small phenotypic effect, so that a segregating population shows a continuous scale of resistance from susceptible to resistant. In contrast, monogenic resistance governed by one major gene displays a discontinuous range of variation in resistance; therefore susceptible and resistant genotypes can easily be distinguished. Except for a few cases that demonstrate that *R* genes (for example *mlo* and *Lr34*) can confer durable resistance, most dominant monogenic resistances last for a short time, because the pathogens overcome the resistance by evolution of the corresponding pathogen *Avr* genes. Polygenic resistances are usually considered to be non-race specific and durable, although this is not true for all polygenic resistances (Ribeirro do vale et al., 2001). Even though polygenic resistance occurs at different levels to nearly all pathogens in most cultivars of most crops (Ribeirro do vale et al., 2001), it is still poorly exploited, while monogenic resistance, often introgressed from wild species, is very common in crop breeding. The main reason for the lack of the use of polygenic resistance in cultivars is both the often not complete nature of this resistance, and the difficulty to pyramid all QTLs into one single cultivar. Development of molecular markers flanking the QTLs will facilitate pyramiding of multi QTLs into one cultivar (Bai, 2005) and combining polygenic and monogenic resistances into a single cultivar may improve the resistance level and its durability (This thesis). Hence, although it is more difficult to study many genes with a small effect than one gene with a large effect, for plant breeding polygenic resistance certainly deserves attention.

Dominant and recessive *R* genes

Substantial research on plant disease resistance has focused on dominant *R* genes and their corresponding resistance pathways (Iyer et al., 2004). The related work has been well reviewed by Martin et al (2003), and Hammond-Kosack and Parker (2003). In this introduction the focus is on recessive *R* genes. From the over 50 cloned *R* genes (Coaker et al., 2005), the majority are dominant genes, most of which share a short conserved domain, the leucine rich repeat (LRR). In contrast to dominant *R* genes, few recessive *R* genes have been identified. Fortunately, with more attention being paid to the mechanisms of susceptibility, a wide range of recessive resistance loci conferring resistance to different pathogens in different plant species have recently been identified. So far, three recessive resistance genes have been isolated, *xa5* (Iyer and McCouch, 2004), *RRS1-R* (Deslandes et al., 2002) and *mlo* (Büschges et al., 1997). The cloned rice *xa5* gene for disease resistance to *Xanthomonas oryzae* pv*. oryzae* encodes the gamma subunit of transcription factor IIA (TFIIA gamma), and does not belong to any class of cloned *R* genes (Iyer and McCouch, 2004). The cloned dominant (*RRS1-S*) and recessive (*RRS1-R*) alleles from susceptible and resistant *Arabidopsis* accessions to *Ralstonia solanacearum* encode highly similar predicted proteins differing in length, which present a novel structure combining domains found in plant TIR-NBS-LRR resistance proteins and a

WRKY motif characteristic of some plant transcriptional factors (Deslandes et al, 2002). However, these two cloned recessive *R* genes are not absolutely recessive; in transgenic plants, *RRS1-R* behaves as a dominant gene and xa5 may be partial dominant rather than absolute recessive, because of the intermediate disease level displayed by heterozygous F_1 plants. Both of these recessive genes were claimed to fit the guard hypothesis (a complementary hypothesis for the gene-for-gene model) and to have a function in the R protein complex (Deslandes et al, 2002; Iyer and McCouch, 2004).

By contrast, the cloned *mlo* gene mediates a loss-of-function resistance (Kim et al., 2002), resulting in papilla formation rather than activation of plant defense. Strictly, *mlo* should not be considered as an *R* gene but as a loss-of-function allele of the *Mlo* locus (probably a compatibility factor). Screening of mutants that enhance resistance in *Arabidopsis* resulted in the identification of a range of recessive genes like *mlo*, such as *pmr4, pmr5 and pmr6* (Vogel et al., 2002; Nishimura et al., 2003; Vogel et al., 2004) (Table 1), allowing us to have a fresh view into recessive *R* genes. The commonplace characteristic of these recessive genes is that the according dominant alleles encode negative regulators of defense pathways or components required for establishing compatibility (Table 1). There are a number of recessive genes leading to suppression of resistance responses, because the corresponding dominant alleles code for regulators or signaling components of plant defense pathways. These recessive genes (mutants) are very useful for the understanding of resistance and susceptibility.

Table 1 List of several representative recessive mutants known to enhance resistance at different infection stages during plant-biotroph interaction and two mutants related to HR.

Mutant	Function of the dominant allele	Infection stage	Pathosystem	Citation
mlo	Negative regulator of cell death and defense reactions	Penetration	Barley- *Blumeria graminis f. sp. Hordei (Bgh)*	Büschges et al., 1997; Kim et al., 2002
ol-2	Unknown	Penetration	Tomato-*Oidium neolycopersici*	Bai et al., 2005; This thesis
pmr5	Modification of pectin in cell wall or extrahaustorial matrix	Penetration or nutrient -uptake	*Arabidopsis-Erysiphe cichoracearum*	Vogel et al., 2004
pmr6	Pectate lyase-like protein	Nutrient -uptake	*Arabidopsis-Erysiphe cichoracearum*	Vogel et al., 2002
pmr4	Putative callose synthase	Penetration	*Arabidopsis-Erysiphe cichoracearum*	Nishimura et al., 2003
edr1	Negative regulator of SA-inducible defense response	Posthaustorial	*Arabidopsis-Erysiphe cichoracearum*	Frye et al., 2001
dnd	Component of signal pathway leading to HR	Not applicable	*Arabidopsis-Pseudomonas syringae*	Yu et al., 1998
lsd1	Negative regulator of cell death	Not applicable	*Arabidopsis* lesion mimic mutant	Dietrich et al., 1997

Although much progress has been made on the research of dominant *R* gene mediated resistance in the past years, the understanding of non-host resistance, polygenic resistance and susceptibility is still poor. Because dominant *R* genes often lack durability in terms of resistance researchers start to pay attention to other less-studied plant resistances. In this thesis, susceptibility, monogenic (dominant and recessive) and polygenic resistances were investigated in the interaction between tomato and *O. neolycopersici*. This pathosystem is described in more detail below.

Tomato and *O. neolycopersici* - pathosystem in this thesis

Tomato

Tomato is one of the most widely grown vegetable crops in the world. Until 2004, the global production of tomatoes increased up to approximately 1.16 x 10^8 million-ton/year (FAOSTAT data, 2004). Tomatoes belong to the Solanaceae (nightshades), which is a medium-sized family with approximately 90 genera and 3000-4000 species with incredible morphological and chemical diversity and a worldwide distribution. Economically, the Solanaceae represent the third most important plant taxon. It includes the sole plant fueling the tobacco industry (Nicotiana), the tuber-bearing potato, a number of fruit-orientated vegetables (tomato, eggplant and peppers), ornamental plants (Petunias, *Schizanthus*, *Salpiglossis* and *Browallia*), plants with edible leaves (*Solanum aethiopicum* and *S. macrocarpon*) and medicinal plants (*Datura* and *Capsicum*) (Figure 2). A number of Solanaceae plants act as model experimental organisms, for instance, tomato for fruit ripening and plant defense, tobacco for tissue culture, transformation, plant defense and VIGS (Virus Induced Gene Silencing), and potato for starch research and petunia for the biology of anthocyanin pigments. All these characteristics make the Solanaceae one of the most research-worthy groups of the angiosperms.

Recently the phylogenetic relationships in Solanum (Solanaceae) were updated based on the sequences of the chloroplast gene *ndhF* (Bohs and Olmstead, 1997). The genera *Lycopersicon* and *Cyphomandra* were integrated into the *Solanum* genus (Bohs and Olmstead, 1997). Further studies on phylogenetic inference in wild tomatoes through comparison of AFLP fingerprinting with other markers resulted in the new nomenclature for tomato species (Table 2, Figure 2) (Spooner et al., 2005 & in press).

Cultivated tomatoes (*Solanum lycopersicum* L) are the descendants of wild *S. lycopersicum*. Wild tomato species (*Solanum* section *Lycopersicon*) originate from western South America (Spooner et al., 2005). The accessions of *S. esculentum* var. *cerasiforme*, which were imported to Europe in the 15th century, had already reached an advanced stage of cultivation in Mexico the center of domestication. Initially, in Europe, selection and breeding was carried out mainly in Italy, which led to increased popularity of the tomato due to its appearance, taste and its nutritional value. All the tomato species are diploid (2*n*=24; Rich 1979; except that rare tetraploid forms occur) and most of them can be crossed (sometimes with difficulty) to the cultivated tomato (Spooner et al., 2005). Therefore, tomato becomes the genetic cornerstone of the Solanaceae family due to its modest-sized diploid genome (950 Mb), tolerance to inbreeding and intercrossability to wild tomato species. As a result, a wealth of knowledge has accumulated on tomato

genetics, cytogenetics and physiology.

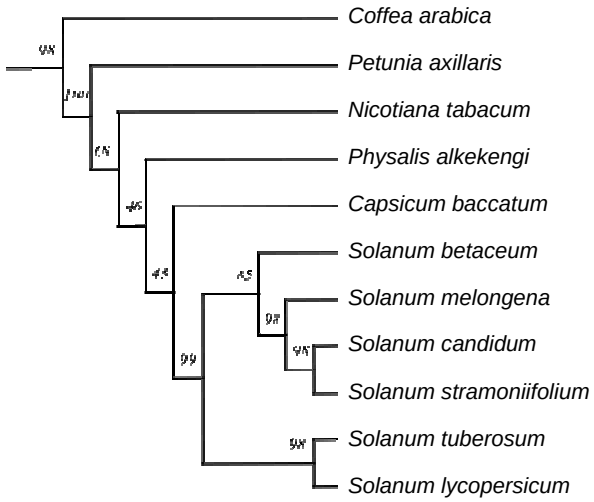
Phylogenic tree of the Solanaceae (including coffee)	Common name
	
Coffea arabica	Coffee
Petunia axillaris	One of the parents of Petunia
Nicotiana tabacum	Tobacco
Physalis alkekengi	Chinese lantern & Winter cherry
Capsicum baccatum	Bird pepper
Solanum betaceum	Tree tomato
Solanum melongena	Eggplant
Solanum candidum	Buffalo bur
Solanum stramoniifolium	Red-fruited nightshade, Coconilla
Solanum tuberosum	Potato
Solanum lycopersicum	Tomato

Figure 2 An overview of the phylogeny of the Solanaceae. Adapted from the Solanaceae genomics network (http://www.sgn.cornell.edu/help/about/about_solanaceae.html), provided by Feinan Wu, based on Bohs and Olmstead, (1997).

Table 2 List of wild tomato species (*Solanum* section *Lycopersicum*) and allies - the "Tomato clade" (with equivalents in the previously recognized genus *Lycopersicon*, now part of a monophyletic *Solanum*). Some important traits for breeding are also listed. [Adapted from the Solanaceae genomics network (http://www.sgn.cornell.edu/help/about/solanum_nomenclature.html), provided by Prof. Sandra Knapp, based on Peralta, Knapp and Spooner, unpublished monograph in *Systematic Botany Monographs*; Spooner et al, 2005; and the Tomato Genetics Resource Centre (http://tgrc.ucdavis.edu)]

No.	New name in ***Solanum***	***Lycopersicon* equivalent**	**Importance for breeding purpose**
1	*Solanum lycopersicum* L.	*Lycopersicon esculentum* Miller	Moisture tolerance, resistances to wilt, root-rotting, and leaf-spotting fungi
2	*Solanum habrochaites* S. Knapp & D.M Spooner	*Lycopersicon hirsutum* Dunal	Cold and frost tolerance, insect resistance (glandular hairs), and other resistance
3	*Solanum neorickii* D.M. Spooner, G.J. Anderson & R.K. Jansen	*Lycopersicon parviflorum* C.M. Rick, Kesicki, Fobes & M. Holle	Fungal resistance (This thesis and other projects at WU)
4	*Solanum pimpinellifolium* L.	*Lycopersicon pimpinellifolium* (L.) Miller	Contributed to improve color and fruit quality. Insect, nematode, and disease resistance

No.	New name in *Solanum*	*Lycopersicon* equivalent	Importance for breeding purpose
5	*Solanum pennellii* Correll	*Lycopersicon pennellii* (Correll) D'Arcy	Contributed to drought resistance; dense pubescence of glandular hairs imparts insect resistance
6	*Solanum chmielewskii* (C.M. Rick, Kesicki, Fobes & M. Holle) D.M. Spooner, G.J. Anderson & R.K. Jansen	*Lycopersicon chmeilewskii* C.M. Rick, Kesicki, Fobes & M. Holle	Contributed to higher sugar content in the crop
7	*Solanum chilense* (Dunal) Reiche	*Lycopersicon chilense* Dunal	Drought resistance
8	*Solanum cheesmaniae* (L. Riley) Fosberg	*Lycopersicon cheesmaniae* L. Riley	Salt tolerance, Lepidoptera and virus resistances
9	*Solanum galapagense* S. Darwin & Peralta	Part of *Lycopersicon cheesmaniae* L. Riley	Salt tolerance, Lepidoptera and virus resistances
10	*Solanum 'N peruvianum'* (4 geographic races: humifusum, lomas, Marathon, Chotano-Yamaluc)	Part of *Lycopersicon peruvianum* (L.) Miller (incl. var. humifusum and Marathon races)	Virus, bacteria, fungi, aphid, and nematode resistance
11	*Solanum 'Callejon de Huaylas'* to be described by Peralta	Part of *Lycopersicon peruvianum* (L.) Miller (from Ancash, alogn Río Santa)	Virus, bacteria, fungi, aphid, and nematode resistance
12	*Solanum corneliomuelleri* J.F. Macbr. (1 geographic race: Misti nr. Arequipa)	Part of *Lycopersicon peruvianum* (L.) Miller; also known as *Lycopersicon glandulosum* C.F. Mull.	Virus, bacteria, fungi, aphid, and nematode resistance
13	*Solanum peruvianum* L.	*Lycopersicon peruvianum* (L.) Miller	Virus, bacteria, fungi, aphid, and nematode resistance
14	*Solanum juglandifolium* Dunal	*Lycopersicon juglandifolium* (Dunal) J.M.H. Shaw	Flood tolerance (probably a general feature)
15	*Solanum ochranthum* Dunal	*Lycopersicon ochranthum* (Dunal) J.M.H. Shaw	Flood tolerance (probably a general feature)
16	*Solanum sitiens* I.M. Johnst.	*Lycopersicon sitiens* (I.M. Johnst.) J.M.H. Shaw	Drought tolerance
17	*Solanum lycopersicoides* Dunal	*Lycopersicon lycopersicoides* (Dunal in DC.) A. Child ex J.M.H. Shaw	Chilling tolerance

Currently, the genetic map of tomato as a model plant for the Solanaceae family is among the most saturated of eukaryotic species; the map of “Tomato-EXPEN 2000” harbors 1668 markers (http://www.sgn.cornell.edu). Comparative linkage maps of pepper, eggplant, potato and tomato exemplify the inter-specific synteny between all the four genomes (Tanksley et al., 1992, Livingstone et al., 1999, Doganlar et al., 2002a&b). These comparative maps have not only shed light on the nature of genome evolution, but have also facilitated comparative mapping studies of qualitative and quantitative traits. As a result, comparative genetics of specific traits, for example disease resistance, allowed

identification/cloning of genes or loci using the knowledge of other Solanaceae plants (Grube, et al, 2000; Thorup et al., 2000; Brouwer et al., 2004 and Huang et al., 2005). These comparative maps also provide a platform on which the sequence, marker and trait information of all Solanaceae species can be exchanged and employed. Thanks to the consensus mapping population (referred as F2.2000) developed in Tanksley's lab, large amounts of DNA and cuttings of F2.2000 are available for distribution along with data for approximately 2000 segregating markers as well as plant phenotypes (http://www.sgn.cornell.edu). This *S. esculentum* x *S. pennellii* F2 population is currently being linked to the "seed" BACs for the international sequencing project. Tomato introgression lines, characterized mutants, numerous cytogenetic stocks and wild species accessions, which are maintained at the Tomato Genetics Resource Center in Davis (http://tgrc.ucdavis.edu/), are public available, representing valuable genetic resources to the tomato community. In addition, tomato mutant seeds can be ordered from the mutant library (http://zamir.sgn.cornell.edu/ mutants/), which is derived from inbred variety M82 mutated by using EMS and fast-neutrons. Tomato germplasm is also maintained and can be ordered from the USDA-ARS Plant Genetic Resources Unit (http://www.ars-grin.gov/ npgs/orders. html).

Research groups from ten nations are sequencing the tomato genome and the updated information about the progress can be obtained at SGN (Solanaceae genomics network; http://www.sgn.cornell.edu/). SGN is designed to provide a common entry point to various sources of data and analysis tools, integrating both local and remote resources. In addition, tomato BAC libraries constructed by Rod Wing and co-workers are available for mapping and gene isolation, and more than 160,000 tomato ESTs from 24 cDNA libraries are deposited in SGN and TIGR (The Institute for Genomic Research) are playing an important role in tomato sequence annotation. In conclusion, because of the economical importance, wide cross ability, Solanaceae status, rich genetic and genomic resources, relatively small genome, and the sequencing efforts, tomato is absolutely a model crop.

Powdery mildew and tomato powdery mildew

Erysiphales, so-called "powdery mildews", which colonies display a white and powdery appearance on the leaves, stems, or fruits of plants, belong to the phylum Ascomycota. These obligate biotrophs can infect over 9,000 dicot and 650 monocot plant species, and are among the most common plant diseases (Saenz and Taylor, 1999; Chaure et al., 2000; Takamatsu, 2004). Powdery mildews cause the greatest losses, in term of crop yield, of all the single types of plant diseases (Chaure et al., 2000). Braun (1987) described 18 genera and 435 species of the powdery mildew in his monograph (cited by Takamatsu, 2004). Morphological data using light microscopy, SEM (Scanning Electron Microscopy) and molecular analyses [ITS (internal transcribed spacer) and ribosomal DNA sequences], as well as the infectivity (host range), were used to clarify the phylogeny and evolution of the powdery mildews (Cook et al., 1997; Saenz and Taylor, 1999; Takamatsu, 2004; Mori et al., 2000). However, drawing a fine conclusion on the evolution and phylogeny of the powdery mildews is still difficult since the data do not always agree with each other. The fact that only the asexual stage of the life cycle is known of most powdery mildews also attributes

to the difficulties to make a clear classification of the powdery mildews.

For tomato powdery mildews (*Oidium lycopersici* and *O. neolycopersici*) a sexual stage is unknown. *O. neolycopersici* with non-catenate conidia is widespread in Europe, Africa, North and South America and Asia. By contrast, *O. lycopersici* with catenate conidia occurs in Australia (Kiss et al., 2001). In this thesis, the tomato powdery mildew fungus used was collected from infected commercial tomato cultivars in the Netherlands and was classified as *O. neolycopersici* based on the morphology of the conidiophores and ITS sequence analyses (Kiss et al., 2001). Tomato powdery mildew caused by *O. neolycopersici* was first reported in 1986 in the Netherlands [Simonse, 1987; Paternotte 1988, cited in thesis C. Huang (2000)] and has spread rapidly around the world (Kiss et al., 2001). Tomato powdery mildew (*O. neolycopersici*) has recently become a very serious worldwide disease of tomato, especially in greenhouse cultivation (Jones et al., 2001). It is one of the most fungicide-sprayed diseases of tomato. The spreading of this plant disease has caused and is causing large damage to tomato production, and it is causing serious environmental pollution due to fungicide use.

Fortunately, many research groups in different parts of the world are working on either the fungal side or the plant side of this serious plant disease. We showed that the *O. neolycopersici* – tomato interaction likely fits the gene-for-gene model (Bai et al, 2005). Collecting isolates and identification of *Avr* genes will increase our understanding of the interaction between *O. neolycopersici* and tomato. However, the difficulties to maintain this obligate biotrophic fungus on media are hindering the employment of molecular tools on the fungal side to reveal the pathogenicity mechanisms. Nevertheless, major advances in comprehending the interaction between tomato and *O. neolycopersici* have been made from the tomato side, the details of which are described below.

Resistance to *O. neolycopersici* in tomato

Although most modern tomato cultivars are susceptible to *O. neolycopersici*, screening of wild tomato species resulted in the detection of resistance resources (Lindhout et al., 1994 a&b), such as *Solanum habrochaites* (former *L. hirsutum*), *S. peruvianum* (former *L. peruvianum*) and *S. neorickii* (former *L. parviflorum*). So far, six *Ol* genes and three major QTLs, which confer resistance to *O. neolycopersici* in tomato, have been identified (Lindhout et al., 1994b; Ciccarese et al., 1998; Bai et al., 2003 and 2005), and several cultivars that carry monogenic *R* genes are now on the market. The monogenic dominant resistance genes *Ol-1* and *Ol-3* introgressed from *S. habrochaites* G1.1560 and G1.1290 respectively, have been fine-mapped on the long arm of chromosome 6 (Lindhout et al., 1994 a&b; Huang et al., 2000 a&b; Bai et al., 2005). The resistance in *S. lycopersicum* var *cerasiforme* is contributed by a recessive gene *ol-2* that maps on chromosome 4 (Ciccarese et al., 1998 and 2000; De Giovanni et al., 2004). The *Ol-4* gene, which maps on the short arm of chromosome 6, originates from *S. peruvianum* LA2172 (Bai et al., 2004 and 2005). *Ol-5* is derived from *S. habrochaites* PI247087 and locates on the long arm of chromosome 6 (Bai et al., 2004). *Ol-6* with unknown origin maps closely to *Ol-4* (Bai et al., 2004). Three resistance QTLs were introgressed from *S. neorickii* G1.1601 and have been mapped on chromosomes 6 and 12 (Bai et al., 2003).

The monogenic *Ol-1, Ol-3, Ol-4, Ol-5* and *Ol-6* genes and the three *Ol*-QTLs have been

introgressed into the tomato cultivar Moneymaker (MM) and the resistance mechanisms have been studied microscopically. Previous microscopic observations (Bai et al., 2005) revealed that the resistance responses caused by *Ol-1, Ol-3* and *Ol-5* are strongly associated with slow-HR, while resistance responses mediated by *Ol-4* and *Ol-6* are associated with fast-HR (Huang et al., 2000 a&b; Bai et al., 2005, chapter 3). The resistance in *S. neorickii* governed by three major resistance QTLs was less associated with HR (Huang et al., 2000 a&b). Resistance tests of those near isogenic lines carrying different resistance genes worldwide suggest that the tomato – *O. neolycopersici* interaction is governed by a gene-for-gene model and that *O. neolycopersici* isolates in different geographic regions harbour different *Avr* genes (Bai et al., 2005).

Transcript profiling - plant functional genomics

Scientists are facing the tremendous raw data of genomic information. So far, 263 genome-sequencing projects have been completed that include 33 eukaryotic genomes, and about five hundred eukaryotic species are being studied in whole-genome sequencing or EST sequencing projects (Cited from http://www.genomesonline.org/, till 21 may 2005). Plant genomes tend to be bigger and more complicated than mammalian genomes and it is difficult to get funding for plant projects compared to Human or Mouse genome projects (Gura, 2000). Nevertheless, genome-sequencing projects of *Arabidopsis*, rice and diatoms were finished in 2000, 2002 and 2004, respectively. Projects aimed at sequencing the genomes of major crop and model plants, including maize, cotton, tomato, potato, barley, soybean, coffee, alfalfa, Brassica etc, have been initiated and will bear fruits in several years.

With the existing and coming overwhelming DNA sequencing data, plant scientists are facing the question how to unravel the functions of the “newly annotated genes”. Once a plant genome has been sequenced to completion, most of the genes can be annotated and their putative function may be predicted through BLAST searching (Holtorf et al., 2002). However, the *in-silico* candidate functions of these genes need to be validated through studying the expression and analyzing gene product activity. Gene activity can be tracked by recording the abundance of its transcript during biological processes; high-throughput transcript profiling can monitor the activities of many genes simultaneously. Since transcript abundance does not always reflect protein level and activity (Gygi et al., 1999). Proteomics and metabolomics, the profiling of expressed proteins and metabolites respectively, can complement the data. In recent years transcript profiling has been used to study many aspects in the plant sciences. Profiling methods are sequencing-based (SAGE, MPSS) (Matsumura et al., 1999; Meyers et al., 20004), cDNA fragment-based (DDRT-PCR, cDNA-AFLP) (Benito et al., 1996; Bachem et al., 1996) and hybridization-based (macro- and micro array) (reviewed by Aharoni and Vorst, 2002). All these methods have their own drawbacks and advantages (Table 3), and researchers can select the method suitable for their situation or use two methods simultaneously.

Why was cDNA-AFLP used in this thesis?

In this thesis, cDNA-AFLP was selected for the following reasons: Firstly, cDNA-AFLP is

an open method, which allows the detection of new genes. Secondly, designed tomato-genome arrays were not available in 2001 - 2005. Thirdly, little is known about gene expression of the interaction of tomato – *O. neolycopersici,* thus developing a dedicated array for this pathosystem is not plausible. Furthermore, cDNA-AFLP is a mature and stable method in our lab and the employment of both the LICOR sequencer and the Odyssey machine make excision of bands from PAGE gels very feasible. In addition, the data generated using cDNA-AFLP will be compared with a subproject (TRI-2) of the CBSG (Center of BioSystem Genomics, http://www.cbsg.nl/), in which transcript profiles of the same pathosystem will be studied by using a tomato array (Affymatrix).

Table 3 Major advantages and limitations of the main transcript profiling technologies. MPSS, massively parallel signature sequencing; SAGE, serial analysis of gene expression; SSH, suppression subtractive hybridization; cDNA-AFLP, cDNA amplified fragment length polymorphism analysis; Oligo chips, oligonucleotide-based arrays; DD, differential display reverse transcription – PCR; "+" indicates that the technology has the characteristics, "-" not.

Technology characteristics	**Sequencing based**		**Hybridization based**			**Fragment-size based**	
	MPSS	**SAGE**	**SSH**	**Oligo chip**	**cDNA array**	**cDNA -AFLP**	**DD**
False positive	-	-	+	-	+	-	+
Low sensitivity and reproducibility	-	-	+	-	-	-	+
Knowledge of prior sequence	-	-	-	+	-	-	-
Quantitative	+	+	-	+	+	+/-	-
Automation	+	+	-	+	+	+/-	-
Enrichment of low-abundance transcripts	-	-	+	-	-	-	-
Cross-hybridization problem	-	-	-	-	+	-	-
Set-up cost (general, also dependent on experiment scale)	High	High	Low	High	High	Low	Low
Coverage	Scale -based	Scale -based	Low	High	High	Enzyme -based	Low
Total RNA required (ug)	>50	>50	> 50	> 50	> 50	5-20	5

Scope of this thesis

As described above, tomato powdery mildew (*O. neolycopersici*) is an important fungal disease, and a good understanding of the susceptibility and resistance mechanisms of tomato to this obligate biotroph will not only enrich the scientific knowledge but also give clues to breed durable powdery mildew resistant tomato cultivars. In this thesis, susceptibility, monogenic (dominant and recessive) and polygenic resistances were investigated at macroscopic, microscopic and transcriptional levels.

We aim to answer the following questions: -What are the differences and similarities of the transcript profiles of susceptible, monogenic- and polygenic resistance responses to *O. neolycopersici* in tomato? -Do *ol-2*-based (recessive, associated with papilla formation), *Ol-1*-based (dominant, associated with slow HR) and *Ol-4*-based (dominant, associated

with fast HR) resistance responses show different transcript profiling and use different defense signaling pathways? -What are the performances of the tomato lines with different combinations of resistance QTLs (*R*-QTLs*)* responding to *O. neolycopersici* at the macroscopic, cytological and transcriptional levels? and -What is the effect of pyramiding *R*-QTLs into one tomato line and will the genetic background make a difference in the resistance appearance?

Chapter 2: Susceptible genotype - Moneymaker (MM - *S. lycopersicum*), a resistant tomato BC_1S_2 line with the dominant *R* gene *Ol-1* introgressed from *S. habrochaites* in the genetic background of MM, and a resistant accession of *S. neorickii* carrying three *R*-QTLs were used for transcript profiling (cDNA-AFLP) analysis of their responses to tomato powdery mildew. It is shown that the main difference between the responses is the expression timing and generally the susceptible response is slower than the resistant ones. While the monogenic resistance response displays an expression peak, which is not shown in the polygenic resistance response.

Chapter 3: Three types of tomato resistance to *O. neolycopersici* using cDNA-AFLP with selected primer combinations that detected differentially expressed transcript derived fragments (DE-TDFs) in previous experiments (Chapter 2): *ol-2*-based resistance associated with papilla formation, *Ol-1*-based resistance associated with slow-HR and *Ol-4*-based resistance associated with fast HR. Also expression of genes that are components of known defense pathways was studied by RT-PCR. Both cDNA-AFLP and RT-PCR data suggest that *Ol-1, Ol-4* and *ol-2* mediated resistances employ different pathways. These findings are discussed.

Chapter 4: The tomato near isogenic lines containing different numbers of *R*-QTLs inoculated with *O. neolycopersici* were evaluated macroscopically and microscopically, and compared with resistant lines carrying *ol-2* and *Ol-1* and the susceptible control MM. We revealed that HR is a major factor in the *R*-QTL-meditated resistance and different individual *R*-QTL(s) may employ different HR mechanisms. While the pyramiding of *R*-QTL(s) resulted in a higher frequency of qualitatively different necrotic cells, which leads to a high-level resistance comparable to *Ol-1* mediated resistance. It was also concluded that penetrated papillae, vesicle accumulation in the cells and structural changes in extra-haustorial matrix may perform roles in the *R*-QTL-meditated responses, but are not specific to individual *R*-QTL(s). It was observed that tomato leaves are composed of a mosaic of “compatible” and “incompatible” cells in their interaction with *O. neolycopersici*.

Chapter 5: The transcript profiles of resistance responses to *O. neolycopersici* from the same set of tomato lines as observed in Chapter 4*,* were investigated by using cDNA-AFLP with selected primer combinations based on previous experiments (Chapters 2 and 3). Data suggest that pyramiding of *R*-QTLs only alters the defense pathways qualitatively rather than quantitatively. A number of DE-TDFs were also *in-silico* mapped, and interesting DE-TDFs are discussed.

General discussion: The combined results of Chapters 2-5 are discussed in relation to relevant literature. We demonstrated that tomato defense barriers to *O. neolycopersici* are correlated with different infection stages during interaction. Conservation and quantitative nature of pathogen-induced transcriptomes of compatible and incompatible

interactions of tomato and *O. neolycopersici* are proposed. The different phenotypic responses of QTL-NILs containing the same *R*-QTLs are discussed; genes required for the resistance mediated by *R* genes/QTLs in tomato are hypothesized. The experimental result that *Arabidopsis* is a host of *O. neolycopersici* is presented and *Arabidopsis* and tobacco as model plants for studies on the interaction of tomato and *O. neolycopersici* are discussed. Future work is indicated according to the messages of the thesis.

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Chapter 2

Tomato defense to the powdery mildew fungus: differences in expression of genes in susceptible, monogenic- and polygenic resistance responses are mainly in timing

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Abstract *Oidium neolycopersici* is the causal agent of tomato powdery mildew. In this paper, gene expression profiles were investigated of susceptible, monogenic- and polygenic resistant tomato genotypes in response to *O. neolycopersici* infection by using cDNA-AFLP. Around 30,000 TDFs (Transcript Derived Fragments), representing ~22% of the transcriptome based on *in-silico* estimation, were identified and 887 TDFs were differentially expressed (DE-TDFs) upon inoculation with *O. neolycopersici* spores. 256 of these DE-TDFs were further studied to determine their temporal patterns. About 53% of the DE-TDFs were only associated with the susceptible interaction and most of them were up regulated at later time-points, implying a possible role in susceptibility. Forty-two percent of the further studied DE-TDFs were similarly regulated in both the compatible and incompatible interactions. All of these commonly induced DE-TDFs displayed an expression peak at seven days post inoculation in monogenic resistant response but sustained up-regulation in the susceptible and in the polygenic resistant interaction. More than half of these DE-TDFs showed earlier timing in resistant interactions compared to susceptible interaction. Only two percent of the identified DE-TDFs were specific to either the monogenic or the polygenic resistant response and three percent showed other patterns. By annotation of the 174 sequenced DE-TDFs we found that 32% of the corresponding transcripts were known to be involved in plant defense, whereas the other transcripts played general roles in signal transduction (13%), regulation (18%), protein synthesis and degradation (13%), energy metabolism (13%) including photosynthesis, photorespiration and respiration. Transcripts of the DE-TDFs, showing earlier up-regulation in incompatible interactions compared to the compatible interaction or being resistance specific, predominantly execute putative roles in plant defense and signal transduction. By contrast, transcripts showing similar temporal patterns in compatible and incompatible interactions are often associated with housekeeping functions and regulation. We propose that the host plants employ similar components of the defense pathways but differ in timing during the compatible and incompatible interactions of tomato and *O. neolycopersici*.

Keywords: *Solanum lycopersicum*, *O. neolycopersici*, cDNA-AFLP, basal defense, monogenic and polygenic resistance, differentially expressed transcript derived fragment (DE-TDF).

Introduction

In nature, plants have to face the attacks from a variety of intruders, such as viruses, bacteria, fungi and insects because they cannot move away from these natural enemies. Most plants can protect themselves against non-specific pathogens with passive defense mechanisms including cell wall thickness and waxy, anti-microbial components. To protect themselves against attack of specific pathogens and pests, active defense systems are very important whereby resistance genes play pivotal roles. More than 50 plant disease resistance (*R*) genes have been cloned (Coaker et al., 2005), which comprise *R* genes that match the corresponding avirulence (*Avr*) genes of pathogens according to the well-known gene-for-gene model (Flor, 1971). Typically, the race-specific resistance response is associated with HR (Hypersensitive Response) microscopically and/or macroscopically. Several race non-specific resistance genes like RPW8, RPG1 and FLS (reviewed by Hammond-Kosack and Parker, 2003), have also been cloned. The mechanisms of both race-specific and race non-specific resistance responses are well studied in some famous plant-pathogen model systems like the barley - *Blumeria graminis-f.sp.hordei (Bgh)* pathosystem (Schulze-Lefert and Vogel, 2000) and tomato - *Cladosporium fulvum* pathosystem (Joosten and De Wit, 1999). However, far less is known of the mechanisms underlying quantitative resistance governed by a number of genes.

Fungal diseases occur very often and are one of the biggest threats for plant health. Tomato powdery mildew caused by the biotrophic fungus, *O. neolycopersici* has recently become a very important disease worldwide of tomato (*S. lycopersicum*). There are two known species of tomato powdery mildew in the *Oidium* genus, *O. lycopersici* occurring in Australia and *O. neolycopersici* occurring in the rest of the world; conidia form mainly a chain for *O. lycopersici* and single spores for *O. neolycopersici* (Jones et al., 2001). The disease has caused large damage in the European tomato production, especially in the glasshouse production. Although the cultivated tomato is susceptible to the fungus, resistance occurs in many wild species of tomato (Lindhout et al., 1994a&b), such as *S. habrochaites (*former *Lycopersicon hirsutum)* and *S. neorickii* (former *L. parviflorum*). Several cultivars that carry monogenic *R* genes are now on the market. The monogenic dominant resistance genes *Ol-1* and *Ol-3* introgressed from *S. habrochaites* G1.1560 and G1.1290 respectively have been fine-mapped on the long arm of Chromosome 6 (Lindhout et al., 1994a&b; Huang et al., 2000 a&b; Bai et al., 2005). Three resistance QTLs were introgressed from *S. neorickii* G1.1601 and have been mapped on Chromosomes 6 and 12 (Bai et al., 2003). Both the monogenic *Ol-1* gene and the three *Ol*-QTLs have been introgressed in the tomato cultivar Moneymaker (MM) and the resistance mechanisms have been studied microscopically. Previous studies showed that the resistance response caused by *Ol-1* is strongly associated with HR (Huang et al., 2000 a&b; Bai et al., 2005), while the resistance in *S. neorickii* governed by three major resistance QTLs is less associated with HR (Huang et al., 2000 a&b).

cDNA-AFLP is a genome-wide expression analysis technology that does not require prior knowledge of gene sequences. This PCR-based technique combines a high sensitivity with a high specificity, allowing detection of rarely expressed genes and distinction between

homologous genes (Bachem et al., 1998; Reijans et al., 2003). Since the first introduction of cDNA-AFLP to profile genes involved in potato tuber development (Bachem et al., 1996), more than 50 papers have been published on different biological processes using this platform. Based on these results, cDNA-AFLP is considered as a reliable, available technique for laboratories, especially for organisms with little sequence information.

In this paper, cDNA-AFLP was employed to compare gene expression profiles in the susceptible genotype (Moneymaker), a monogenic resistant line and a *S. neorickii* accession, which is the donor of the *Ol*-QTLs in response to infection with *O. neolycopersici*. The outcome will increase our understanding of the mechanisms of the tomato - *O. neolycopersici* interaction. Our data indicate that a large part of the differences between basal defense in the compatible interaction and *R*-gene (*R*-QTL) mediated responses in the incompatible interactions of tomato and *O. neolycopersici* is mainly due to the timing of the expression of genes involved. The monogenic resistant response results in an expression peak of DE-TDFs at 7 days post inoculation, while in both the susceptible MM and the polygenic resistant *S. neorickii* accession these DE-TDFs are constantly up-regulated.

Materials and methods

Plant materials

Three tomato genotypes were used in the cDNA profiling experiments: *S. lycopersicum* cv. Moneymaker (here after referred to as S-MM), as susceptible genotype; BC_1S_2 plants homozygous for the resistance gene *Ol-1* (hereafter referred to as R-Ol-1), generated by backcrossing MM with a breeding line harboring *Ol-1* introgressed from *S. habrochaites* G1.1560 and being selected using linked molecular markers; *S. neorickii* G1.1601, a wild tomato accession (hereafter referred to as R-QTL), which harbors three major *Ol*-QTLs.

Fungal material and inoculum preparation

O. neolycopersici was collected from infected tomato plants in the Netherlands (Lindhout et al. 1994a) and is continuously maintained on S-MM plants in growth chambers at 20±2℃, relative humidity (RH) 70% and 16 hours day -length. Fresh spores were washed from seriously infected leaves with water to prepare the inoculum (2×10^4 spores/ml). Water was sprayed as mock inoculation.

Experimental set-up of and sampling

All plants were grown in climate cells with optimal temperature, photoperiod and light conditions (20±2℃, 16 hours daytime, light intensi ty 150 μmol/m^2,s). Two independent inoculation experiments were performed as biological controls for cDNA-AFLP analysis. The experimental design consisted of two randomized blocks for both experiments with S-MM as borderlines and controls for spontaneous infection. Four-week-old plants were used for whole-plant inoculation as described by Bai et al. (2003). The second and third true leaves were collected and directly put into liquid N_2 and the remaining plant was kept for macroscopic disease evaluation. For each leaf sample another plant was used. In experiment one, samples were collected from inoculated and mock-inoculated plants of S-MM, R-Ol-1 and R-QTL at 0, 5, 24, 29, 48, 72 HPI (hours post inoculation). In

experiment two, samples were collected at 0, 1, 2, 3, 4, 7 DPI (days post inoculation) for both resistant genotypes and at the same time points plus 9 and 14 DPI for S-MM.

cDNA-AFLP

RNA isolation and cDNA synthesis were accomplished according to the cDNA-AFLP protocol of Bachem et al., 1998 (also can be found at http://www.dpw.wau.nl/pv/). In brief, the "hot-phenol" method was used to isolate RNA. The concentration and integrity of total RNA were measured with the spectrophotometer (Eppendorf, Germany) and checked on 1% agarose gel. For mRNA purification and enrichment, polyA^{+}-RNA was extracted from 20 µg of total RNA using poly-d[T]$_{25}$V oligonucleotides coupled to paramagnetic beads (Dynal A.S. Oslo, Norway). Double-strand cDNA was synthesized using SuperScriptII RNase H^{-} reverse transcriptase, RNase H and DNA polymerase I (*E.coli*) (all purchased from Invitrogen life technology, USA). Double-strand cDNA was extracted with phenol: chloroform (1:1), ethanol-precipitated and dissolved into a suitable volume sterilized H_2O. The cDNA quality was checked on 1% agarose gel and the concentration was measured by using a spectrophotometer (Eppendorf, Germany). Template preparation followed the standard AFLP protocol (Vos et al., 1995; Bachem et al., 1996). Two restriction enzyme combinations *AseI*/*TaqI* and *Mse*I/*Eco*RI were used (sequence details of primers and adaptors see Bachem et al., 1996; Vos et al., 1995). Primer pairs of *Eco*RI+3/*Mse*I+2 and *Ase*I+2/*Taq*I+2 were used for selective amplification. Selective amplification was conducted with one of the two primers labeled with IRD700 or IRD800 (LICOR, USA). PCR products were separated on 6% PAGE gel and visualized with a LICOR sequencer (LICOR, USA).

Excision and sequencing of interesting fragments

Interesting DE-TDFs were excised from PAGE gel using the Odyssey machine (LICOR, USA), and then re-amplified with *M13r_M00* (5'-GGATAACAATTTCACACAGGGAT-GAGTCCTGAG-AA) and *M13f_E00* (5'-TTTCCCAGTCACGACGTTGGACTGCGTACC-AATTC) or AseI00 and *Taq*I00 and purified over G50 columns (Amersham Bioscience, USA). The PCR products were sequenced directly (Greenomics, the Netherlands).

Sequence analyses, primer designing and RT-PCR

The BLAST results were obtained against TIGR (http://www.tigr.org/tdb/tgi/plant.shtml) tomato/ *Arabidopsis* TC databases using BLASTN and TBLASTX. Primers were designed based on the obtained DE-TDF sequences using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/). The primer sequences of actin were obtained from literature (Ditt et al., 2001). Semi-quantitative RT-PCR was conducted with the designed primers following the PCR program: 94 ℃ 1 minute (min); 94 ℃ 30 second (s), 60 ℃ 30 s and 72 ℃ 1min for 30 cycles; 72 ℃ 7 min. The PCR products were displayed on 1.2% agarose gels.

Results

Tomato plants grow optimally under natural light conditions in the glass houses, however

since the light condition is seasonally and experiments cannot be repeated under identical conditions, we decided to use the climate cell to carry out the inoculation experiments. Four experiments were accomplished to optimize the growth conditions for tomato plants and disease tests in climate cells (Wageningen University). The optimal conditions are described in the materials and methods section. Based on the microscopic observations of the infection process (Huang et al., 1998), macroscopic observation of the disease progress and protein analysis of intercellular fluid (data not shown), time-points for sample collection after spore-suspension and mock inoculation were chosen. For experiment one leaf material was collected from 0 till 72 hours post inoculation, for experiment two from 0 till 7 days after inoculation (dpi) for the resistant lines and from 0 till 14 dpi for the susceptible MoneyMaker.

Specificity, *in-silico* transcriptome coverage and TDF redundancy of cDNA-AFLP

Very little difference in cDNA-AFLP patterns was revealed between the two blocks of experiment one using eight primer combinations. Therefore, the samples from one block were used for full scale gene expression profiling and the samples of the other block were stored in the -80 ℃ freezer. Since constitutive TDFs of all samples showed uniform intensity by using ten random primer combinations, the samples collected at different timepoints can be pooled for efficient large-scale cDNA-AFLP screening without causing false differentials. For the pooling, pre-amplification products of all the time-points were bulked per genotype-treatment prior to selective amplification: hereafter referred to as bulk time-point analysis. In experiment one, 72 primer combinations were used to screen the bulks. Since only five weakly differential TDFs were found, it was decided to focus on experiment two, in which samples were collected at later time-points, to obtain DE-TDFs. In total, there are 256 possible primer combinations for *Ase*I+2/*Taq*I+2, and 1024 possible primer combinations for *Eco*RI+3/*Mse*I+2 (Table 1). In experiment two, totally 768 primer combinations (*Ase*I+2/*Taq*I+2 and *Eco*RI+3/*Mse*I+2) were used for bulk time-point analyses, and 331 primer combinations resulted in DE-TDFs (Table 1). On average, each primer combination revealed 40 clear bands, so that approximately 30,000 TDFs were surveyed. A computer program, RE-Predictor, was written to estimate transcriptome coverage (unpublished data). Tomato ESTs (average length of ESTs is 450 bp) downloaded from the NCBI database have been assembled into 15,098 contigs with a mean length of 900 bp. By using RE-Predictor and the tomato contig database, transcriptome coverage of *Mse*I/*EcoR*I and that of *Taq*I/*Ase*I in cDNA -AFLP were estimated, provided that the fragment size limit was set at 50 to 500 bp, which coincides with the fragment range in an actual cDNA-AFLP image. As a result, when digesting cDNA with *Ase*I and *Taq*I prior to selective amplification, 18% of the transcriptome is covered, while digestion with *Eco*RI and *Mse*I results in 23% coverage. When both enzyme combinations are used, the total coverage is 36% (Table 1), which is less than the sum of 23% and 18% because of overlap between the partial transcriptome maps. In the cDNA-AFLP screening described in this paper, not all selective primer combinations (768 out of 1280) were employed and the proportional coverage of the used primer combinations was 22% (Table 1). The *in-silico* TDF redundancies for *Ase*I+2/*Taq*I+2 and for *Eco*RI+3/*Mse*I+2, which refer to the number of AFLP fragments per contig estimated

by using RE-predictor with the tomato contig database, are 1.29 and 1.47 respectively, but the joint *in-silico* redundancy increases to 1.58 (Table 1), since both enzyme combinations have an overlapping coverage.

Table 1 Overview of cDNA-AFLP analysis in bulk time-point analysis, *in-silico* estimation of transcriptome coverage and predication of TDF redundancy using RE-predictor

	PC* number		percentage of PC giving DE-TDF	DE-TDF number displayed by used PCs	Transcriptome coverage of PCs		TDF redundancy$
	Total	Used			Total-PC#	Used-PC##	
*Ase*I+2/*Taq*I+2	256	128	31%	95	18%	9%	1.29
*Eco*RI+3/*Mse*I+2	1024	640	45%	792	23%	14%	1.47
Total	1280	768	43%	887	36%	22%	1.58

* PC: primer combination.

The coverage was estimated based on total number of PCs.

##The coverage was estimated based on the number of used PCs in cDNA-AFLP analysis.

$ Redundancy was estimated based on total PCs using RE-predictor, the redundancy of used PCs was supposed to be the same

Differentially expressed TDFs identified in bulk time-point analyses

Among the visualized TDFs, 887 up-regulated DE-TDFs were detected (Table 1) and no obviously down-regulated DE-TDFs were observed. The up-regulated DE-TDFs revealed in bulks showed several kinds of expression patterns (Figure 1). Generally, they can be divided into four classes. About 53% of the 887 DE-TDFs displayed induction only in the compatible interaction (class I), while being absent or constitutively expressed in incompatible interactions (Figure 1, panel A - C). The DE-TDFs of class II (42% of the identified DE-TDFs) were induced in inoculated pools of compatible S-MM and incompatible R-Ol-1 and R-QTL or induced in the inoculated pools of S-MM and one of the incompatible pools (R-Ol-1 or R-QTL) (Figure 1, panel D - F). Very few monogenic resistance-specific (~0.5%) or polygenic resistance-specific (~1.5%) DE-TDFs (class III) were detected (Figure 1, panel G-I). Class IV consisted of DE-TDFs (~3%) that were not induced by fungi as above three classes, but they may still be associated with resistance because of the differential expression pattern or level between the compatible pools (S-MM) and incompatible pools irrespective of the treatment (J-L in Figure 1).

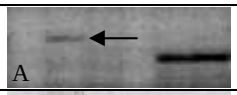
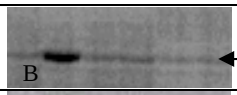
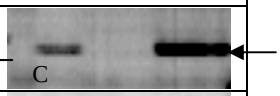
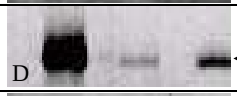
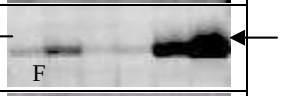
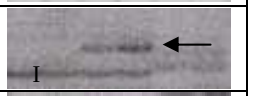
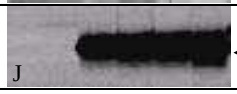
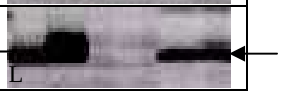
Time course and pattern of DE-TDFs identified in individual time-point analysis

For each time-point, leaf tissue was collected from one unique tomato plant to avoid that wound responses mask the pathogen-induced responses. The different plants can however, also be considered as biological repeats within each genotype. To exclude the DE-TDFs caused by developmental processes from the DE-TDFs caused by pathogen-induced responses, samples from mock-inoculated plants were always compared to leaf samples of inoculated plants in individual time-point analyses (Figure 2).

One hundred and ten primer combinations, which identified 248 DE-TDFs in the bulks, were chosen for individual time-point analyses to confirm the identity and display the

timing of DE-TDFs. In individual time-point analyses, samples of all time-points of both inoculated and mock-inoculated genotypes, which comprise 46 interactions (genotypes × treatments × time-points), were investigated. All the 248 DE-TDFs found in bulk time-point analyses were identified again in individual time point analyses.

Figure 1 The cDNA-AFLP image sections of twelve representative TDFs that showed difference between genotypes and/or treatments in bulk time-point analyses.

Expression pattern	1 2 3 4 5 6	1 2 3 4 5 6	1 2 3 4 5 6
Class I: Induced in inoculated S-MM plants	 A	 B	 C
Class II: Induced in both inoculated S- and R-plants	 D	 E	 F
Class III: Induced in inoculated R-plants	 G	 H	 I
Class IV: Differential between S-plants and R-plants	 J	 K	 L

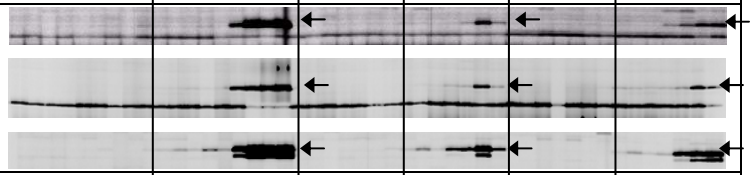
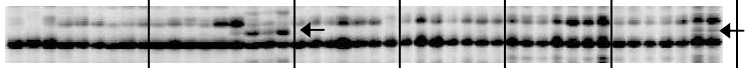
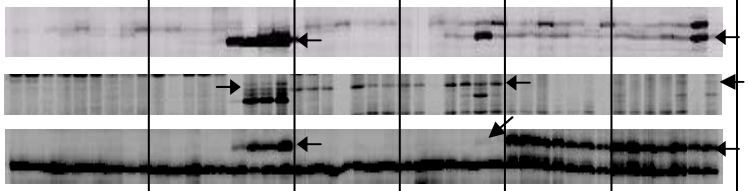
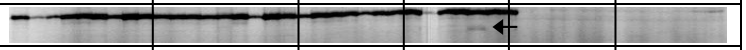
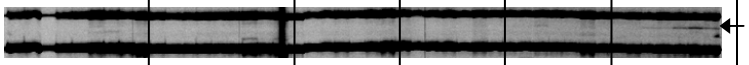
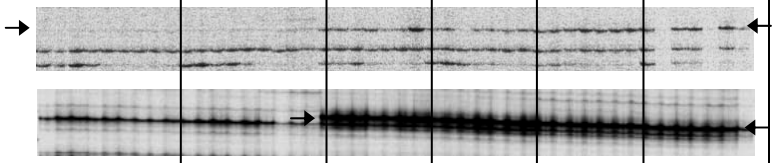
1 to 6 represent the pools of all the time-point samples: 1 mock-inoculated S-MM, 2 inoculated S-MM, 3 mock inoculated R-Ol-1, 4 inoculated R-Ol-1, 5 mock inoculated R-QTL and 6 inoculated R-QTL. Arrows point at different DE-TDFs including one allele or different alleles from different genotypes. Panel A to L represent different sections of cDNA-AFLP images displayed by different primer combinations or from the same primer combination.

In total, 129 DE-TDFs of class I in the bulks were only induced in inoculated S-MM at seven DPI or later in individual time-point analyses. The DE-TDFs of class II in the bulks were subclassified into classII-1, 2, 3 and 4 in individual time-point analyses. About 60% (53) of the 89 DE-TDFs of class II-1, class II-2 and class II-3, started expression earlier or had obviously higher expression level at the starting time-point in the monogenic R-Ol-1 and/or polygenic R-QTL compared to S-MM. The other 40% (25) of the 89 DE-TDFs displayed similar timing in S-MM, R-Ol-1 and/or polygenic R-QTL. In addition, all the DE-TDFs associated with R-Ol-1 showed an expression peak at 7 DPI in R-Ol-1. Twelve class-II-4 DE-TDFs are induced in inoculated S-MM and in the incompatible interaction R-Ol-1 or R-QTL but constitutively expressed in the other incompatible interaction.

DE-TDFs, which belong to class III in the bulks, were displayed as class III-1 and 2 in individual time-point analyses. Four Class III-1 DE-TDFs were only induced in inoculated R-Ol-1 plants and only two class-III-2 DE-TDFs were induced in inoculated R-QTL. Twelve DE-TDFs of class-IV in the bulks, which were not associated with inoculation but showed different expression levels or patterns between R-Ol-1 and R-QTL, were confirmed as Class IV DE-TDFs in individual time-point analyses.

The cDNA-AFLP fingerprints in individual time-point analyses showed that all the constitutively expressed TDFs have a very uniform intensity among different inoculated and mock-inoculated genotypes. A semi-quantitative RT-PCR of all the samples with actin-derived primer pairs further proved the uniformity of templates (Figure 3). The reliability of the DE-TDFs was proven by RT-PCR with primer pairs designed based on the sequence of a DE-TDF, which showed the same expression timing (Figure 3).

Figure 2 Different classes of the DE-TDFs confirmed in individual time-point analysis are classified based on the response specificity, which are illustrated by representative DE-TDFs in cDNA-AFLP image sections

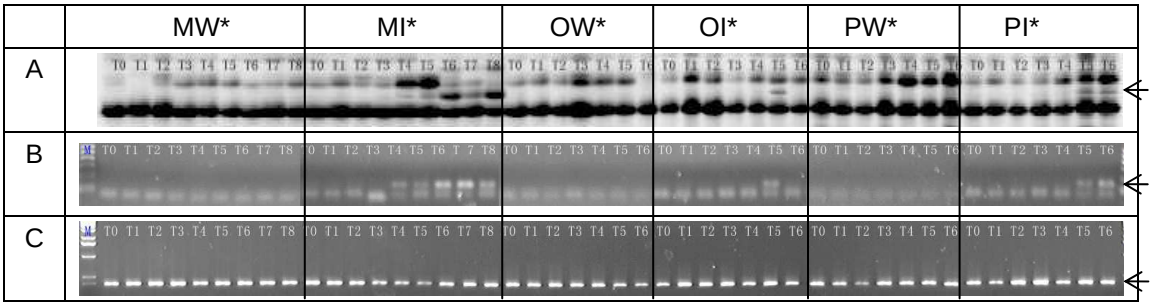
Class	**Expression pattern***	**Number of DE-TDF**	**Further description of expression pattern**	**Expressional timing of DE-TDFs in different genotypes/treatments**
Class I	MI	129	Only induced in inoculated S-MM	
Class II-1	MIOIPI	64 (38#)	Induced in inoculated S- and R-plants .In R-OI-1 there is always a high-level expression peak at 7 DPI&	
Class II-2	MIOI	8 (5#)	Induced in inoculated S-MM and R-OI-1. In R-OI-1 there is always an expression peak at 7 DPI	
Class II-3	MIPI	17 (9#)	Up-regulated in inoculated S-MM and R-QTL	
Class II-4	MI(OW)OI(PW)PI	12	Induced in inoculated S-MM, constitutively expressed and/or induced in R-OI-1 or R-QTL	
Class III-1	OI	4	Specific expression in inoculated R-OI-1	
Class III-2	PI	2	Specific expression in inoculated R-QTL	
Class IV	Constitutively differential	12	Constitutively expressed in S- and R-plants with higher expression level in R-OI-1 and/or R-QTL or only constitutively expression in R-plants	
Total		248		

* I: inoculated, W: mock inoculated; O: R-OI-1 and P: R-QTL.

Number in brackets refers to DE-TDFs giving earlier expression in R-OI-1 and R-QTL.

& Days post inoculation

Figure 3 Comparison of cDNA-AFLP image of a DE-TDF (A) and semi-quantitative RT-PCR with primer pair designed based on the sequence of the DE-TDF (B). Semi-quantitative RT-PCR of actin was used as a constitutive control (C).



* I: inoculated, W: mock-inoculated; O: R-OI-1 and P: R-QTL

Arrows point at the target DE-TDF (A), corresponding band in RT-PCR (B) and actin derived band in RT-PCR (C).

Sequence information

One hundred and seventy four DE-TDFs were successfully sequenced and annotated by Blasting against EST database of TIGR and NCBI (Table 3 and Appendix 1). Based on the possible origin of the transcripts (plant/pathogen) and the putative function of the transcripts, we divided them into nine groups (Table 2). About 40% (69) of the sequenced DE-TDFs had no match in the databases (Group I). One hundred and five of the 174 sequences had homologous information from the databases. Among the 105 sequenced DE-TDFs with hits in databases, 5 TDFs are likely from pathogen origin (Group G) because they have good hits in fungal EST databases and not in tomato EST databases; and 30 TDFs are homologous to sequences with unknown (Group H) or known functions not associated with defense before (Group F). Eighty of the 105 TDFs showed homology to plant ESTs with known functions and represented transcripts with a role in known defense, which refers to transcripts with known evidence that they are involved in defense (Group A), or with more general roles. For the latter class, we made a division into transcripts involved in signaling (Group B) and regulation (Group C) and into transcripts with housekeeping functions, like protein synthesis and degradation (Group D) and energy metabolism (Group E). We calculated about 32% (26) of the 80 function-informative transcripts, which were homologous to sequences with known function from plants, were directly involved in plant defense, while approximately 13% (10), 18% (14), 13% (10) and 13% (10) of these transcripts were associated with signal transduction, regulation, protein synthesis and degradation, energy metabolism including photosynthesis, photorespiration and respiration, respectively (Table 2).

By linking the blast results and functional classification to expression pattern and timing we predicted the general function of different sets of DE-TDFs (Table 3). We concluded that a large part of the set of function-informative DE-TDFs, which showed earlier timing in resistant genotypes compared to S-MM (Table 3, No. 1-7), have putative functions involved in known defense. While the set of function-informative DE-TDFs displaying the same temporal pattern between compatible and incompatible interactions (Table 3, No. 8-13) are more likely involved in housekeeping function and regulation. However, this conclusion needs more evidence to be testified. Signaling related DE-TDFs are observed in both the earlier-timing and same-timing sets (Table 3, No. 6, 11 & 26). In addition, DE-TDFs specific to the monogenic R-OI-1 (Table 3, No. 25-28) are indicated to

be associated with signaling or known defense. However, DE-TDFs specific to polygenic R-QTL (Table 3, No. 29-32) are more often related with known defense or basic function but not with signaling.

Table 2 Classification of 174 sequenced DE-TDFs based on BLAST results

	Blast results of DE-TDF sequences	**Group**	**Number**
Function-informati	Known defense responses (secondary metabolate synthesis, cell	A	26
ve (with functional	wall associated and oxidative burst, etc.)		
information from	Signal transduction (GTP-binding proteins, kinases, etc.)	B	10
plant EST	Regulation (transcription factors, heat shock proteins, etc.)	C	14
databases)	Ubiquination pathway and protein synthesis related	D	10
	Photosynthesis, photorespiration and respiration	E	10
	other*	F	10
Subtotal			80
No functional	Pathogen derived#	G	5
information from	Unknown**	H	20
plant EST	No hits$	I	69
databases			
Subtotal			94
Total			174

* Genes that encode proteins with functions not associated with defense before.

** Genes that encode proteins of unknown functions

Good-match found in fungal databases but not in plant databases

$ No homologous match in databases

Table 3 List of the DE-TDFs with homologies (e value<5e-02)

No.	**DE-TDF name$**	**Pattern*, Class** and Earlier timing?***			**Group#**	**e value**	**Homology annotation&**
1	M13E49-176	MIOIPI	II-1	+	A	8.1e-10	Snakin2 {*Solanum tuberosum*}, complete, --antimicrobial peptide.
2	M21E34-182	MIOIPI	II-1	+	A	6e-20	Malate dehydrogenase mRNA, complete cds; nuclear gene for mitochondrial product
3	A16T13-235	MIOIPI	II-1	+	A	2.1e-6	Ascorbate peroxidase
4	A13T13-400	MIOIPI	II-1	+	A	4.9e-17	Aspartic proteinase –related
5	A13T24-230	MIOWOIPI	II-4	+	A	1.4e-7	Tomato genome clone BH144711.1 homology to Apoptosis inhibitor {*Arabidopsis thaliana*}
6	M13E49-150	MIOIPI	II-1	+	B	2e-5	Putative GTP-binding protein {Oryza sativa (japonica cultivar-group)}
7	A13T24-226	MIOIPI	II-1	+	F	2.2e-05	Adenylosuccinate synthetase
8	M13E48-251	MIPI	II-3	-	B	3e-8	Putative GTP-binding protein
9	A16T13-262	MIOIPI	II-1	-	C	4e-17	Glucose-regulated protein 78
10	M14E47-332	MIOIPI	II-1	-	C	1.4e-20	J8-like protein {*Arabidopsis thaliana*}

No.	DE-TDF name[$]	Pattern*, Class** and Earlier timing?***			Group[#]	e value	Homology annotation[&]
11	M15E34-170	MIOIPI	II-1	-	D	1e-14	40S ribosomal protein S4. [Potato] {*Solanum tuberosum*}
12	M13E48-195	MIPI	II-3	-	D	4.2e-18	hexameric polyubiquitin {*Nicotiana sylvestris*}
13	A13A13-85	MIPI	II-3	-	D	5.1e-10	Yippee like protein
14	M20E58-200	MI	I	NA	A	1e-4	>gb|CA781179.1| 031E09AF Infected Arabidopsis Leaf Arabidopsis thaliana cDNA, mRNA sequence
15	M17E49-195	MIOIPI	II-1	NA	A	3e-14	P69C protein
16	M17E62-160	MIOIPI	II-1	NA	A	1.4e-2	S-adenosyl-l-homocysteine hydrolase
17	M22E47-430	MIOIPI	II-1	NA	A	3e-33	pyruvate dehydrogenase kinase {*Arabidopsis thaliana*}
18	M21E47-170	MIOIPI	II-1	NA	B	2.1e-6	Ras-related GTP-binding protein (RAN3) identical to atran3 [*Arabidopsis thaliana*] GI:2058280
19	M21E48-190	MIOIPI	II-1	NA	C	2.8e-9	RNA-binding protein {*Arabidopsis thaliana*}, partial
20	M12E62-180	MIOIPI	II-1	NA	E	9e-20	Putative heme A farnesyltransferase homolog {*Oryza sativa* (japonica cultivar-group)}
21	M16E58-205	MIOIPI	II-1	NA	D	1e-7	Chloroplast protease {*Capsicum annuum*}
22	M21E52-220	MIOIPI	II-1	NA	F	1.6e-2	Oxidoreductase short-chain dehydrogenase/reductase family-like protein {*Arabidopsis thaliana*}
23	M16E75-185	MIOIPI	II-1	NA	H	3.5e-5	Putative protein
24	M21E56-370	MIOIPI	II-1	NA	H	6e-19	Unknown protein {*Arabidopsis thaliana*}
25	M23E55-430	MIOWOI	II-4	+	A	1e-29	N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase THT1-3 [*Lycopersicon esculentum*]
26	A18T23-108	OI	III-1	+	A	4.9e-4	Cytochrome P450 family protein
27	M19E61-189	OWOI	III-1	+	D	6e-16	60S ribosomal protein L6 (YL16-like).
28	M14E72-209	OWOI	III-1	+	B	4.9e-2	GTP-binding protein Rab6 -common tobacco
29	M22E55-229	PI	III-2	+	A	7e-21	Putative senescence-associated protein {*Pisum sativum*}
30	M13E53-319	PI	III-2	+	A	7e-6	putative senescence-associated protein {*Pisum sativum*}
31	M14E47-310	PI	III-2	+	E	3.5e-16	Chlorophyll A-B binding protein 13 chloroplast precursor (LHCII type III CAB-13). [Tomato]
32	M11E69-190	PWPI	III-2	+	H	4.1e-27	hypothetical protein F22K20.8

[$] DE-TDFs were name with primer combination-fragment size

* Abbreviations in this column represent the expression pattern and size of DE-TDFs, MI, OI, OW, PI and PW have the same indication as those in Figure 2.

** Classes in this table have the same indication as those in Figure 1 and 2.

*** The "earlier timing" refer to whether the DE-TDF were earlier expression in resistant genotypes compared to S-MM; in this column, "+" represents that the DE-TDFs showed earlier timing in resistant genotypes or specific to resistance genotypes; "-" represents that same temporal pattern of the DE-TDF was displayed between resistant and susceptible genotypes. "NA" means that the corresponding DE-TDFs were only identified in bulk time-point analyses but no time-course data available.

The functional groups have same interpretation as those in Table 2

& Homologies are the BLASTN against no-redundant EST database in NCBI and tomato no-redundant EST in TIGR or TBLASTX against tomato no-redundant EST in TIGR.

Discussion

Tomato powdery mildew is a recently appeared fungal disease (Jones et al., 2000). Nothing is known of transcriptional responses during the interaction of tomato with *O. neolycopersici*. To elucidate the tomato defense responses during the interaction of tomato and *O. neolycopersici*, we carried out a comprehensive study of the fungal-inoculation-induced changes at the transcriptional level to identify co-regulated genes. cDNA-AFLP was used to detect genes induced in the susceptible interaction, monogenic- (associated with HR) and polygenic resistance (with yet unknown mechanism) responses. Compared with the DNA chips methodology, cDNA-AFLP is an unbiased method, which can be used to reveal altered expression of any gene that carries the suitable restriction site (Durrent et al., 2000). In addition, cDNA-AFLP has a very high reproducibility, which was confirmed using RNA gel blots (Bachem et al., 1996), we also confirmed the template uniformity with actin and the differential pattern for three sequenced TDF. Amplification of fragments from constitutively expressed genes provided internal control bands for every primer combination (Durrent et al., 2000). Our results showed the same phenomena, TDFs from constitutively expressed genes have uniform intensity and serve as internal controls for differentially expressed TDFs. We have sequenced one constitutive TDF homologous to a constitutively expressed gene ferredoxin NADP reductase, which is one component of photosynthesis complex.

Most responses occur late in the infection process

In experiment one, leaves were sampled at earlier time-points to detect genes involved in early responses of tomato against powdery mildew fungi. Using 72 primer combinations in bulk time-point analyses only five weak DE-TDFs were detected. This result may coincide with the fact that the resistance responses of both R-Ol-1 and R-QTL are post-haustorial, and haustorium formation occurs at 24- 41 hours post inoculation (Huang et al., 1998; Bai et al., 2005). This may also be explained by the fact that powdery mildew fungi interact solely with epidermal cells of tomato where the earlier expression of genes in attacked epidermal cells could be diluted by the uninfected mesophyll cells in the whole-leaf samples. The use of epidermal strips in future gene expression studies may increase the sensitivity to detect earlier interaction transcriptional events.

In experiment two, late time-points were added for sampling and a large-scale cDNA-AFLP screening was conducted to detect DE-TDFs. Almost all DE-TDFs induced in inoculated resistant genotypes were also induced in inoculated S-MM, showing that gene expression changes between compatible and incompatible interaction overlap to a great extent. However, ~60% of these DE-TDFs showed an earlier induction in resistant genotype(s) compared to S-MM (Figure 2). Apparently, the initiation of defense response in S-MM is too slow to stop the spread of *O. neolycopersici*. Similar results were obtained in gene expression studies in *Arabidopsis* (Maleck et al., 2001). The whole-leaf sampling strategy used in the cDNA-AFLP analysis of the present study may account for the difficulty to get a theoretical "absent" or "present" expression profiling between the compatible and incompatible interaction of tomato and *O. neolycopersici*. In barley, *B. graminis* attack induces indistinguishable expression profiles in both resistant and susceptible whole-leaf samples (Gregersen et al., 1997), while epidermal cells of leaves from susceptible and resistant genotypes show a mosaic of responses with respect to forming effective papillae or allowing pathogen penetration (Gjetting et al., 2004). Similarly, microscopic observations on the interaction between tomato and *O. neolycopersici* indicated that both S-MM and R-Ol-1 leaves display a mosaic of attacked epidermal cells that show resistant or susceptible reaction to the fungi. However, the proportions of "resistant" and "susceptible" cells are different between susceptible and resistant plants (data not shown), which may explain the phenomenon that earlier timing of gene expression in resistant genotypes compared to S-MM.

The differences in expressional timing of DE-TDFs between the compatible and incompatible interaction do not clarify which genes are specific to "resistant" or "susceptible" leaf cells. A single-cell analysis method has been established to generate transcript profiles from individual epidermal cells in barley and proven useful for analyzing papilla-resistant and successfully infected cells separately (Gjetting et al., 2004). The single-cell analysis method may be helpful to check the specificity of interesting DE-TDFs found in the whole-leaf interaction of tomato and *O. neolycopersici*.

Coverage and number of genes in tomato - *O. neolycopersici* interactions

By using the computer program-RE-predictor and the database in which average length of contigs is 900 bp, it was estimated that the *in-silico* redundancy of TDFs surveyed by *Mse*I/*Eco*RI and *Taq*I/*Ase*I is 1.58 (Table 1). In *Arabidopsis thaliana*, the average length of the randomly selected 5000 full-length cDNAs was 1080 bp (Maleck K et al., 2000). Since nearly 70% of the tomato unigenes have significant matches at the amino acid level to one or more genes of *Arabidopsis* (van der Hoeven et al., 2002), we assume that tomato has similar average cDNA length to *Arabidopsis*. Considering the average length difference between EST contig (900 bp) and speculated tomato cDNA, the *in-silico* redundancy of TDFs surveyed by *Mse*I/*Eco*RI and *Taq*I/*Ase*I can be corrected from 1.58 to 1.90 (1.58×1080/900). In the present study, 887 TDFs are differentially expressed (Table 1), of which 44% (390 TDFs) are associated with incompatible interactions of tomato and *O. neolycopersici*. Taking the redundancy (1.90 times) into account, about 210 non-redundant genes are likely represented by the 390 TDFs. These 210 genes resulted from cDNA-AFLP displayed by 768 *Mse*I+2/EcroRI+3 and *Taq*I+2/*Ase*I+2 primer

combinations, covering ~22% (60% of 36%, Table 1) of the transcriptome. Thus ~960 (210/22%) non-redundant genes are potentially involved in the resistance responses. EST contigs predicted that the tomato genome encodes ~35,000 genes (Van der Hoeven et al., 2002). Hence about 3% (960/35,000) of all the tomato transcripts are thought to be altered in abundance during the incompatible interaction of tomato and *O. neolycopersici.* This percentage of 3% is in the same order of magnitude as the percentages found in other studies: cDNA-AFLP analysis showed that approximately 1% of tobacco genes are differentially transcribed in Avr9-triggered defense responses in cultured *Cf9*-cells (Durrant et al., 2000); 1.5% of the total *A. thaliana* gene set is co-regulated with SAR and in response to infection of pathogens (Maleck et al., 2001); 2% of the total numbers of genes (35,000) were estimated to be differentially expressed in tomato leaves of RG-PtoR plants four hours after *Pseudomonas* infection in comparison to RG-ptoS/RG-prf3 plants (Mysore et al., 2002).

More genes induced in compatible interaction compared to the incompatible interactions

Interestingly, more TDFs were monitored to be induced in susceptible interaction compared to incompatible interactions of tomato and *O. neolycopersici*: 42% of the 887 DE-TDFs were induced in both interactions, 53% of the 887 DE-TDFs are only associated with susceptible interaction, while only 2% of the DE-TDFs are specific to resistance responses. From studies on the mechanism of *MLO* in barley, it is assumed that the powdery mildew fungus has evolved means to exploit host defense signaling to its own advantage (Panstruga, 2003). There is even evidence that powdery mildew fungi actively suppress host-cell death during compatible interaction, causing the “green island” effect’ (Schulze-Lefert and Vogel, 2000). The “green island” effect of a compatible interaction between barley and the powdery mildew fungus (a biotroph) illustrates massive pathogen-induced changes of cell death regulation resulting in cell death suppression in invaded cells and leaf senescence suppression (Hückelhoven et al., 2003). In this study, the tomato powdery mildew fungus used, is also a biotroph, not only combating plant defense, but also suppressing plant cell death, which may explain why more than half of the DE-TDFs involved in compatible interaction are only associated with the compatible interaction of tomato and *O. neolycopersici*. The genes specific to the susceptible interaction are induced late, about 98% DE-TDFs of Class I (only associated with inoculated S-MM), appeared at or after seven DPI (Figure 2), suggesting that they may play a role in susceptibility. The genes represented by the overlapped DE-TDFs between the compatible and incompatible interactions could be responsible for the basal defense in S-MM, which limit the pathogen infection to some extent. However, it cannot be excluded that some of the DE-TDFs are of fungal origin.

Expression peak in R-Ol-1 may coincide with formation of HR

The expression peak was detected at seven DPI in the R-Ol-1 for all the 64 Class II-1 and eight Class II-2 DE-TDFs. This may correspond to the timing and pattern of full HR in the R-Ol-1, as fungal growth starts to be arrested at seven DPI. It will be interesting to see, whether the expression peak will be earlier in inoculated *Ol4* lines, since in these lines cell

death at primary haustoria is very effective and there is generally no continued hyphae growth after 3 days post inoculation (Bai et al., 2005). In R-QTL, the 64 class-II-1 and 17 Class II-3 DE-TDFs showed a continuously up-regulated expression comparable to that in S-MM, except that about 55% of these DE-TDFs (Figure 2) showed earlier expression in inoculated R-QTL compared to inoculated S-MM. Although at the sampling time-points used we did not detect a induction peak for genes involved in polygenic resistance response, we still assume an expression peak exists at certain later time-point not included in the present study, from which fungal growth start to be arrested, coinciding with the previous result that less HR is associated with polygenic resistance response to *O. neolycopersici* in R-QTL compared to *Ol1*-mediated monogenic resistant response (Huang et al., 1998). Interestingly, it was monitored that most of the class-II-1 DE-TDFs showed higher expression levels in compatible interaction at nine DPI compared to in incompatible interactions and up-regulation at 11 and 14 DPI. These two time-points cannot be compared to the resistant genotypes, as these were not evaluated. However, the observation that the response in S-MM is slow but constantly increases at the late time-points may still be explained by the fact that more fungal haustoria were formed in S-MM compared to in resistant genotypes (unpublished data).

Expressional timing difference of the overlapping components between the response pathways of compatible and incompatible interaction is crucial

In this study, among the genes induced in both susceptible and resistant interactions, it appears that genes involved in known defense and signaling predominately have earlier timing in incompatible interactions. At the initiation of response to the fungal pathogen, the induction speed of these tomato genes could be crucial for the difference between compatible and incompatible interactions. Based on the expression pattern and Blast results, we conclude that most of the sequenced function-informative DE-TDFs, which showed earlier timing in incompatible interactions or were resistance specific, could be involved in known defense and signaling (Table 3). For the involved genes that play roles in housekeeping functions and regulation displayed the same temporal pattern among the susceptible and resistant responses (Table 3). Most likely, resistant responses are quicker or stronger than susceptible responses at the initiation stage, while the known defense and signaling related genes may be activated quicker in resistant interactions compared to the compatible interaction of tomato with *O. neolycopersici*.

In the interaction between *Arabidopsis* and the pathogenic oomycete *Peronospora parasitica*, many genes defined by earlier and transient increase in expression encode proteins that execute defense roles (Eulgem et al., 2003). This is similar to our results. However, in the interaction between *Arabidopsis* and *P. parasitica*, genes exhibiting a sustained or delayed expression induction often encode putative signaling proteins (Eulgem et al., 2003). This is different from the interaction of tomato with *O. neolycopersici* where signaling proteins are induced both earlier and same timing between compatible and incompatible interaction. During the interaction of *Arabidopsis* and bacterial pathogen *Pseudomonas syringae*, a large part of the difference between the incompatible and compatible interaction can be explained quantitatively (Tao et al., 2003). Based on the result of the *Arabidopsis* and bacterial pathogen *P. syringae*, a quantitative model was

proposed and further discussed to decipher the difference between *R*-gene mediated defense and basal defense in compatible interaction (Tao et al., 2003; Eulgem., 2005). This quantitative model is consistent with the tomato - *O. neolycopersici* system in this study, since we found that expression of genes involved in the compatible and incompatible interactions mainly differs in timing. However the question arises for the origin of the quantitative nature, whether the timing difference is derived from the quantitative difference between the basal defense in compatible interaction and the *R*-gene (or *R*-QTL) mediated response in incompatible interactions of tomato and *O. neolycopersici* or from the mosaic nature of “resistant” and “susceptible” cell existing in both resistant and susceptible plant? To answer this question, further evidence is needed.

Based on the several detected and sequenced specific DE-TDFs, we suggest that different specific defense components employed by the mongenic and polygenic resistances may influence the common defense pathways. Transcripts of two DE-TDFs specific to monogenic resistance code Cytochrome P450 family protein and GTP-binding protein (Table 3, No. 26 & 28), which play a role in secondary metabolism pathway and signaling, respectively. By the contrast, DE-TDFs specific in the polygenic resistance response are more likely related to the downstream components of defense pathway like senescence-associated protein or some proteins with housekeeping function (Table 3, No. 29, 30 & 31). Downstream components may not influence common defense pathway as effectively as those upstream components, which effect may be enlarged many times. This may explain why the polygenic resistance is more often partial compared to the monogenic resistance response.

In conclusion: In tomato - *O. neolycopersici* interaction, twice as many genes are induced in the compatible interaction as in the incompatible interactions. Genes involved in basal defense of the compatible interaction and *R*-gene mediated response of the incompatible interactions overlap to a great extent. The expression differences of these genes in basal defense of compatible interaction, monogenic and polygenic resistance responses are mainly in timing.

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Chapter 3

Transcript profiling of genes involved in powdery mildew induced defense responses in tomato mediated by papilla formation, fast or slow hypersensitive responses

(Manuscript in preparation)

Abstract Resistance in tomato lines containing the recessive resistance gene *ol-2*, the dominant *R* genes *Ol-1* or *Ol-4* is mediated by papilla formation, slow HR and fast HR respectively. Both the susceptible interaction and resistance responses to powdery mildew (*Oidium neolycopersici)* controlled by *ol-2, Ol-1* and *Ol-4* were investigated using cDNA-AFLP and reverse transcription PCR (RT-PCR). The transcript profiles of these responses are different. Generally, the transcript profiles of *Ol-1* mediated resistance and susceptible responses are similar. A large part of the common up-regulated genes in *Ol-1* mediated resistance and susceptible responses displayed no expression in the *Ol-4* mediated resistance response or later up regulation in *ol-2* mediated resistance response. The sequence information of some differentially expressed transcript derived fragments (DE-TDF) specific to *Ol-4* and *ol-2* mediated resistance responses further suggests that these responses are different from the *Ol-1* mediated resistance response. The RT-PCR analyses suggested that *ol-2* mediated resistance involving papilla formation is independent of SA, JA and ethylene pathways. An isofome of lipoxygenase (LOX) plays a role in resistance responses mediated by *Ol-4* (fast HR) and *ol-2* (papilla formation). *Ol-4* mediated resistance response is associated with the ethylene pathway but not with JA and SA pathways. *Ol-1* mediated resistance is likely associated with the SA and ethylene pathways.

Keywords: tomato (*Solanum lycopersicum*), powdery mildew (*O. neolycopersici)*, cDNA-AFLP, RT-PCR, Near Isogenic Lines (NILs), *Ol* genes, recessive, dominant, fast HR, slow HR, papilla formation.

Introduction

Biotrophic fungi need to be successful in all the infection stages to finish their life cycle, including spore deposition, spore germination and germ tube development, finding a stoma, stoma recognition and appressorium formation, stoma penetration/cell wall penetration, haustorium formation, colonization and sporulation (Niks and Rubiales, 2002). Tomato powdery mildew is a serious disease caused by a biotrophic fungus (*O. neolycopersici*), which infection stages include all the above described stages except for stomata recognition and penetration. It appears that tomato can arrest the biotrophic fungal growth at any of the infection stages. However, the tomato resistances to *O. neolycopersici* that have been studied are mainly associated with papilla formation (cell wall penetration) and the hypersensitive response (HR) (post-haustorium stages) (Bai et al., 2005). Six *R* genes and three major QTLs, which confer resistance to *O.*

neolycopersici in tomato, have been identified (Lindhout et al., 1994b; Ciccarese et al., 1998; Bai et al., 2003 and 2005). The monogenic dominant resistance genes *Ol-1* and *Ol-3,* introgressed from *S. habrochaites* G1.1560 and G1.1290 respectively, have been fine-mapped on the long arm of chromosome 6 (Lindhout et al., 1994 a&b; Huang et al., 2000 a&b; Bai et al., 2005). The resistance in *S. lycopersicum* var *cerasiforme* is contributed by a recessive gene *ol-2* that maps on chromosome 4 (Ciccarese et al., 1998 and 2000; De Giovanni et al., 2004). The *Ol-4* gene, which maps on the short arm of chromosome 6, originates from *S. peruvianum* LA2172 (Bai et al., 2004 and 2005). The *Ol-5* gene is derived from *S. habrochaites* PI247087 and maps on the long arm of chromosome 6 (Bai et al., 2004). *Ol-6* with unknown origin maps to the *Ol-4* locus (Bai et al., 2004). Three resistance QTLs were introgressed from *S. neorickii* G1.1601 and have been mapped on chromosomes 6 and 12 (Bai et al., 2003).

The resistance mediated by *ol-2* is associated with papilla formation and is expressed at the cell wall penetration stage, while the attacked host cells stay alive. *Ol-4*-based resistance activates a necrotic response in the attacked host cells upon penetration, so called fast HR or single-cell HR. *Ol-1*-based resistance is manifested at later stages; while most primary haustoria are formed in cells that remain alive, the secondary haustoria trigger programmed cell death in the attacked host cells. This resistance type is named slow HR or multiple-cell HR. Systemic acquired resistance (SAR) can be triggered by HR following the recognition of pathogen AVR proteins by host R proteins. SAR can also be triggered by the so-called basal defense responses in a compatible interaction, which is defined as the basal defense system limiting the extent of disease caused by virulent pathogen races during the compatible interaction (Maleck and Dietrich, 1999; Eulgem, 2005). A set of pathogenesis related (*PR*) genes accompany SAR. Some of these *PR* genes are specific to the well-defined defense pathways activated by SA, jasmonic acid (JA), or ethylene, and can be used as a measure to monitor each defense pathway (Smart et al., 2003). *PR-1* (encoding a protein of unknown function) is a creditable marker gene of SAR in *Arabidopsis* (Maleck and Dietrich, 1999; Maleck et al., 2001) and is responsive to the applications of JA, ethylene or SA in tomato (Van Kan et al., 1995), while *PR-2* (β - 1, 3 – glucanase, *GluB*) is induced by analogues of JA and ethylene in tomato (Van Kan et al., 1995). Tomato proteinase inhibitors I and II (*Pin1* and *Pin2*) were used to monitor the JA signaling pathway in tomato responses to aphid attacks (Martinez et al. 2003). The tomato homologue of coronatine insensitive 1 (*Coi1*) is required for the JA mediated defense pathway (Lashbrook et al., 1998; Li et al., 2004) and *ETR1,* the receptor gene of ethylene, is suitable to monitor the ethylene pathway. NIF1 is an interaction protein of NPR1, which mediates the expression of *PR-1* in the SA signaling pathway (Maleck and Dietrich, 1999; Eulgem, 2005). Besides these *PR* genes, there are some specific genes associated with HR, for example the tomato homologues of *HSR203* and *DAD1* act as active participant and suppressor of programmed cell death respectively (Hoeberichts et al., 2001). Bax inhibitor (BI1) acts as an anti-apoptotic factor in animal cells and is implicated in the regulation of cell death in plants (Panstruga, 2003). Oxidative degradation of membranes may be a causative factor in hypersensitive cell death, which may result from AOS (active oxygen species) or lipoxygenase (LOX, EC 1.13.11.12) activities (Jalloul et al., 2002). *LoxD* encodes a tomato chloroplast LOX gene that may act as a component of the octadecanoid defense signaling pathway (Heitz et al.,

1997). Therefore, the expression altitude of *LoxD* could be used to monitor oxidative degradation of membranes and octadecanoid defense signaling. HR is usually associated with resistance in the gene-for-gene interaction; however, the discovery of *DND* (defense no death) genes proves evidence that gene-for-gene interaction can be separated from HR (Glazebrook, 1999; Jurkowski *et al*., 2004). SGT1 is required for the disease resistance mediated by *R* genes in plants (Muskett and Parker, 2003) probably involving the ubiquitination pathway (Austin et al., 2002). Besides the progress made on these defense or HR-related genes, several interesting genes have been identified through analyzing induced or natural mutants in disease development, which may increase our understanding of compatibility and resistance. The genes *PMR5* and *PMR6* that are compatibility factors and *PMR4*, a negative regulator of SA pathway, were also monitored (Vogel et al., 2002; Nishimura et al., 2003; Vogel et al., 2004). *PEN1* (Homologous to *ROR2*) (Colins et al., 2003) and *MLO* (Büschges et al, 1997) enrich our knowledge on the mechanism of resistance mediated by papilla formation.

Monitoring the transcript changes of the above listed genes in resistance responses to *O. neolycopersici* mediated by papilla formation, fast HR, slow HR or basal defense in tomato will help to understand the underlying resistance mechanisms. Additionally, monitoring of the tomato-powdery mildew interaction using cDNA-AFLP, which screens a random set of genes and allows the discovery of new genes involved, will increase our knowledge of the resistance mechanisms employed in this pathosystem. In the present study, we investigated gene expression profiles by using cDNA-AFLP responding to *O. neolycopersici* of the resistant near isogenic tomato lines bearing different *Ol* genes (*Ol-1*, *ol-2* and *Ol-4*) in comparison to that of the susceptible tomato cultivar (Moneymaker). Additionally, semi-quantitative RT-PCR expression patterns of tomato genes or homologues, which represent marker genes of the different defense pathways (like JA, SA and ethylene pathway), central components in resistance signaling or compatibility establishment during the interaction of tomato and *O. neolycopersici,* are monitored.

Materials and methods

Plant materials

Four tomato genotypes were used in this study. *S. lycopersicum* cv. Moneymaker (MM) was used as a susceptible control (hereafter named S-MM) and as the recurrent parent in development of NILs. NIL-Ol-1 (BC_3S_2 of a breeding line carrying the *Ol-1* gene with S-MM as recurrent parent*)*, NIL-Ol-4 ((BC_3S_2 of a breeding line carrying the *Ol-4 gene* with S-MM as recurrent parent*)* and F3-ol-2, a F_3 line of *S. lycopersicum* cv. Marmande × *S. lycopersicum* var. *cerasiforme* carrying homozygously the recessive *ol-2* gene, are used as resistant lines (details are described in Bai *et al*., 2005). Resistances in these NILs are associated with slow HR (NIL-Ol-1), fast HR (NIL-Ol-4) and papilla formation (F3-ol-2), respectively.

Fungal material and inoculum preparation

O. neolycopersici was collected from infected tomato plants in the Netherlands (Lindhout et al., 1994a), and is continuously maintained on S-MM plants in growth chambers at

20±2℃, a relative humidity (RH) of 70% and 16 hour s day-length. Fresh spores were washed from the infected leaves with water to suspensions with concentrations of 2×10^4 spores/ml. Water was sprayed as mock inoculation.

Experimental set-up and sampling

All plants were grown in climate cells with optimal temperature, photoperiod and light conditions (20±2℃, 16 hours daytime, light intensi ty 150 μmol/m^2, s). The experimental design consisted of two randomized blocks with S-MM as borderlines and controls for spontaneous infection. Four-week-old plants were inoculated by spraying spore suspensions on whole plants. From each plant, leaves were only once harvested, so leaf samples of different time-points were harvested from different plants. For each time-point after inoculation, the second and third true leaves were collected and directly put into liquid N_2; the remaining plant was kept to evaluate symptom development. Samples were collected at 1, 3, 5, 7 and 9 dpi (days post inoculation) for all the genotypes and treatments.

cDNA-AFLP and sequencing of interesting transcript-derived fragments

The cDNA-AFLP protocol was as described in Chapter 2. Target DE-TDF were excised from PAGE gels using the Odyssey machine (LICOR, USA), re-amplified with standard AFLP primers *Mse*I00 and *Eco*RI00 and purified over G50 columns (Amersham Bioscience, USA). The PCR products were sequenced directly (Baseclear, the Netherlands).

Sequence analysis

The BLAST results were obtained against TIGR (http://www.tigr.org/tdb/tgi/plant.shtml) tomato/ *Arabidopsis* TC databases using BLASTN and TBLASTX. The sequenced DE-TDFs were mapped onto the tomato genome through blasting against the Solanaceae genomics network (SGN) unigene and marker databases (http://www.sgn.cornell.edu/). The DE-TDFs that were homologous to markers or unigenes with known locations on the tomato-Expen map series deposited in SGN were mapped to the corresponding loci.

Primer design and RT-PCR

The sequences of marker genes of different disease resistance pathways are obtained from websites (TIGR and NCBI databases). Primers (Table 1) were designed using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/). The primer sequences of actin were obtained from literature (Ditt et al., 2001). Semi-quantitative RT-PCR was carried out with the designed primers following the PCR program: 94 ℃ 1 minute (min); 94 ℃ 30 second (s), 60 ℃ 30 s and 72 ℃ 1min for 25 – 33 cycles; 72 ℃ 7 min. The PCR products were displayed on 1.2% agarose gels.

Table 1 The sequences of primer pairs used in RT-PCR analyses and TM value

Primer name	Forward primer	Reverse primer	TM Value (℃)
LeETR1	5'AACTGCTGTCATGCTTGTGC	5'GCAAAAGTGGGACCCTAACA	60
LeSGT1	5'GGAGTTCCTCTCCATCACCA	5'CCACCGACAGATTAGGCAAT	60

Primer name	Forward primer	Reverse primer	TM Value (℃)
NIF1	5'TGTCTTGTCAGGGATGTGGA	5'TGAAAGGGCATCTTCTGCTT	60
LeMLO5	5'TTCCCCACATTTGTCTATTTCC	5'TAGCACAAACCAAAGCCACA	60
LeROR	5'CTGGTTGTGGACCTGGAAGT	5' CTGAGCACCACCTCTGACAA	60
LeDND2	5'GTGACGATGACATGGACGAG	5'TGGTCTTGATGTAGTCGTGGA	60
Le PMR4	5'CTCCTTGCTCCCTTCCTCTT	5'ATCAGAATCACCAGGGTTGC	60
LeCOI1	5'TGATAATGGTGTGCGTGCTT	5'GCTGGATGCTCCGAGACTAC	60
LeBI1	5' GCAACCGCTGGAGTTATGAT	5' ATGGAACCACCAAAAATGGA	60
LePMR6	5'AAAATGGCCGAATTTACGTG	5'AACCGTCGTGGCAATTAGAC	60
LePMR5	5'TCACGGGTGACCCTCTATCT	5'CCGTAACACGTCTTCGTTGA	60
PR1	5'TCTTGTGAGGCCCAAAATTC	5'GGATATCAATCCGATCCCACT	60
GluB	5'TGGAATGATGGGGAACAACT	5'TGCACGTGTATCCCTCAAAA	60
Pin2	5'TGGCTGTTCACAAGGAAGTT	5'GCCTTGGGTTCATCACTCTC	60
LoxD	5'CCATCTATGGCCAGCAT	5'GTGACAACACGTTTGGATCG	60
LeDAD1	5'GTTCTGCTTATGCCGCAACT	5'CCGAAAGCCTAACAAAATCC	60
LeHSR203	5'GGCGGTGGTTTTTGTATCAG	5'AGGGGGTTTGTTCCTGTTCT	60

Results

Disease evaluation

Disease symptoms were evaluated and described by Bai et al (2005). Briefly, the susceptible genotype (S-MM) displayed heavy sporulation (DI=3). In NIL-Ol-1 leaves some white mycelium was detected at early time-points, but at later time-points, white mycelium disappeared and obvious yellow/brown lesions were observed on the inoculated leaves (Chapter 4, Figure 1). Resistance in F3-ol-2 and NIL-Ol-4 was absolute: both on F3-ol-2 and NIL-Ol-4 inoculated leaves, no symptoms like mycelium or lesions were visible.

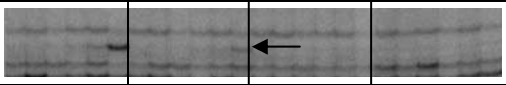
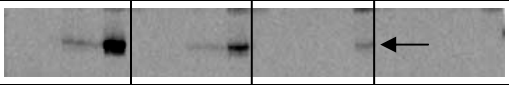
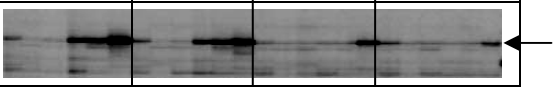
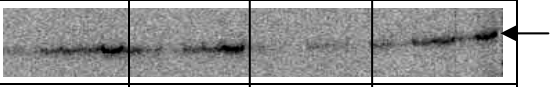
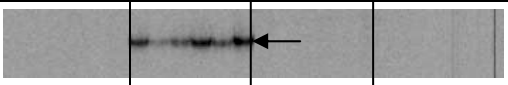
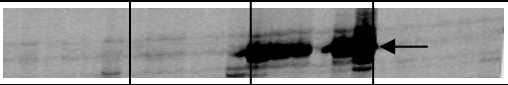
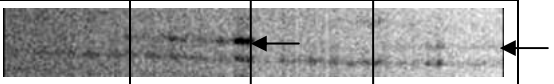
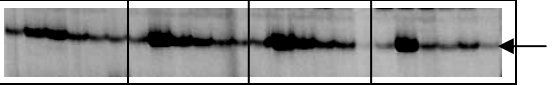
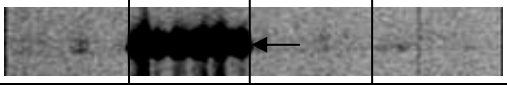
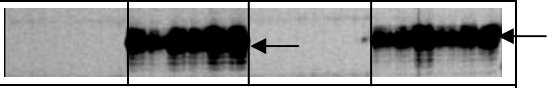
cDNA-AFLP analyses

The transcript profiles of MM, NIL-Ol-1, F3-ol-2 and NIL-Ol-4 were investigated using cDNA-AFLP. Sixty-nine primer combinations were selected that displayed DE-TDFs in a large scale screening of inoculated and mock-inoculated susceptible (S-MM), monogenic resistant (BC_1S_2-Ol-1 line) and polygenic resistant (*S. neorickii*) genotypes in the experiment of Chapter 2. In this Chapter, these selected 69 primer combinations displayed about 3000 bands; a total of 180 DE-TDFs were induced in the inoculated plants compared to mock-inoculated plants (Figure 1, class I - IV). Approximately 16% (29) of the induced DE-TDFs are only up-regulated in resistant genotypes (Figure 1, class III). About 17% (30) of the induced DE-TDFs show a transient expression patterns in MM, NIL-Ol-1, F3-ol-2 and NIL-Ol-4 (Figure 1, class IV). The remaining 67% (121) of the induced DE-TDFs were up regulated in both the susceptible and resistant genotype(s) (Figure 1, class I - II).

The 121 DE-TDFs that are up regulated in compatible and incompatible interaction(s) are divided into two classes (I and II) based on their expression timing. Class I DE-TDFs are later or not induced in NIL-Ol-4 and F3-ol-2 compared to S-MM and NIL-Ol-1. Of this

class, 46 DE-TDFs were only up regulated in S-MM and NIL-Ol-1 (Figure 1, class I-1); 47 were only up-regulated in S-MM, NIL-Ol-1 and F3-ol-2 (Figure 1, class I-2) and only 6 were up-regulated in all lines: S-MM, NIL-Ol-1, F3-ol-2 and NIL-Ol-4 (Figure 1, class I-3). It was remarkable that almost all (43 of 47) of the class I-2 DE-TDFs displayed a later expression timing in inoculated F3-ol-2 compared to inoculated S-MM, and that all six class I-3 DE-TDFs displayed a later expression timing in F3-ol-2 and NIL-Ol-4 compared to S-MM and NIL-Ol-1. In contrast to this, 12 of the 22 DE-TDFs of class II displayed an

Figure 1 Different expression classes of the DE-TDFs in the cDNA-AFLP analysis are presented: susceptible genotype (S-MM) and resistant genotypes: NIL-Ol-1, F3-ol-2 and NIL-Ol-4

Class	**Number of DE-TDF**	**Description of expression pattern**	S-MM	NIL-Ol-1	F3-ol-2	NIL-Ol-4
			# w inoc	w inoc	w inoc	w inoc
			* P 1 3 5 7 9	P 1 3 5 7 9	P 1 3 5 7 9	P 1 3 5 7 9
I-1	46	Up regulated in S-MM and NIL-Ol-1				
I-2	47	Up regulated in S-MM, NIL-Ol-1 and F3-ol-2, but most are later in F3-ol-2				
I-3	6	Up regulated in all genotypes, but later in F3- ol-2 and NIL-Ol-4				
II	22	Up regulated in all genotypes, with similar expression timing				
III-1	5	Up regulated only in NIL-Ol-1				
III-2	14	Up regulated only in F3-ol-2				
III-3	5	Up regulated only in NIL-Ol-4				
III-4	5	Up regulated in NIL-Ol-1 and NIL-Ol-4				
IV	30	Early and transient expression in all lines; generally higher in resistant lines				
V	9	Differentially expressed between genotypes but not between treatments				
VI	20	Polymorphic bands associated with resistant genotypes				
Total	209					

Refers to treatments, w - mock inoculation, inoc - inoculation; * P represents the pooled mock inoculated samples harvested at 1, 3, 5, 7 and 9 days post inoculation; 1, 3, 5, 7 and 9 refers to the number of days post inoculation of inoculated samples; Arrows point the target TDFs.

earlier expression pattern in F3-ol-2 and NIL-Ol-4 compared to S-MM and NIL-Ol-1, while the remaining 10 displayed similar timing in all four interactions. In summary, less than 25% (28/121) of the up-regulated DE-TDFs in both compatible and incompatible interactions (class I and II) are associated with NIL-Ol-4 (class I-3 and II); about 60% of

the up-regulated DE-TDFs are associated with F3-ol-2 (class I-2 & 3 and II), from which more than two third (class I-2 & 3) displayed a weak and later-timed expression in F3-ol-2 compared to S-MM and NIL-Ol-1. Of the DE-TDFs of classes I and II, fourteen, seven and eight DE-TDFs display earliest timing or highest level of expression in NIL-Ol-1, F3-ol-2 and NIL-Ol-4 respectively.

The DE-TDFs that display a transient expression pattern are grouped into class IV. Remarkably, all DE/TDFs in this class are induced in all four genotypes. In this class IV, 1, 20 and 4 DE/TDFs display highest expression level in NIL-Ol-1, F3-ol-2 and NIL-Ol-4 respectively.

Next to the DE-TDFs that are induced in inoculated plants compared to mock inoculated plants, 9 DE-TDFs display a differential expression level between genotypes (Figure 1, class V). Additionally, 20 bands (0.7%) of the total 3000 bands displayed were specific to one or two resistant genotypes, which may indicate that the corresponding genes map in the introgressed region, carrying the *R*-gene/*R*-QTL, or are regulated by the resistance loci. These class V and VI TDFs represent putative candidate genes for the resistance genes *Ol-1, ol-2* and *Ol-4.*

Sequence analyses

Target DE-TDFs of both classes I - IV (induced upon inoculation) and of class V and VI (associated with the *R* gene) were selected for sequencing. A total of 62 TDFs were successfully sequenced. BLAST results were obtained against TIGR (http://www.tigr.org/tdb/tgi/plant.shtml) tomato and *Arabidopsis* tentative consensus databases. On the basis of the BLAST results, the DE-TDFs were classified into several functional groups (the standard employed is the same as that in Chapter 2) (Table 2).

Table 2 Functional groups of 68 sequenced DE-TDFs based on the BLAST results against TIGR TC database

	Blast results of DE-TDF sequences	**Group**	**Number**
Function-informative[&]	Known defense responses (secondary metabolites synthesis, cell wall associated, oxidative burst etc.)	A	9
	Signal transduction (GTP-binding protein, kinases etc)	B	7
	Regulation (transcription factor, heat shock protein etc)	C	6
	Ubiquination pathway and protein synthesis related	D	6
	Photosynthesis, photorespiration and respiration	E	3
	Other*	F	0
No functional information from plant EST databases	Pathogen derived[#]	G	0
	Unknown**	H	8
	No (good) hits[$]	I	23
Total			62

& with functional information from plant EST databases; * Genes that encode proteins with functions not associated with defense before; ** Genes that encode proteins of unknown functions; # Good-match found in fungal databases but not in plant databases; $ No homologous match in databases or hit with e value>5.0e-2

Nine of the 39 TDFs had homology to plant ESTs that are directly involved in defense (group A). For the remaining DE-TDFs, a division was made into transcripts involved in

signaling (seven DE-TDFs in group B) and regulation (six in group C) and into transcripts with housekeeping functions, like protein synthesis and degradation (six in group D) and energy metabolism (three in group E). Twenty-three sequenced DE-TDFs had no/no good match (e value > 5e-2) in the databases (group I). Among the 39 sequenced DE-TDFs with hits in databases, none are likely to have a pathogen origin (group G), and 8 DE-TDFs are homologous to sequences with unknown function (group H).

In Table 3, the BLAST results, functional classification, expression patterns and timing, are presented of successfully sequenced DE-TDFs. The sequenced DE-TDFs that are induced in both susceptible and resistant genotypes and that show a similar time course in NIL-Ol-1 and the susceptible genotype (Class I) are involved in housekeeping, signaling, regulation and known defense (Table 3, No. 1-13). The sequenced DE-TDFs induced in both susceptible and resistant genotypes, that display earlier timing or a higher expression level in resistant genotypes compared to S-MM, are also involved in housekeeping, signaling, regulation and known defense (Table 3, No. 14-19). Transcripts of class-III DE-TDFs, which are specific to incompatible interaction(s), are mainly associated with regulation of transcription and translation (4 TDFs), but also with energy metabolism (2 TDFs), with the possible exception of TDFs that are homologous to genes with unknown function (Table 3, No. 20-30). Two transiently expressed DE-TDFs of class IV displaying highest expression levels in F3-ol-2 compared to the other genotypes are homologous to genes involved in the regulation of transcription (Table 3, No.31-32). The remaining two sequenced class-IV DE-TDFs, are associated with housekeeping functions (Table 3, No.33-34). In addition, several interesting class-VI DE-TDFs displaying constitutive expression patterns in resistant NILs were sequenced. These TDFs (Table 3, No. 35-38) were homologous to transcripts involved in signaling, the ubiquintination pathway and photosynthesis. In this set of DE-TDFs, there is some redundancy (Table 3, No 18 and 19).

Table 3 The putative functions or defense pathways involved of the sequenced DE-TDFs of different classes (Figure 1), based on the BLAST results against TIGR tomato/*Arabidopsis* TC database (only the DE-TDFs with BLAST e value < 5.0e-2 are listed).

No.	**$PC-size**	***Expression class**	****Earlier Timing?**	**e value/ Identity**	**#Group**	**Homology annotation&**
1	M12E58-290	I-1	-	8.9e-44/ 98%	A	>tomato|TC153678 similar to UP|CHIC_LYCES (Q05538) Basic 30 kDa endochitinase precursor (PR-2)
2	M12E62-186	I-1	-	2.8e-19/ 96%	A	>tomato|TC161002 weakly similar to UP|Q6PUG0 (Q6PUG0) 3-dehydroquinate dehydratase / shikimate dehydrogenase isoform 2
3	M18E41-260	I-1	-	5.3e-1/ 82%	A	>tomato|TC155487 weakly similar to UP|Q86GL5 (Q86GL5) Peroxiredoxin 3,

No.	$PC-size	*Expression class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
4	M23E55-430	I-2	-	1e-29	A	>TC116150 N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase THT1-3 [Lycopersicon esculentum]
5	M22E61-510	I-3	-	1.1e-34/ 95%	A	>tomato|TC162516 similar to UP|Q93WS1 (Q93WS1) Selenium binding protein
6	M13E49-150	I-1	-	2e-5	B	>TC116965 similar to GP|22093651|dbj|BAC06946. Putative GTP-binding protein {Oryza sativa (japonica cultivar-group)}, partial (92%)
7	M21E49-265	I-1	-	1.1e-3/ 62%	B	>tomato|TC163331 similar to GB|AAN72183.1|25084133|BT002172 protein kinase-like protein {Arabidopsis thaliana;}
8	M21E53-310	I-2	-	4.2e-4/ 61%	C	>tomato|TC162485 weakly similar to UP|NUCL_HUMAN (P19338) Nucleolin (Protein C23)
9	M23E55-196	I- 1	-	8.4e-15/ 94%	D	>tomato|TC153698 homologue to gb|AF036493.1|AF036493 Tragopogon dubius large subunit 26S ribosomal RNA gene, partial sequence, partial (80%)
10	M15E34-170	I-2	-	8.9e-10/ 82%	D	>arab|BU634848 similar to SP|O59950|RS4_ 40S ribosomal protein S4 (S7).[Candida lipolytica] {Yarrowia lipolytica}
11	M16E58-205	I-2	-	1e-7	D	>TC117131 homologue to GP|3808101|emb|CAA09935.1 chloroplast protease {Capsicum annuum}, complete
12	M21E56-370	I-1	-	6e-19	H	>BG123848 weakly similar to GP|22136960|gb unknown protein {Arabidopsis thaliana}, partial (17%)
13	M12E42-265	I-1	+/1>S	2.5e-18/ 89%	A	>tomato|TC162024 similar to UP|C762_SOLME (P37122) Cytochrome P450 76A2 CYPLXXVIA2) (P-450EG7)
14	M12E62-196	II	-	1.3e-17/ 93%	B	>tomato|TC162880 homologue to UP|O22402 (O22402) GDP dissociation inhibitor

No.	[$]PC-size	*Expression class	**Earlier Timing?	e value/ Identity	[#]Group	Homology annotation[&]
15	M15E71-220	II	+/1>S>2, 4	1.1e-27/ 95%	C	>tomato|TC154081 UP|Q43529 (Q43529) Homeobox, complete
16	M12E62-800	II	+/1, 4>S>2	4.4e-115/ 89%	D	>tomato|TC153558 UP|Q39257 (Q39257) Ubiquitin
17	M13E64-215	II	+/2>1, 4, S	1.5e-25/ 100%	C	>tomato|TC167295 similar to UP|ENO_LOLPE (O02654) Enolase (2-phosphoglycerate dehydratase)
18	M13E51-460	II	+/4>1>S>2	1.3e-71/ 99%	A	>tomato|TC162242 homologue to UP|GTX1_SOLTU (P32111) Probable glutathione S-transferase
19	M21E53-455	II	+/4>1>S>2	1.1e-64/ 99%	A	>tomato|TC162242 homologue to UP|GTX1_SOLTU (P32111) Probable glutathione S-transferase
20	M22E35-520	III-1	RS	1.3e-21/ 66%)	A	>arab|TC275227 UP|Q8H960 (Q8H960) Tobamovirus multiplication 2B
21	M11E69-195	III-1	RS	3.9e-20/ 80%	E	>tomato|TC116384 homologue to SP|P09114|ILV2_TOBAC Acetolactate synthase II chloroplast precursor (EC 4.1.3.18) (Acetohydroxy-acid synthaseII), partial (10%)
22	M15E71-245	III-2	RS	1.9e-4/ 69%	B	>tomato|TC155307 weakly similar to UP|ZOG_PHALU (Q9ZSK5) Zeatin O-glucosyltransferase (Trans-zeatin O-beta-D-glucosyltransferase)
23	M19E37-408	III-2	RS	1.1e-52/ 98%	B	>tomato|TC162602 similar to UP|Q9FXT0 (Q9FXT0) Elicitor responsible protein(TCIP), partial (43%) Length = 729
24	M12E62-620	III-2	RS	5.9e-55/ 95%	E	>tomato|TC153545 UP|CB2B_LYCES (P07370) Chlorophyll a-b binding protein 1B, chloroplast precursor (LHCII type I CAB-1B) (LHCP),
25	M16E75-175	III-2	RS	1.1e-06/ 72%	H	>tomato|BF050774,
26	M19E35-148	III-2	RS	5.4e-10/ 96%	H	>tomato|TC156146 similar to TIGR_Ath1|At1g15980.1 68414.m01917 expressed protein
27	M19E37-131	III-2	RS	5.4e-10/ 96%	H	>tomato|TC156146 similar to TIGR_Ath1|At1g15980.1 68414.m01917 expressed protein

No.	$PC-size	*Expression class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
28	M19E37-148	III-2	RS	5.4e-10/ 96%	H	>tomato|TC156146 similar to TIGR_Ath1|At1g15980.1 68414.m01917 expressed protein
29	M16E35-365	III-3	RS	7.9e-06/ 59%	B	>At1g14280.1 68414.m01693 phytochrome kinase,
30	M14E42-465	III-4	RS	1.4e-71/ 93%	C	>tomato|TC153824 UP|ENO_LYCES (P26300) Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), a bi-function transcription factor
31	M12E60-245	IV	+/2>1>4, S	2.2e-22/ 76%	C	>At5g08610.1 68418.m01024 DEAD box RNA helicase (RH26) translation initiation factor kinase.
32	M13E64-315	IV	+/2>1>4>S	6.0e-10/ 65%	C	>tomato|BE462175 transcription factor {Lycopersicon esculentum >PIR|T07398|T07398 myb-related transcription factor TH
33	M13E64-325	IV	-	2.1e-47/ 97%	D	>tomato|TC161777 homologue to UP|O82529 (O82529) Ribosomal protein L27a
34	M13E64-370	IV	+/1>4>2>S	7.4e-33/ 81%	E	>tomato|TC162533 weakly similar to TIGR_Ath1|At1g22400.1 68414.m02801 UDP-glucoronosyl/UDP-glucosyl transferase family protein contains Pfam profile: PF00201
35	M16E75-180	VI (*ol-2*)	NI	2.3e-18/ 99%	H	>tomato|AW737782,
36	M16E75-135	VI (*Ol-4*)	NI	5.9e-08/ 96%	B	>tomato|TC154636 weakly similar to TIGR_Ath1|At1g56720.1 68414.m06523 protein kinase family protein contains protein kinase domain, Pfam:PF00069
37	M20E37-306	VI (*Ol-4*)	NI	8.8e-43/ 94%	D	>tomato|TC156116 homologue to gb|AF223066.1|AF223066 Humulus lupulus 26S ribosomal RNA gene, partial sequence

No.	$PC-size	*Expression class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
38	M18E41-220	VI (*Ol-1* and *Ol-4*)	NI	2.8e-34/ 95%	H	>tomato|TC159169 similar to TIGR_Ath1|At1g73060.1 68414.m08448 expressed protein, partial (66%) *In-silico* mapping: TG231 (8.6 cm, near GP79 – a marker linked to *Ol-4*)
39	M13E64-170	VI (S-MM and *ol-2*)	NI	5.1e-05/ 70%	H	>dbj|BAB11508.1 unnamed protein product [Arabidopsis thaliana]

$ Primer combination and fragment size are listed

* Classes in this Table refer to expression classes presented in Figure 1, for class VI TDFs the NIL-specificity is listed.

** "Earlier timing" refers to earlier expression in resistant genotype(s) compared to S-MM; in this column, "+" means that the DE-TDFs showed earlier timing in resistant genotypes or are specific to resistance genotypes; "-" means that DE/TDF has same temporal pattern in resistant and susceptible genotypes. "NI" means that the TDF is not induced. "RS" means that the corresponding TDF is specific to resistance line(s). "S, 1, 2 and 4" represent S-MM, NIL-Ol-1, F3-ol-2 and NIL-Ol-4 respectively; and ">" means earlier timing or higher level of expression.

The functional groups are described in Table 2

& Homologies are the BLASTN results against TIGR tomato/*Arabidopsis* TC database.

Signaling/resistance pathway analysis using semi-quantitative RT-PCR

In order to clarify the mechanisms of the compatible interaction and resistance responses associated with slow HR, fast HR and papilla formation, RT-PCR was conducted to monitor the expression patterns of pathway dependent defense genes or tomato homologues of mutant genes affecting resistance or susceptibility in other pathosystems.

In Figure 2, it is shown that upon inoculation *GluB*, a downstream component of JA and ethylene pathways displayed a similar up-regulation in S-MM and NIL-Ol-1 with an expression peak at 7 dpi, a weak constitutive expression in F3-ol-2, and an expression peak in NIL-Ol-4 at 3 dpi, followed by up regulation till 9 dpi. Similarly, *PR1*, a downstream component of SA, JA or ethylene pathways was up regulated in S-MM and NIL-Ol-1, not detectably expressed in F3-ol-2 and transiently expressed in NIL-Ol-4 with peaks at 3 dpi and 9 dpi. The expression of *LoxD*, which is associated with peroxide production and a component of the octadecanoid defense signaling, was high in water pools of S-MM and NIL-Ol-1, but very weak in the inoculated plants of these two genotypes; in contrast to S-MM and NIL-Ol-1, a strong expression peak of *LoxD*, was observed at 3 dpi in both inoculated F3-ol-2 and NIL-Ol-4. *LeMlo5* and *lePMR5,* representing genes required for compatibility, were transiently expressed in all the four interactions. *LePMR6,* also a gene required for compatibility*,* was up regulated in all the four interactions at 9 days post inoculation, with an extra expression peak in NIL-Ol-4 at 3dpi. Upon inoculation, *LeHSR,* a marker for activation of programmed cell death, was up regulated in inoculated S-MM, NIL-Ol-1 and NIL-Ol-4 and constitutively expressed in F3-ol-2 interactions. *LeETR1,* encoding an ethylene receptor, was weakly up regulated in inoculated S-MM, NIL-Ol-1 and F3-ol-2, but displayed a different time course in NIL-Ol-4 with an expression peak at 3 dpi flowed by up-regulation till 9 dpi as for *GluB* and *PR-1*. The expression of *Pin2* a

marker gene for the JA pathway was slightly induced in all the four interactions at 9 days post inoculation.

Figure 2 The agarose-gel images displaying RT-PCR products amplified with primer pairs designed according to sequence of marker genes of different defense signaling pathways

Name	**Putative function, roles in plant-pathogen interaction or defense pathway**	**Accession/ TC No.$**	S-MM	NIL-Ol-1	F3-ol-2	NIL-Ol-4
			#w inoc	w inoc	w inoc	w inoc
			*P 1 3 5 7 9	P 1 3 5 7 9	P 1 3 5 7 9	P 1 3 5 7 9
GluB	Basic β-1,3-glucanase (PR2), response to JA and ethylene	M80608				
PR1	PR1, marker gene of SAR, response to SA, JA and ethylene	M69274				
LoxD	Lipoxygenase, lipid peroxidation and lipoxygenase pathway	U37840				
LePMR5	PMR gene, may code a compatibility factor	TC157427				
LeMLO	Membrane protein Mlo5, penetration required	BG137076				
LePMR6	Pectate lyase, may be a compatibility factor	TC157680				
LeHSR	HSR203J, a active participant of HR	AB022689				
LeETR1	Ethylene receptor 1, ethylene pathway	U47279.1				
PIN2	Proteinase inhibitors II, response to methyl-JA-based pathway	K03291				
LePMR4	Glycosyl transferase family 48 protein, callose synthesis	TC156118				
LeNIF1	NPR1-interactor protein 1, downstream of SAR	AF143442.1				
LeROR2	Syntaxin-related protein Nt-syr1, for penetration resistance	TC166265				
LeDND2	CNGC4, mutation of causes defense-no-death symptom	TC169687				
LeBI1	Bax inhibitor, required for penetration and compatibility	AY380778.1				
LeSGT1	Ubiquitin ligase-associated protein, ubiquitination pathway	TC162726				
LeCOI1	Coronatine-insensitive 1, component of JA pathway	AY423550.1				
LeDAD	DAD1, defending against apoptotic death, suppressor of HR	AJ250003				
Actin	Constitutively expressed gene	–				

[#] Refers to treatments, w - mock inoculation, inoc - inoculation; * P represents the pooled mock inoculated samples harvested at 1, 3, 5, 7 and 9 days post inoculation; 1, 3, 5, 7 and 9 refers to the number of days post inoculation of harvested inoculated samples.

No obvious expression changes were observed in all the four interactions for *lePMR4,* a negative regulator of SA pathway and *leBI1,* involved in the regulation of cell death in plants and *leROR2,* an essential component for *mlo* mediated disease resistance*, leCoi1,* a marker for the JA pathway, *leSGT1,* an important component in resistance signaling*, LeDAD1*, a marker for suppression of cell death*, leNIF1*, involved in the regulation of *PR1* expression in the SA pathway, and *leDND2,* an essential component of HR.

In summary, the expression patterns for S-MM and NIL-Ol-1 interactions were very similar for all genes investigated, with only minor differences in timing (Figure 2). Remarkably, *GluB, PR1* were not induced in F3-ol-2, and expressed differently between NIL-Ol-4 and S-MM and NIL-Ol-1. The expression of *LoxD* was very early induced in F3-ol-2 and NIL-Ol-4 interactions and suppressed in S-MM and NIL-Ol-1 interactions. *LeMlo*, *lePMR5* and *lePMR6* displayed obvious expression changes with similar patterns in all the four genotypes upon inoculation. The remaining genes displayed slight expression changes or no expression changes in all the four genotypes upon inoculation.

Discussion

Transcript profiles of resistance responses mediated by *ol-2, Ol-1* and *Ol-4* are different

cDNA-AFLP profiling clarified that three quarters of the differentially expressed genes up-regulated in the compatible interaction and in resistance responses mediated by *Ol-1* (involving slow HR) are not associated with the resistance response mediated by *Ol-4* (involving fast HR). Likewise, one third of these genes are not associated with the resistance responses mediated by *ol-2*. From the up-regulated DE-TDFs that are common between inoculated S-MM, NIL-Ol-1 and F3-ol-2 and/or NIL-Ol-4 (class I-2, I-3 and II), two thirds are later induced or weaker expressed in the *ol-2* mediated resistance (involving papilla formation) compared to the other interactions. These classes of DE-TDFs, common to both compatible and incompatible interactions, could constitute basal defense genes or genes for the establishment of compatibility that are expressed in compatible cells (cells that allow successful invasion by the powdery mildew fungus). Since in F3-ol-2 leaves, 20% of the attacked leaf cells appear compatible at the cellular level, expression of these genes is five times lower compared to the signal of the compatible S-MM interaction. In NIL-Ol-4, no cells were observed to interact compatibly with *O. neolycopersici* (Bai et al. 2005). If compatible cells express basal defense genes or genes for the establishment of compatibility, then it is not surprising that only 25% of the class I and II DE-TDFs were also expressed in NIL-Ol-4.

Class-III DE-TDFs are up regulated only in incompatible interactions. Also here, the different resistant interactions induce different genes. Fourteen of the twenty-nine genes are unique for the F3-ol-2 interaction; five each are specific for the NIL-OL-1 and NIL-OL-4 interaction, while five are shared between the NIL-Ol-1 and NIL-Ol-4 interactions. That

ol-2-based resistance responses employ different defense mechanism is expected, since the resistance mechanism is unique, papillae are formed and HR plays no role (Bai et al., 2005; Chapter 4). That the defense transcriptome of the NIL-Ol-4 interaction is so different from the defense transcriptome of NIL-Ol-1 was unexpected, since both interactions involve HR based resistance responses. One explanation for this outcome is that fast-HR mediated resistance employs a different defense mechanism compared to slow-HR mediated resistance; another explanation is that the amplitude of transcripts of inoculated *Ol-4* plants is below detection by cDNA-AFLP, owing to the fact that much less leaf cells interact with the pathogen in *Ol-4* plants compared to *Ol-1* plants (Chapter 4).

Sequence information of the DE-TDFs, which are specifically up regulated in *Ol-4* and *ol-2* mediated resistance responses, also implicates that different mechanisms are employed in these resistance responses from that in *Ol-1* mediated resistance. Certain F3-ol-2 specific DE-TDFs are homologous to genes involved in upstream signaling components, suggesting that specific pathways might be induced. For example, DE-TDF 23 (M17E37-408; table 3) is homologous to an elicitor responsible protein, and DE-TDF 29 (M16E35-365; table 3) is homologous to a phytochrome kinase. Unfortunately, another four *ol-2* interaction specific DE-TDFs are homologous to ESTs with unknown function. Sequence determination of the remaining DE-TDFs from this class may increase our understanding of the mechanism of *ol-2*-based resistance.

The DE-TDFs that displayed earlier timing or a higher expression level in *Ol-4*-based and *ol-2*-based resistance responses are mainly associated with genes involved in regulation of transcription, which may influence the speed or amplitude of defense pathways rather than the nature. DE-TDF (Table 3, No. 17) is homologous to enolase, a bi-functional transcription factor, and is much higher expressed in inoculated *ol-2* plants compared to the three other genotypes.

Glutathione S-transferase (GST) is an enzyme that regulates the redox state of glutathoine pools, GST transcription is induced during the oxidative burst and closely linked to H_2O_2 production; it can serve as an indirect measure of ROIs (Zeier et al., 2004). Very interestingly, two DE-TDFs (Table 3, No.18 & 19) homologous to GST are earlier expressed in inoculated NIL-Ol-4 compared to NIL-Ol-1, MM and F3-ol-2, which indicates that H_2O_2 accumulates earlier in *Ol-4* mediated resistance responses. The tendency of the expression timing of these two DE-TDFs is: NIL-Ol-4 > NIL-Ol-1 > S-MM > F3-ol-2 (> means earlier). *Ol-4* resistance involves a fast HR, while *Ol-1* resistance involves a multi-cell or slow HR, suggesting that H_2O_2 production is associated with HR, like in the barley response to *Bgh* (Hückelhoven et al., 1999). In *mlo*-mediated resistance against *Bgh* in barley, early H_2O_2 production (18 hours post inoculation) is associated with papilla formation (Hückelhoven et al., 1999). The correlation between H_2O_2 production and papilla formation in F2-ol-3 cannot be revealed in this study because the earliest time-point investigated is one day post inoculation, and that may be too late to detect H_2O_2 production associated with papilla formation (Hückelhoven et al., 1999). The role of the weakly and late expressed GST in *ol-2* mediated resistance needs further investigation. We will use DAB staining (Hückelhoven et al., 1999) of infected leaf samples to investigate timing and location of H_2O_2 production in the four interactions.

Four DE-TDFs of class IV that are transiently expressed in all four interactions, were

homologous to genes involved in transcription regulation, translation regulation and housekeeping (Table 3, 31-34). These genes most likely affect defense pathways quantitatively rather than qualitatively. Several sequenced TDFs of class VI (Table 3, No. 35-38) that are specific to certain resistance NIL(s) are homologous to transcripts with roles in signaling, the ubiquintination pathway and photosynthesis. These DE-TDFs are candidates for the corresponding *R* genes. Interestingly, one of these DE-TDFs (Table 3, No. 38) specific to NIL-Ol-4, mapped to the *Ol-4* region on the tomato EXPEN 2000 linkage map deposited in SGN through *in-silico* mapping. Further functional analysis will help to clarify whether it is a good candidate of *Ol-4.* DE-TDF M16E75-135 (Table 3, No. 36) is homologous to a protein kinase family protein, which makes it an interesting candidate for the *Ol-4* gene.

Resistance mediated by the recessive gene *ol-2* is independent of defense pathways mediated by SA, JA and ethylene

Microscopic observations indicate that during the tomato - *O. neolycopersici* interaction, *ol-2* mediated resistance is associated with papilla formation and suppression of spore germination, which is very different from the HR-associated resistance responses mediated by all other dominant *Ol-* genes (Bai et al., 2005; Chapter 4). The transcript profiles and RT-PCR analyses in this study suggest that *ol-2*-based resistance is independent of the known defense pathways mediated by ethylene, JA and SA. The genes *GluB* and *PR1* were not induced in *ol-2* mediated resistance response to *O. neolycopersici,* indicating that the resistance is independent of SA, JA and ethylene pathways. A similar observation was made in *pmr5* mutants (Vogel et al. 2004). Likewise the *mlo*-mediated resistance in barley to the powdery mildew fungus (*Blumeria graminis f.sp.hordei*, *Bgh*), which is also mediated by papilla formation, is not associated with the SA pathway (Hückelhoven et al, 1999). The expression pattern of *leETR1* in the compatible interaction (S-MM) and *ol-2*-based resistance is similar*,* which additionally indicates that *ol-2*-based resistance response does not depend on the ethylene pathway. Also *leCoi1* was not differentially expressed in any of the studied interactions, which does not suggest a role for JA in *ol-2* mediated resistance. The expression of DE-TDF M12E62-186 (Table 3, No. 2), which is homologous to shikimate dehydrogenase, is not induced in *ol-2*-based resistance. The fact that shikimate dehydrogenase is the enzyme responsible for the synthesis of shikimate, which is the general precursor of the SA biosynthesis pathways (Shah, 2003), strengthens the conclusion that *ol-2* mediated resistance is independent of SA.

The lipoxygenase pathway is involved in both *ol-2* based and *Ol-4* based resistance responses

Various lipoxygenase (LOX) isomers are known in plants (Agrawal et al., 2004). 13-LOX, which utilizes linolenic acid to synthesize 13-hydroperoxylinolenic acid, a precursor of jasmonic acid, forms a major branch of the LOX pathway which is involved in defense signaling. Another isoform of lipoxygenase, such as 9-LOX, is also involved in several defense responses by the synthesis of divenyl esters (Jalloul et al., 2002; Smart et al., 2003). *LoxD,* which expression is monitored in this study, encodes a tomato chloroplast

LOX that might be a component of the octadecanoid defense signaling pathway (Heitz et al., 1997). *LoxD* is suppressed in inoculated S-MM and NIL-Ol-1, while it is transiently expressed, reaching a peak at 3 dpi, in *ol-2*- and *Ol-4*-mediated resistance responses. We propose that an isoform of LOX other than 13-LOX is activated in *ol-2*-based resistance responses, because the JA pathway is not activated. We cannot exclude the possibility of the involvement of 13-LOX in *ol-2* and *Ol-4* mediated resistance responses, since 13-hydroperoxylinolenic acid is not only a precursor of JA via allene oxide synthase (AOS) but can also be converted into traumatin under the activity of hydroperoxide lyase (HPL) (Smart et al., 2003). Further investigation on the expression of the tomato *HPL* gene during the tomato and *O. neolycopersici* interaction will elucidate whether traumatin is involved in these resistances. It is also very interesting to testify whether the isoform is 9-LOX is induced in *Ol-4* and *ol-2* mediated resistance, since 9-LOX is associated with defense responses involving HR and partial resistance (Jalloul et al., 2002; Smart et al., 2003). *LoxD* was not expressed in inoculated S-MM and NIL-Ol-1 leaves, but was expressed in mock-inoculation pools. Since *LoxD* responds to wounding as well as pathogens (Heitz et al., 1997; Jalloul et al., 2002), we assume that some mock- inoculated samples were more exposed to wounding. Further biological replications will be needed to confirm this assumption.

***Ol-4* mediated resistance appears independent from JA and SA pathways**

Ol-4 mediated resistance, involving fast-HR, is probably associated with the ethylene pathway. *GluB*, which responds to JA and ethylene in tomato (Van Kan et al., 1995), was much earlier induced by *O. neolycopersici* in NIL-Ol-4 compared to both S-MM and NIL-Ol-1. In NIL-Ol-4 transient expressed *GluB* reaches a peak at 3 dpi, while the highest expression peak in S-MM and NIL-Ol-1 is at 5 dpi. *LeCoi1* expression suggests that this gene is not induced in both compatible and incompatible interactions studied. *LeCoi1* expression, the fact that the JA pathway is generally not directly involved in biotrophic interactions (Hammond-Kosack and Parker, 2003) and the fact that the compatible interaction of *O. neolycopersici* and tomato is independent of the JA pathway (Thaler et al, 2004), urges us to conclude that the JA pathway is not relevant in the tomato - *O. neolycopersici* interaction, and that the early induction of *GluB* is caused by ethylene.

PR1, which can be induced by JA, SA and ethylene (Van Kan et al., 1995), was also much earlier induced in *Ol-4*-based resistance response compared to the S-MM and NIL-Ol-1 responses. Since DE-TDF M12E62-186 (Table 3, No. 2) homologous to shikimate dehydrogenase is not induced in both the *Ol-4*-based- and the *ol-2*-based resistance response, we presume that *Ol-4*-based resistance is independent of the SA pathway. As mentioned above, *Ol-4* mediated resistance is independent of JA pathway; we therefore assume that ethylene induces *PR1* in inoculated NIL-Ol-4, even though the *leETR1* expression profile in NIL-Ol-4 is not clearly different from that in the other interactions.

SA and ethylene play a role in *Ol-1* mediated resistance.

Based on cDNA AFLP and RT-PCR data, we suggest that SA and ethylene pathways are commonly employed by basal defense of compatible interaction and slow-HR mediated

resistance response, because the homologue of shikimate dehydrogenase (indirect marker of SA synthesis), *leETR1, GluB* and *PR1* were up-regulated in compatible interaction and slow-HR mediated resistance response with a similar timing.

Future research

In the discussion above, we suggest that *ol-2, Ol-4* and *Ol-1* mediated resistance responses employ different pathways. Further conformation of this finding will be from resistance tests with tomato lines or mutants deficient in SA, JA and ethylene biosyntheses. NILs with the studied *R* genes will be crossed with *NahG* and *def1* tomato lines that cannot accumulate SA and JA respectively, to study the role of SA pathway in the respective resistances and testify the conclusion JA is no role in tomato and *O. neolycopersici* interaction. The tomato NILs will also be crossed with ethylene insensitive tomatoes (*Never-ripe*) to study the role of the ethylene pathway in resistance.

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Chapter 4

Tomato defense against powdery mildew: quantitative resistance is mainly mediated by the hypersensitive response

(Manuscript in preparation)

Abstract Tomato near or nearly isogenic lines (NIL) with dominant, recessive resistance genes, or different combinations of genes for quantitative resistance to *Oidium neolycopersici* were investigated macroscopically and microscopically upon *O. neolycopersici* infection. Resistant plants with the recessive *ol-2* gene showed complete resistance to the pathogen, mediated by papilla formation. Resistance tests with NILs containing different number of resistance QTLs (*R*-QTLs) showed that the number of *R*-QTLs is correlated with the level of resistance, indicating an additive effect of individual *R*-QTLs. Microscopic analyses suggest that micro HR (hypersensitive response) plays an important role in both the dominant *Ol-1* gene and *R*-QTL mediated resistance. The average incidence of micro-HR cells in NILs with all three *R*-QTLs was comparable to that in NIL containing *Ol-1* and much larger than the sum of incidences of NILs with *R*-QTL2 & 3 and NIL with *R*-QTL1, indicating that the effects of *R*-QTL1 and *R*-QTL2 & 3 are not only additive and suggesting an interaction between the *R*-QTLs. Different types of micro-HR cells are also associated with individual *R*-QTL(s) and pyramiding of individual *R*-QTL(s) in one genotype leads to novel types of micro-HR cells. Penetrated papillae, vesicle accumulation and structural changes in extra-haustorial matrix perform basal roles in the resistance mediated by *Ol-1* and *R*-QTL(s), but are not specific to individual *R*-QTL(s) or *Ol-1*.

Keywords: tomato (*Solanum lycopersicum*), *O. neolycopersici*, near/nearly isogenic lines, *R*-QTL, papilla formation, hypersensitive response (HR), vesicle, extra-haustorial matrix and histology.

Introduction

When attacked by pathogens, plants respond to the intruder with different defense mechanisms, while specialized plant pathogens attempt to exploit the host and circumvent these defense mechanisms. Many plant pathogenic fungi causing large damage in crop production are biotrophic parasites such as mildew, rust, and smut fungi (Voegele et al., 2001). Powdery mildew caused by *O. neolycopersici* is a fungal disease of greenhouse tomato affecting yield and quality. Five dominant *R* genes (*Ol-1, Ol-3, Ol-4, Ol-5* and *Ol-6*) and one major resistance QTL (*R*-QTL1) against *O. neolycopersici* have been mapped to tomato chromosome 6 and two other major *R*-QTLs (*R*-QTL2 & 3) have been mapped to chromosome 12 (Bai et al., 2003 & 2005). A recessive resistance gene *ol-2* has been mapped to chromosome 4 (Ciccarese et al., 1998 and 2000; De Giovanni et al., 2004). The mechanisms of *R*-gene-mediated resistance response are studied extensively

(Hückelhoven et al., 1999; Joosten and de Wit, 1999; Vanacker et al., 2000; Schulze-Lefert and Vogel, 2000; Rooney et al., 2005), but very little is known of the mechanisms underlying quantitative resistance governed by a number of *R*-QTLs and the interaction between the major *R*-QTLs that determine the resistance response.

Plant cells responding to fungal attack undergo large morphological alterations, along with rapid and extensive metabolic reprogramming (Schmelzer, 2002). Plants can inhibit the pathogen growth at almost all stages of the infection process (Niks and Rubiales, 2002). HR and papilla formation (Thordal-Christensen et al., 1997; Hückelhoven et al., 1999; Vanacker et al., 2000) represent mechanisms that are extensively studied because of the availability of useful model pathosystems. For example, in a well-investigated pathosystem, barley and *Blumeria graminis* f. sp. *hordei* (*Bgh*), dozens of resistance gene loci render the plant resistant against different *Bgh* isolates (Schulze-Lefert and Vogel, 2000). These genes govern fungal arrest at different stages of the interaction: at the penetration stage while the attacked cells stay alive (*mlo*); at the penetration stage in cells that subsequently undergo a single-cell HR (*Mlg*); or after fungal penetration by a subsequent multi-cell HR (*Mla12*) (Hückelhoven et al., 1999 and 2001). However, besides these clear mechanisms additional resistance mechanisms at different infection stages, such as spore germination, germ tube development and resistance during colonization and sporulation may also be relevant (Niks and Rubiales, 2002).

In the tomato – *O. neolycopersici* interaction, *ol-2* mediated resistance to *O. neolycopersici* is associated with papilla formation, which is similar to the *mlo* dependent resistance against *Bgh*. *Ol-4* mediated resistance to *O. neolycopersici*, phenotypically similar to *Mlg* mediated resistance against *Bgh*, triggers a single-cell HR upon fungal penetration. Additionally, *Ol-1* mediated resistance to the tomato powdery mildew fungus is associated with multi-cell HR, similar to *Mlal2* dependent resistance to the barley powdery mildew fungus (Bai et al., 2005; Hückelhoven et al., 1999; Chapter 4). The present study aims at studying the cytological resistance mechanisms associated with quantitative resistance to *O. neolycopersici* in tomato.

We developed a set of NILs containing one, two or three major resistance *R*-QTLs to *O. neolycopersici*. Six of these NILs (two for each *R*-QTL composition) were analyzed macroscopically and microscopically, and compared with two resistance controls: an F_3 line carrying the recessive *ol-2* gene (F3-ol-2) and a NIL carrying the dominant monogenic resistant gene *Ol-1* (NIL-Ol-1). By microscopic analyses of the infection processes we identified several resistance mechanisms, like the well-known HR and papilla formation, but also less common barriers including germination suppression, vesicle accumulation and structural changes of the extra-haustorial matrix. We also demonstrate that in the tomato - *O. neolycopersici* interaction, these response mechanisms are common in both the compatible and incompatible interactions but differentiate in timing.

Materials and methods

Plant materials

Nine tomato genotypes were used for the microscopic and macroscopic observations. *S. esculentum* cv. Moneymaker was used as a susceptible control (hereafter named S-MM)

and as the recurrent parent in the development of NILs. NIL-Ol-1 (BC_3S_2 of a breeding line carrying the *Ol-1* gene with S-MM as recurrent parent*)* and F3-ol-2, a F3 line of *S. lycopersicum* cv. Marmande × *S. lycopersicum* var. *cerasiforme* carrying homozygously the recessive *ol-2* gene (details are described in Bai *et al*., 2005) were used as monogenic dominant and recessive resistant controls, respectively. Six lines homozygous for three combinations of *R*-QTLs (two lines for each combination) were used (Table 1), which were generated by backcrossing *S. neorickii* (harboring three major *R*-QTLs) two times to S-MM. Presence of *R*-QTLs was monitored by flanking molecular markers and disease tests; the genetic background was monitored using AFLP markers. Flanking markers CT184 and Aps1 were used for the selection of *R*-QTL1 on chromosome 6 (NILs hereafter named N1Qa and N1Qb). Similarly, the lines N2Qa and N2Qb contain both *R*-QTL2 and 3 on chromosome 12, selected by using CT129, CT99 & TG111. All the mentioned markers were used to monitor the presence of all three *R*-QTLs in N3Qa and N3Qb. The BC_2 lines were selfed twice to create BC_2S_2 lines, homozygous for the introgressed *R*-QTLs.

Fungal material and inoculum preparation

O. neolycopersici was previously collected from infected tomato plants in the Netherlands (Lindhout et al., 1994a), and is continuously maintained on S-MM plants in growth chambers at 20±2℃, a relative humidity (RH) of 70% and 16 hours day-length. Fresh spores were washed from the infected leaves with water to suspensions with concentrations of 2×10^4 spores/ml or 3×10^5 spores/ml. The lower-concentration and higher-concentration suspensions were sprayed on four-week-old tomato plants for macroscopic evaluation and microscopic analyses, respectively. Water was sprayed as mock-inoculation for both analyses.

Experimental set-up and sampling

All the plants were grown in climate cells with optimal temperature, photoperiod and light conditions (20±2℃, 16 hours daytime, light intensi ty 150 µmol/m^2, s). Six plants from each genotype were grown in two replicates for macroscopic observation. In the experiments for microscopic observations, plants were grown in two blocks. In each replicate, three leaf segments (1×3 cm^2) were harvested from one plant per time-point of each genotype at 41, 65, and 89 hpi (hours post inoculation). Collected leaf segments were immediately fixed in acetic acid: ethanol (1:1), and stained with chloral hydrate/trypan blue as described by Huang et al. (1998). These leaf segments were used to prepare three microscopic slides, two of which were examined by two different observers.

Macro- and microscopic evaluation

Disease tests were performed to record the disease index (DI) with 0 = no sporulation; 1 = a few fungal spots surrounded by necrosis, but less than 5% foliar area affected; 2 = moderate number of fungal spots, intermediate sporulation, 5%-30% foliar area infected; 3 = abundant sporulation, more than 30% foliar area affected. Symptoms of inoculated plants with a similar infected foliar area as the plants with scores 1, 2 and 3 but with smaller fungal spots compared to S-MM, were recorded as 0.5, 1.5 and 2.5, respectively. Disease development of each plant was evaluated four times (12, 14, 16 and 19 days post

inoculation; dpi), and the average of the four scores was recorded as disease index of the plant. The disease index of each genotype was the average of six investigated plants of the same genotype. In the macroscopic observations of disease development, “macro HR” refers to visible necrotic lesions on infected tomato leaves.

In the microscopic observation, a conidial spore was defined as a germinated spore when it produced either a germ tube ≧ half the length of the spore (Figure 2) or a germ tube with a primary appressorium formed. A germinated spore that produced at least a primary appressorium was defined as an infection unit (Bai et al., 2005). Twenty infection units of each microscopic slide were observed to record the different structures and to quantify the structures under 1000-time magnification under an Axiophot microscope (ZEISS Germany) using Phase 3 condensers or differential interference contrast (DIC.5-1.4). All micrographs were taken with a 400°Asa film. For the microscopi c observations, “attacked cells” refer to epidermal cells with at least a fungal appressorium, while “micro-HR cells” refer to attacked cells showing typical phenomena, like plasmolysis (Figure 2-A), or the appearance of vesicles or particles in the cells (Figure 2-B).

Results

Macroscopic analysis

Disease symptoms

S-MM showed white fungal colonies on the upper side of the leaves and was always scored as 3 (Figure 1, Table 1). Neither sporulation nor yellow lesions were observed on F3-ol-2 leaves; all six plants were scored as 0. In NIL-Ol-1, a few fungal spots (weak sporulation) surrounded by necrosis were detected at 12 dpi and 14 dpi. Fungal spots disappeared and obvious lesions were observed at these sites after 16 dpi. The disease index of NIL-Ol-1 was 0.2 indicating that the resistance is not complete, which coincides with our previous observations (Huang et al., 2000; Lindhout et al., 1994b). In N3Qa and N3Qb, which contain three *R*-QTLs in the genetic background of S-MM, very few fungal spots were detected on two of the six investigated plants of each genotype at 12 dpi, but no sporulation was observed at 14, 16 and 19 dpi. Disease indices of these two genotypes were 0, thus N3Qa and N3Qb are even more resistant than NIL-Ol-1 (Table 1). N2Qa and N2Qb contain two *R*-QTLs (*R*-QTL2 and *R*-QTL3 on chromosome 12) in an S-MM genetic background and both lines are expected to have a similar resistance level. However, the disease index of N2Qb was much higher (1.7) than that of N2Qa (0.8) (Table 1). N1Qa and N1Qb containing *R*-QTL-1 (chromosome 6) showed similar disease indices (0.8 and 1.1; Figure 1, Table 1). The disease indices of the genotypes investigated in this study displayed the following ranking: F3-ol-2 < N3Qa = N3Qb< NIL-Ol-1 < N2Qa < N1Qa < N1Qb < N2Qb < S-MM.

Macroscopic determination of HR

To quantify the macroscopic HR (hereafter referred to as macro-HR), we calculated the macro-HR incidence as the proportion of leaves with visible necrotic lesions displayed of 24 evaluations (six plants x four time-points) of each genotype. In NIL-Ol-1, obvious and

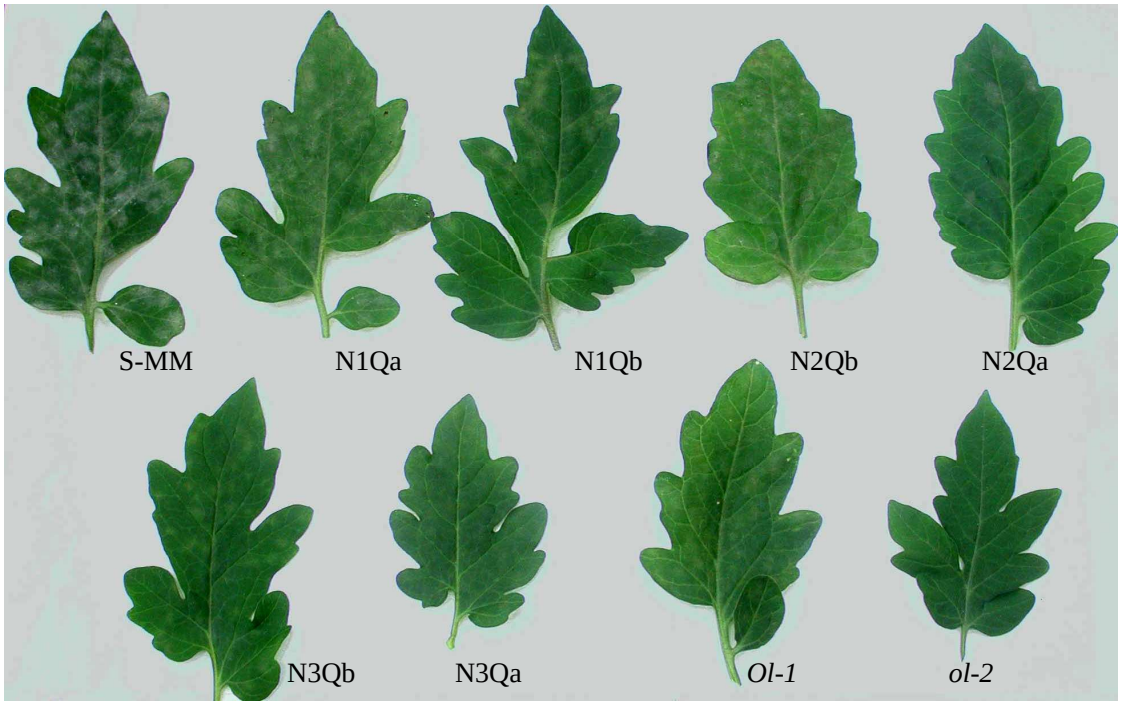

Figure 1 Powdery mildew development on the leaflets of susceptible genotype, F_3 line with a recessive *R* gene, near isogenc lines with a dominant *R* gene and near isogenc lines with different *R*-QTLs at 16 days post inoculation with *O. neolycopersici.*

S-MM refers to the susceptible genotype-Moneymaker; *Ol-1* and *ol-2* refer to NIL-Ol-1 and F3-ol-2 respectively; N1Qa & N1Qb, N2Qa & N2Qb and N3Qb & N3Qa are NILs containing one *R*-QTL, two *R*-QTLs and three *R*-QTLs, respectively.

Table 1 Disease indices, macro-HR incidences of S-MM and near isogenic lines with different quantitative and qualitative resistances inoculated with *O. neolycopersici*

Genotypes	Resistance genes/*R*-QTLs contained in the plants	Phenotype[#]	Disease index[&]	Macro-HR incidence (%) [$]
S-MM (susceptible)	None	S	3	0
NIL-Ol-1[*]	*Ol-1*	R	0.2	96
F3-ol-2	*ol-2*	R	0	0
N1Qa (NIL- 1 *R*-QTL)	*R*-QTL1	R	0.8	71
N1Qb (NIL- 1 *R*-QTL)	*R*-QTL1	IR	1.1	71
N2Qa (NIL- 2 *R*-QTL)	*R*-QTL2 and 3	R	0.8	8
N2Qb (NIL- 2 *R*-QTL)	*R*-QTL2 and 3	IR	1.7	8
N3Qa (NIL- 3 *R*-QTL)	*R*-QTL1, 2 and 3	R	0	29
N3Qb (NIL- 3 *R*-QTL)	*R*-QTL1, 2 and 3	R	0	79

* NIL: near isogenic line, the backgrounds of all the NILs is Moneymaker

* S: susceptible (disease index≥2); IR: intermediately resistant (1<disease index<2); R: resistant (disease index≤1).

[&] Disease index is the mean of the scores of 24 time-point × plant observations per genotype (four-time scores for each of the six investigated plants).

[$] Refers to the relative incidence (%) of macro-HR detected out of the maximum 24 individual time-point × plant observations.

typical HR was seen for almost all evaluations (23 out of the 24). Leaves of N3Qb with three *R*-QTLs displayed obvious lesions, similar to those of NIL-Ol-1. On the leaves of N3Qa macro-HR lesions appeared but were smaller than those on NIL-Ol-1 and N3Qb. Surprisingly, the macro-HR incidence of N3Qa was much lower than that of N3Qb. In N1Qa and N1Qb containing the chromosome 6 *R*-QTL, the lesions were larger than those on the leaves of N3Qb and NIL-Ol-1. The macro-HR incidence of N2Qa & b containing the chromosome 12 *R*-QTLs was low but the lesions had similar phenotypes as the lesions in N1Qa & b. We did not detect macro-HR on the leaves of S-MM and F3-ol-2. The ranking of the macro-HR incidence for all studied NILs was: S-MM = F3-ol-2 = 0 < N2Qa = N2Qb < N3Qa < N1Qa = N1Qb < N3Qb < NIL-Ol-1 (Table 1).

Microscopic analysis

Germination

Germination and growth of conidial spores on leaflets was not completely synchronous; non-germinated and germinated spores with different numbers of hyphae were detected in the same slide. Most of the germinated spores developed into an infection unit, while a small percentage of the germinated spores (<5%) in NILs N3Qa, N3Qb and F3-ol-2 solely formed a long germ tube without appressorium. The conidial spores on leaflets of F3-ol-2 had a lower germination percentage (around 20%) compared to that (35%) of all other lines.

Microscopic determination of Hypersensitive Response

Micro-HR cells, which refer to attacked cells showing typical phenomena, like plasmolysis (Figure 2-A), or the appearance of vesicles or particles in the cells (Figure 2-B), were hardly observed in leaflets of susceptible S-MM and resistant F3-ol-2. Different numbers of micro-HR cells induced per fungal infection unit were observed on the leaflets of plants of NIL-Ol-1 and the six QTL-NILs. The numbers of micro-HR cells per infection unit in the different lines were ranked as: N1Qa ≦ N1Qb < N2Qa ≦ N2Qb < NIL-Ol-1 ≦ N3Qa ≦ N3Qb (Figure 3-A). On average more than three micro-HR cells were detected per infection unit in leaflets of N3Qa & N3Qb and NIL-Ol-1 at 89 hours post inoculation, while the other QTL-NILs had less then one micro-HR cell per infection unit. The percentage of micro-HR cells from the total number of attacked cells gives a better idea of the role of micro-HR in resistance. The percentage of micro-HR cells of all the attacked cells in the different lines showed a similar ranking to the numbers of micro-HR cells per infection unit. These rankings do not agree with the ranking from the macro-HR. N3Qa has clearly a lower macro-HR incidence than N3Qb, while their micro HR incidence is similar. N2Qa&b have a lower macro HR incidence (8%) then N1Qa&b (71%), while the ranking if micro HR is inverse.

Micro HR can take place in cells with primary appressoria/haustoria, which results in arrest of fungal growth, leading to the so-called fast HR. Micro-HR can also take place in cells with secondary appressoria/haustoria, which result in multi-cell/slow-HR. More than 30% of the micro-HR cells in the leaflets of NILs with chromosome 12 QTLs (N2Qa and N2Qb) represented fast HR. In addition, more than half of the micro-HR cells in N3Qa and

N3Qb displayed vesicles along the cell walls (Figure 2-B); this kind of micro-HR cell is rare in N2Qa & b and N1Qa & b.

Papilla formation

During the interaction of tomato and *O. neolycopersici* papillae can arrest the fungal growth at penetration stage, preventing haustorium formation in the attacked epidermal cell. This kind of papillae is referred to as non-penetration papillae (Figure 2-C). In contrast to this, penetrated papillae (Figure 2-D) do not stop the fungal growth at the penetration stage allowing the formation of haustoria. The non-penetration papillae observed were larger than the penetrated papillae. Eighty percent of the appressoria in the leaf cells of F3-ol-2 were associated with non-penetration papillae, less than 10% of the attacked cells of all six QTL-NILs displayed non-penetration papillae, while non-penetration papillae were hardly detected in susceptible S-MM and NIL-Ol-1 (Figure 3-C). Unlike non-penetration papillae, penetrated papillae were observed in all genotypes investigated including S-MM and NILs with *ol-2, Ol-1* and *R*-QTLs. Highest incidences of penetrated papillae were observed in the attacked leaf cells of the six QTL-NILs at 65 hpi and in S-MM at 89hpi (Figure 3-D), suggesting earlier formation of those penetrated papillae in the QTL-NILs.

Vesicles in attacked cells

Under 1000 times magnification, vesicles (2-3 um) were observed in the attacked tomato leaf cells. Vesicles stayed separate from each other (Figure 2-E) or aggregated into irregular shapes (Figure 2-F). Very few microscopically visible vesicles were observed in attacked cells of S-MM and F3-ol-2 (<1% of attacked cells). In NIL-Ol-1 vesicles were only detected in micro-HR cells but not in other attacked cells. In all QTL-NILs, visible vesicles (2-3 µm) were observed in cells with abnormal haustoria regardless whether these cells expressed micro HR. Separate vesicles were observed in the infected leaf cells of all the six QTL-NILs, while aggregated vesicles were observed in more than 25% of the attacked cells of N3Qa & N3Qb and in about 10% of the attacked cells of N2Qa, N2Qb, N1Qa and N1Qb. Remarkable, in N3Qa and N3Qb the vesicles accumulated around the haustoria in more than 30% and 10% of the attacked cells, respectively (Figure 2-E). This was not manifested in the other QTL-NILs.

Timing difference in the development of abnormal haustoria

Generally, haustoria appear as round structures (Figure 2-G). Abnormal haustoria were however also observed in leaf cells of all resistant genotypes investigated and in S-MM 65 and 89 hpi. In these abnormal haustoria the extra-haustorial matrix (the structure existing in the space between the fungal cell wall and the plant membrane around the haustoria) was filled with small vesicles (1-2 µm) (Figure 2-F) or haustoria displayed plasmolysis (Figure 2-H). Although 30% of haustoria in S-MM were abnormal at 65 hours post inoculation, the percentage of abnormal haustoria was clearly lower than that in resistant NILs (60%-80%, Figure 3-E). However, at 89 hours post inoculation, similar percentages of abnormal haustoria were observed in both S-MM and all resistant NILs, suggesting that abnormal haustoria develop later in S-MM than in all resistant NILs.

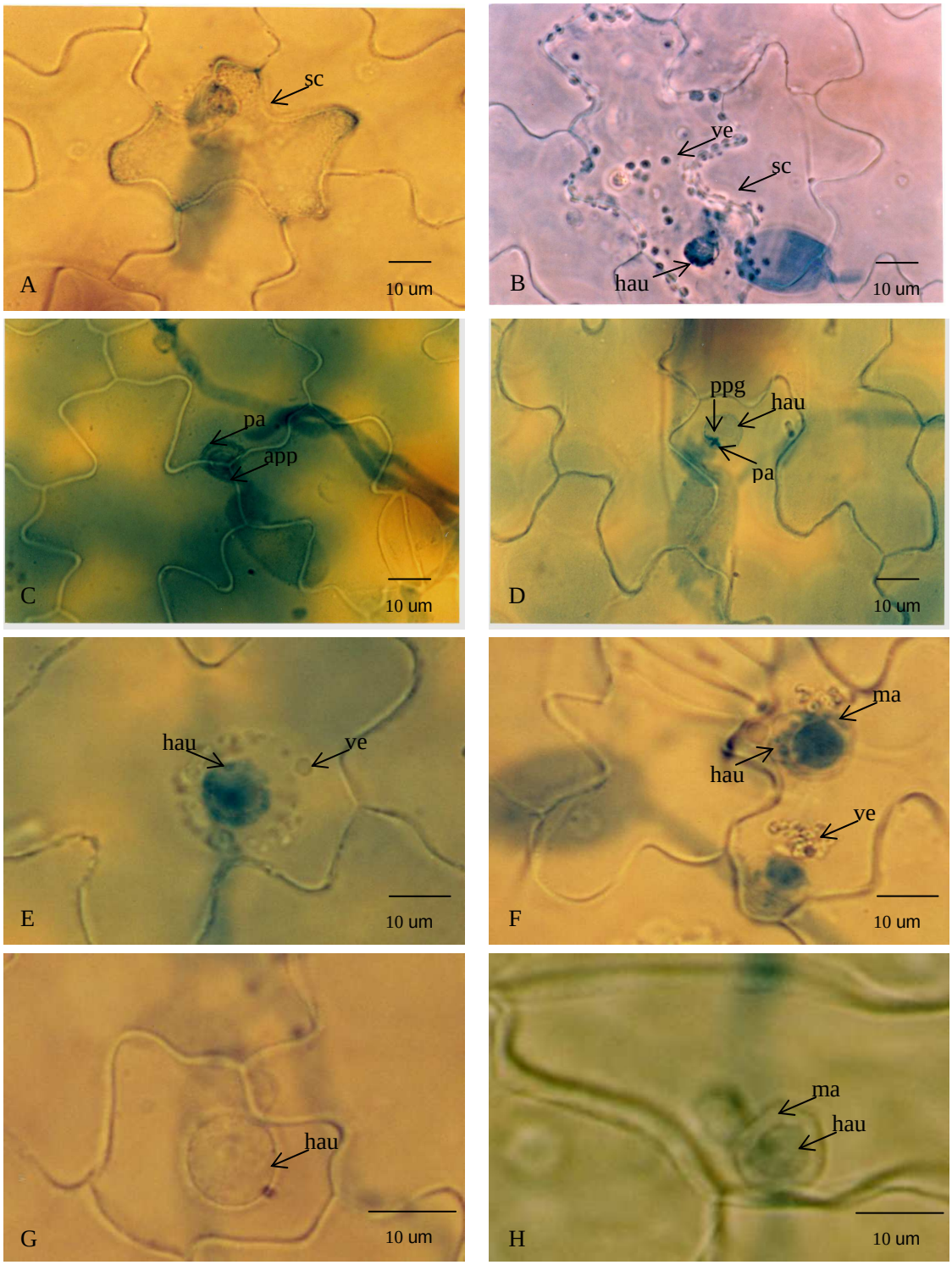

Figure 2 Micrographs (1000×magnification) of the interaction sites between tomato leaf cells and *O. neolycopersici* showing different structures

A: Micro-HR cells without microscopically visible vesicles; B: Micro-HR cells with microscopically visible vesicles were located along the cell wall; C: An infection unit including a germinated spore with a primary appressorium and a non-penetration papilla (no haustorium formed); D: An infection unit including a germinated spore with a primary appressorium and a penetrated papilla with haustorium; E: An attacked epidermal cell with a shriveled haustorium surrounding by microscopically visible vesicles. F: An epidermal cell with two abnormal haustoria with irregular vesicles

attached; G: A normal haustorium formed in the epidermal cell; H: An abnormal haustorium with a disappeared extra-haustorial matrix in the epidermal cell (H). sc: micro-HR cell, ve: vesicle, hau: haustorium, app: appressorium, hau: haustorium, pa: papilla, ppg: penetration peg, ma: extra-haustorial matrix.

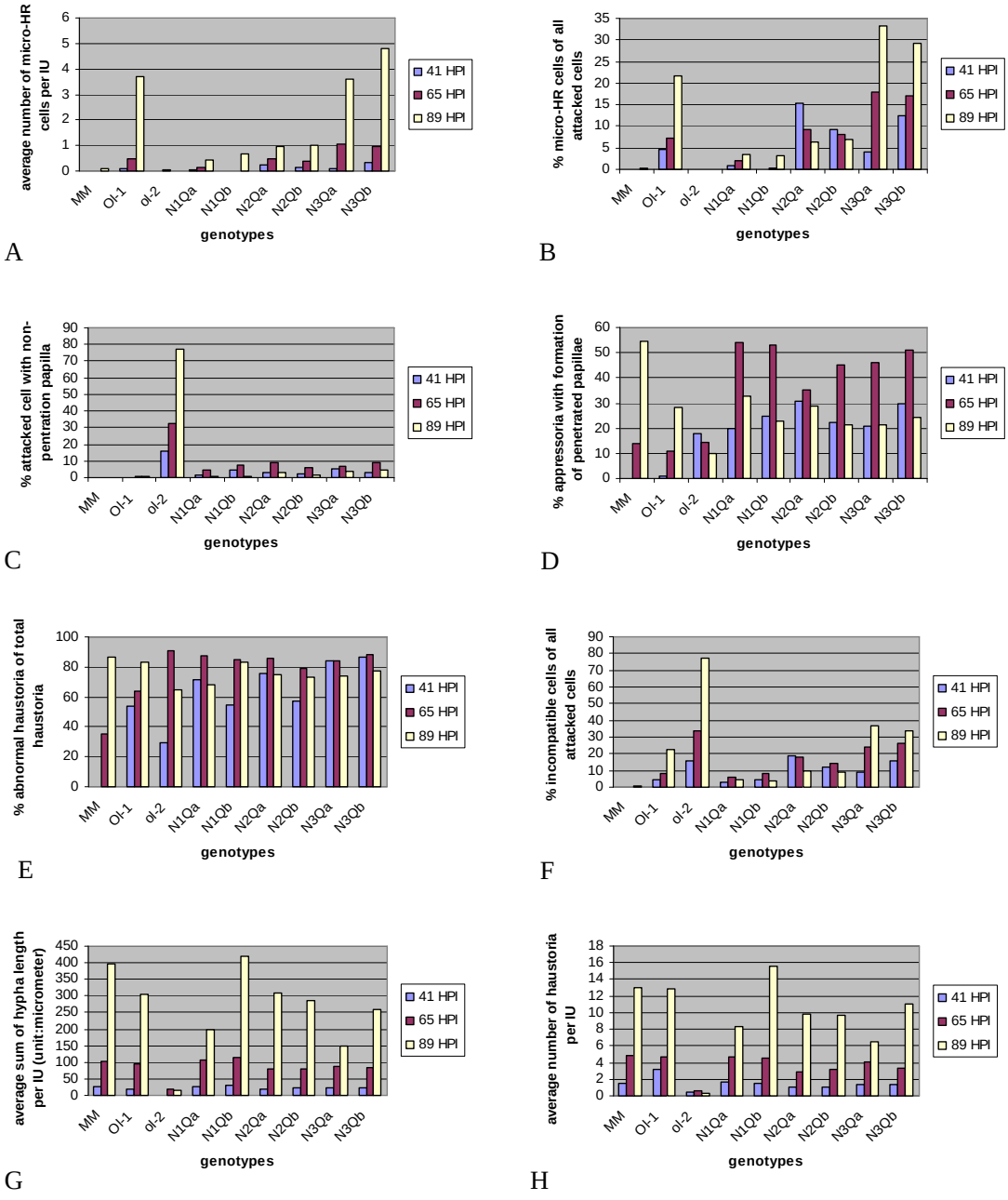

Figure 3 Histological quantification of responses of susceptible genotype (S-MM), F_3 line with a recessive *R* gene (ol-2), near isogenc lines with a dominant *R* gene (*Ol-1*) and near isogenc lines with different combinations of *R*-QTLs at 41, 65 and 89 hours post inoculation with *O. neolycopersici*.

A: The average number of micro-HR cells per infection unit; B: The incidences of micro-HR cells of all attacked cell; C: Percentage of appressoria with formation of non-penetrated papillae of all the formed appressoria; D: Percentage of appressoria with formation of penetrated papillae and haustoria of all the formed appressoria; E: Percentage of abnormal haustoria of all the formed haustoria; F: The incidence of resistant interaction site of total interaction sites; G The average of total hypha length per infection unit; H: Average haustorium number per infection unit.

Attacked epidermal cells form a mosaic of incompatible and compatible cells

Attacked cells with non-penetration papillae or displaying micro HR establish an incompatible interaction with the fungus and are referred to as “incompatible cells”, while the attacked cells with haustoria, but without these cellular responses, are designated “compatible cells”. The percentage of incompatible cells in F3-ol-2 was 20%, 30% and 80% at 41, 65 and 89 hpi, respectively (Figure 3-F). The average incidence of incompatible cells in the six NILs ranges from about 5% to 35% in NILs with increasing numbers of *R*-QTL s (Figure 3-F). In S-MM, less than 1% of the attacked cells displayed a resistant response. While that of NIL-Ol-1 was 20% at 89 hpi (Figure 3-F).

Infection units: hyphae length and number of haustoria

Numbers of haustoria and length of hyphae are measures of infection development. In F3-ol-2, total hyphae length was shorter and the number of formed haustoria was lower per infection unit than in other genotypes (Figure 3-G & H). Generally there is no clear correlation between total hyphae length and number of haustoria per infection unit with resistance level. Interestingly, haustorium numbers of S-MM and NIL-Ol-1 are similar, while average hyphae length of infection units on leaflets of S-MM was larger than that of NIL-Ol-1, indicating that the average hyphae length between neighboring haustoria is longer in MM than in NIL-Ol-1. The ratio between hyphae length and number of haustoria may indicate the success of the haustorium in the sink activity and correlated well with the disease index. The ratio was 30 for S-MM and, and smallest (23-25) for NIL-Ol-1 and N3Q.

Discussion

Based on the results, responses to *O. neolycopersici* in the investigated tomato lines were ranked (Table 2) and discussed in detail below.

Table 2 Summary of typical response to *O. neolycopersici* and the key structural changes of leaf cells in tomato susceptible genotype, F_3 line with recessive *R* gene, NILS with a dominant *R* gene or *R*-QTLs to the pathogen.

Genotypes	Macro HR	Micro HR	Non-penetration papilla	Penetrated papilla	Extra-haustorial matrix	Vesicles in cell	Vesicles around haustorium
S-MM	-	-	-	-	-	-	-
NIL-Ol-1	+++	+++	-	-	+	+	-
F3-ol-2	-	-	+++	++	-	-	-
N1Qa	++	+	+	++	++	++	-
N1Qb	++	+	+	++	++	++	-
N2Qa	+	++	+	++	++	++	-
N2Qb	+	++	+	++	++	++	-
N3Qa	+	+++	+	++	++	+++	+++
N3Qb	++	+++	+	++	++	+++	++

Note: -, +, ++ and +++ refer to no, weak, moderate, and strong correlation respectively between the structural changes and the responses to *O. neolycopersici*.

Resistance mediated by *R*-QTLs and *Ol-1* mainly depends on HR

As expected, the more *R*-QTLs in a NIL, the stronger the resistance, indicating an additive effect of individual *R*-QTLs. Microscopic analyses suggest that micro HR plays an important role in both *Ol-1* and *R*-QTL mediated resistance. The average incidence of micro-HR cells in N3Q with all three *R*-QTLs was much larger than the sum of incidences of N2Q and N1Q, indicating that the effects of *R*-QTL1 and *R*-QTL2 & 3 are not only additive and suggesting an interaction between the *R*-QTLs, which results in a dramatically increased resistance level when the three *R*-QTLs are pyramided in a single plant. Microscopic observations showed that in N3Qa & b micro-HR cells with vesicles are more frequent than in the other QTL-NILs, indicating that pyramiding of the individual *R*-QTLs also created a novel type of necrotic cells that was rare in plants with either *R*-QTL1 or *R*-QTL2 & 3.

HR can be classified into fast HR (single-cell-death HR) and slow HR (multi-cell-death HR) (Hückelhoven et al., 1999 and 2001). In the fast HR, the micro-HR of single cells will generally not develop into a macroscopic lesion. N3Qa with lower macro-HR incidence compared with N3Qb displayed a similar micro-HR incidence to N3Qb. Since the micro HR of N3Qa was not in cells with primary haustoria (fast HR), this cannot explain the lower incidence of macro HR lesions. In NIL-Ol-1 and QTL-NILs, the average number of micro-HR cells with or without visible vesicles shows a general tendency: N3Q > NIL-Ol-1 > N2Q > N1Q (Table 2), which coincides with the macroscopic resistance response (except the N2Qb). This suggests that increasing micro-HR cell numbers could be the pivotal element for the increased resistance when pyramiding *R*-QTLs in one single line.

***ol-2* mediated resistance occurs at germination and penetration stages, unlike *R*-QTL and *Ol-1* mediated resistance.**

The germination ratio of spores on F3-ol-2 was much lower than that on S-MM and NIL-Ol-1, which is in agreement with previous results (Bai et al, 2005). We further demonstrated that the germination ratio of spores on QTL-NILs is similar as that on S-MM and NIL-Ol-1, indicating that on the QTL-NILs spore germination is not suppressed. We therefore hypothesize that a signal from F3-ol-2 suppresses germination of the conidial spores that is absent in the other *R*-NILs. At cell wall penetration stage, the F3-ol-2 resistance is associated with a high incidence of non-penetration papillae (about 70%), decreasing the number of successful haustoria. Accordingly, in QTL-NILs and NIL-Ol-1 haustoria formation is not suppressed; these lines have similar haustoria/appressoria ratios compared to S-MM. Non-penetration papillae were formed in about 5% of the interaction sites in the QTL-NILs, but these non-penetration papillae are generally smaller than those in F3-ol-2. We are not sure whether the corresponding haustoria were not formed yet or the formation of them was suppressed by the smaller non-penetration papillae. Hyphae length – haustoria ratio in QTL-NIL is comparable to that ratio in NIL-Ol-1 without non-penetration papillae, we assume that haustorium formation is not suppressed.

Micro-HR cells were hardly found in investigated F3-ol-2 indicating that the resistance in the F3-ol-2 is mainly expressed at the germination and penetration stages and not associated with HR, thus so-called pre-haustorial resistance (Niks and Rubiales, 2002). In

contrast, NIL-Ol-1 and QTL-NILs resistance is expressed at post-haustorial stage (Niks and Rubiales, 2002), since generally non-penetration papillae and suppression of spore germination are not associated with these partial resistances.

Resistance mediated by *R*-QTLs is associated with penetrated papillae

In general, *R*-QTL(s) mediated resistances are associated with penetrated papillae and HR, in which penetrated papillae may play a basal role and HR makes the difference. In QTL-NILs, most of the formed papillae were penetrated with no significant difference between the six QTL-NILs. Penetrated papillae in QTL-NILs were formed about 24-hour earlier than in S-MM (Figure 3-D), which coincides with the timing difference in the formation of abnormal haustoria (Figure 3-E) in S-MM and in six QTL-NILs, implying that penetrated papillae may be one of the causes for the formation of abnormal fungal haustoria. This also suggests that formation of penetrated papilla and abnormal haustoria in susceptible plants is too late to suppress fungal growth, while the penetrated papillae and abnormal haustoria with earlier timing in the six QTL-NILs could contribute to the suppression of the fungal growth. The study of the *pen1-1* mutant of *Arabidopsis* could support this hypothesis. Upon non-host fungal attack of the barley powdery mildew fungi (*Bgh*), only a two-hour delay in papilla formation in the *pen1-1* mutant of *Arabidopsis* compared to wild type results in the increased penetration of papillae by *Bgh* in this mutant of the nonhost wild type *Arabidopsis* (Assaad et al., 2004). However, the fact that there is no timing difference in formation of penetrated papillae in the QTL-NILs suggests that the formation of penetrated papillae may only play a basal role in the resistance response mediated by *R*-QTLs mediated resistance responses with a similar mechanism.

Sister NILs suggest presence of additional genes required for resistance or *R*-QTLs

N2Qa and N2Qb, with the same *R*-QTL(s), have different levels of resistance (Table 1); N2Qb is incompletely resistant (DI of 1.7), while N2Qa is almost completely resistant (DI of 0.8). The genetic background of the NILs with the same *R*-QTL(s) is not identical, since they are derived from different BC_1 and BC_2 lines; the BC_2S_2 lines still carry on average 12.5% of the *S. neorickii* donor genome. The incomplete resistance of N2Qb can be caused by a lack of genes required for the resistant response. The presence of these genes required for resistance can be verified by genetic analyses. The differences in disease indices between the other pairs of QTL-NILs are very small, suggesting that no other genes required for resistance are lacking in those lines

Appearance of vesicles in attacked host cell and structural changes of extra-haustorial matrix may contribute to *R*-QTL(s) mediated resistance

In S-MM and the resistant F3-ol-2, no obvious vesicles were observed. Vesicle incidences in the six QTL-NILs did positively correlate to both the level of resistance to *O. neolycopersici* and the number of *R*-QTLs. In N3Qa & b, these vesicles were surrounding haustoria, indicating that in N3Qa & b genes regulate this cellular response. In the barley-powdery mildew interaction, vesicles caused by membrane fusion were associated with resistance. One constituent of the vesicles was H_2O_2 (Collins et al., 2003), which has antimicrobial-, cell wall cross-linking- and signaling functions and has a role in resistance

responses mediated by papilla formation and hypersensitive responses (Lamb and Dixon, 1997; Thordal-Christensen et al., 1997). Homotypic vesicle fusion giving rise to larger vesicles results in the fact that vesicles can be visible under the light microscope. PEN1 and ROR2 may play roles in this exocytosis fusion (Collins et al., 2003). Further experiments are needed to study whether the large vesicles in the tomato-powdery mildew system are associated with H_2O_2 (using DAB staining: Thordal-Christensen et al., 1997; Hückelhoven et al., 1999; Vanacker et al., 2000). This might clarify the function of vesicle accumulation in resistance responses. We are especially interested in the vesicle accumulation around haustoria in the attacked leaf cells of N3Qa and N3Qb.

Haustoria are considered as special branches of hyphae formed in the penetrated host cells. They are separated from the host cytoplasm by an extra-haustorial membrane and gel-like layers termed the extra-haustorial matrix located between the extra-haustorial membrane and the haustorial cell wall (reviewed by Panstruga, 2003). HXT1p, a hexose transporter, exclusively located in the haustoria, suggests that the haustorial complex serves as a new sink, enabling the pathogen to compete for nutrients with host cells (Voegele et al., 2001). These results indicate that an intact extra-haustorial matrix may be required for an efficient nutrient uptake. In the present study, two types of changes were observed in haustoria in *R*-NILs: plasmolysis of the haustoria and accumulation of visible vesicles in the extra-haustorial matrix. The earlier timing of the changes in extra-haustorial matrix in the *R*-NILs compared to S-MM implies the association of these changes with resistance. The gene product of *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidoposis*, might be located at the extra-haustorial matrix and result in pectin accumulation in the extra-haustorial matrix, subsequently deceasing nutrient accumulation (Vogel et al., 2002; reviewed by Panstruga, 2003). The tomato ortholog of *PMR6* was up regulated in S-MM, NIL-Ol-1, F3-ol2 and NIL-Ol-4 after inoculation with *O. neolycopersici* (Chapter 3), not indicating a clear correlation of this gene with the compatibility in tomato and *O. neolycopersici* interaction.

Mosaic of incompatible and compatible cells in tomato leave

It has been shown in this study that compatible and incompatible cells exist in both S-MM and all investigated resistant genotypes. A similar phenomenon was observed in the barley - Bgh pathosystem (Gjetting et al. 2004). The different proportion of compatible and incompatible cells may determine the fate of the tomato - *O. neolycopersici* interaction. Single-cell analyses at the transcriptional level, as conducted in the barley - *Bgh* system (Gjetting et al. 2004) will increase our knowledge of the tomato – *O. neolycopersici* system.

In conclusion: HR is involved in resistance responses of both NIL-Ol-1 and QTL-NILs and the incidence of necrotic cells coincides with the resistance level. *ol-2* mediated resistance occurs at germination and penetration stages, unlike *R*-QTL and *Ol-1* mediated resistance (Table 2). Penetrated papillae, vesicle accumulation and structural changes in extra-haustorial matrix perform basal roles in the resistance mediated by *R*-QTL(s), but are not specific to individual *R*-QTL(s). Pyramiding of individual *R*-QTLs in a single tomato line results in a high micro-HR incidence and a high level of resistance comparable to that in the NIL containing *Ol-1*.

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Chapter 5

Transcriptome investigations of powdery mildew challenged tomato lines carrying different combinations of resistance QTLs

(Manuscript in preparation)

Abstract We analyzed the pathogen-induced transcript profiles of tomato lines containing different combinations of resistance QTLs (*R*-QTLs) to *Oidium neolycopersici*, and compared those with the profiles of the susceptible genotype (Moneymaker, S-MM) and a near isogenic line (NIL) carrying the dominant *R* gene *Ol-1*, using cDNA-AFLP. About 4,000 bands were displayed with 78 selected primer combinations. In total, 204 differentially expressed – transcript derived fragments (DE-TDFs) were induced upon *O. neolycopersici* inoculation and 72 DE-TDFs displayed a differential expression level between NILs that was not dependent on inoculation. Transcripts that show similar timing in both compatible and incompatible interactions were associated with basal defense or establishment of compatibility, probably the result of the response of successfully attacked cells. Transcripts that display earlier/higher expression in the incompatible interactions compared to the compatible interaction showed a systemic induction as well. Salicylic acid (SA) and H_2O_2 might be important diffusive signals for both monogenic and polygenic resistance in tomato NILs. The small fraction of differentially expressed genes specific to (partially) resistant genotypes may fine-tune the activation of defense pathways in resistant genotypes through regulating transcription and translation. Pyramiding of *R*-QTLs into a single tomato line leads to a high-level resistance comparable to that mediated by *Ol-1,* and generally the same defense pathways are triggered by these combined *R*-QTLs compared to individual *R*-QTLs. We propose that pyramiding of *R*-QTLs only alters the defense pathways quantitatively rather than qualitatively. The map locations of 11 sequenced DE-TDFs were *in-silico* determined. One DE-TDF is a good candidate for *Ol-1* or *R*-QTL1 because of its specific expression to and co-localization with *Ol-1* and *R*-QTL1.

Keywords: Tomato (*Solanum lycopersicum*), powdery mildew (*O. neolycopersici*), *R*-QTLs, cDNA-AFLP, basal defense, monogenic resistance and polygenic resistance

Introduction

Plant diseases are of great importance to humans because they bring about losses of plants and plant products that humans are living on. The recorded attempt to search for disease control appeared in the mid 1600s, when it was reported that a species or variety was more resistant than another related species or variety (Fokunang et al., 2004). It is conceivable that even before the presence of written reports, plant growers, intentionally or unintentionally, selected the resistant plants to combat plant disease. Co-evolution between plants and pathogens is generally considered to contribute much to the diversity

on earth (Rausher, 2001), while it is also responsible for the fact that plants cannot display resistance to pathogens forever. Qualitative resistance sources, which are often mediated by monogenic resistance genes, have been widely used by plant breeders, because in general this resistance is genetically simple, qualitative and easy to be manipulated. Because of the same reasons, researchers have carried out numerous studies on this form of plant disease resistance. In the past decades, more than 50 resistance (*R*) genes have been isolated (Coaker et al., 2005), and classified into several groups based on the sequence similarities among these genes. However, except for several cases such as resistances mediated by *Lr34* (Kolmer, 1996; Navabi et al., 2005)*, mlo* (Büchges et al., 1997) *and Sr-2* (Hayden et al., 2004), qualitative resistances conferred by single genes are subject to co-evolution between plants and pathogens, and therefore easily overcome by pathogens.

By contrast, quantitative resistances, which are often controlled by more than one gene and influenced by the environment (Young, 1996; Pflieger et al., 2001), are less well studied and sparsely exploited (Ribeirro do vale, et al., 2001). Nevertheless, many plant pathologists believe that quantitative resistance is one of the most durable resistance sources, although there are a few exceptions (Ribeirro do vale, et al., 2001). Hence, a bigger research effort should be made into this largely enigmatic field. In fact, quantitative variation in experimental and natural populations has been a subject of study for a long time. However, knowledge of the molecular basis underlying these quantitative traits is lacking, because the key factors that regulate the variation have hardly been identified (Paran and Zamir, 2003). Recently, several quantitative trait loci (QTLs) in *Arabidopsis*, rice, maize and tomato have been isolated using positional cloning or transposon tagging (Morgante and Salamini, 2003; Paran and Zamir, 2003). It was shown that variation in these quantitative traits is caused by allelic differences in coding or regulatory regions of single genes (Morgante and Salamini, 2003; Paran and Zamir, 2003). To our knowledge, no *R*- QTL has yet been isolated, while the cloning of *R*-QTLs is no doubt of great importance for our understanding of the function and the future engineering of durable resistance in plants. In addition, comprehensive gene expression studies on qualitative resistance are also helpful to understand *R*-QTL mediated defense pathways.

Tomato powdery mildew, caused by *O. neolycopersici*, is a worldwide fungal disease seriously damaging tomato production, especially in the greenhouse. So far, six *Ol* genes and three major *R*-QTLs, which confer resistance to *O. neolycopersici* in tomato, have been identified and mapped (Lindhout et al., 1994; Ciccarese et al., 1998; Bai et al., 2003, 2004 and 2005). The monogenic dominant resistance genes *Ol-1* and *Ol-3,* introgressed from *Solanum habrochaites* (formerly *Lycopersicon hirsutum*) G1.1560 and G1.1290 respectively, have been fine-mapped on the long arm of chromosome 6 (Lindhout et al., 1994 a&b; Huang et al., 2000 a&b; Bai et al., 2005). The resistance in *S. lycopersicum* var *cerasiforme* (formerly *L. esculentum* var *cerasiforme*) is mediated by a recessive gene *ol-2* that maps on chromosome 4 (Ciccarese et al., 1998 and 2000; De Giovanni et al., 2004). The *Ol-4* gene, which maps on the short arm of chromosome 6, originates from *S. peruvianum* LA2172 (Bai et al., 2004 and 2005). *Ol-5* is derived from *S. habrochaites* PI247087 and located on the long arm of chromosome 6 (Bai et al., 2004). *Ol-6* from unknown origin maps closely to *Ol-4* (Bai et al., 2004). Three *R*-QTLs have been

introgressed from *S. neorickii* (formerly *L. parviflorum*) G1.1601 and mapped on chromosomes 6 (*R*-QTL1) and 12 (*R*-QTL2 & 3) (Bai et al., 2003).

The monogenic *Ol-1, Ol-3, Ol-4, Ol-5* and *Ol-6* genes and the three *R*-QTLs have been introgressed into the susceptible tomato cultivar Moneymaker (S-MM) and the resistance mechanisms have been studied microscopically (Bai et al., 2003; Chapter 4). Previous data indicated that the resistance responses caused by *Ol-1, Ol-3* and *Ol-5* are strongly associated with a slow-HR (Hypersensitive Response), while resistance responses mediated by *Ol-4* and *Ol-6* are associated with a fast-HR (Huang et al., 2000 a&b; Bai et al., 2005; Chapter 3 & 4). The resistance response triggered by *ol-2* is mainly associated with papilla formation and affects the germination of fungal spores, but there is no HR involved (Bai et al., 2005; Chapter 4). The resistance in *S. neorickii* governed by three major resistance QTLs is less associated with HR (Huang et al., 2000 a&b). However, we revealed that slow-HR is involved in the resistance responses to *O. neolycopersici* mediated by these *R*-QTLs in a MM background, where the incidence of micro-HR of tomato lines with all three *R*-QTLs together is even higher than that of the near isogenic line containing the *Ol-1* gene (Chapter 4). It was also shown that different individual QTL(s) mediate different HR phenotypes, while the pyramiding of *R*-QTL(s) can result in a new type of necrotic cells and a high-level resistance comparable to *Ol-1*-mediated resistance (Chapter 4).

To obtain a better understanding of the mechanisms of these major *R*-QTLs in the resistance response to powdery mildew, we analyzed the transcript profiles of tomato lines containing different combinations of *R*-QTLs and compared those with the profiles of S-MM and a near isogenic line carrying the dominant *R* gene *Ol-1*, using cDNA-AFLP.

Materials and methods

Plant materials

S. lycopersicum cv. Moneymaker was used as susceptible control (hereafter named S-MM) and as the recurrent parent in the development of NILs. NIL-Ol-1 with S-MM background (Bai et al., 2005) was used as monogenic dominant resistant control. Six lines homozygous for three combinations of *R*-QTLs (two lines for each combination) were used (Table 1), which were generated by backcrossing *S. neorickii* (harboring three major *R*-QTLs) two times to S-MM. Presence of *R*-QTLs was monitored by flanking molecular markers and disease tests; the genetic background was monitored using AFLP markers. Flanking markers CT184 and Aps1 were used for the selection of *R*-QTL1 on chromosome 6 (NILs hereafter named N1Qa and N1Qb). Similarly, the lines N2Qa and N2Qb contain both *R*-QTL2 and 3 on chromosome 12, selected by using CT129, CT99 & TG111. All the mentioned markers were used to monitor the presence of all three *R*-QTLs in N3Qa and N3Qb. The BC_2 lines were selfed twice to create BC_2S_2 lines, homozygous for the introgressed *R*-QTLs.

Fungal material and inoculum preparation

O. neolycopersici was collected from infected tomato plants in the Netherlands (Lindhout et al., 1994a), and is continuously maintained on S-MM plants in growth chambers at 20±2℃, a relative humidity (RH) of 70% and 16 hour s day-length. Fresh spores were

washed from the infected leaves with water to obtain suspensions with concentrations of 2×10^4 spores/ml. Water was sprayed as inoculation control.

Experimental set-up of and sampling

All plants were grown in climate cells with optimal temperature, photoperiod and light conditions (20±2℃, 16 hours daytime, light intensi ty 150 µmol/m^2, s). The experimental design consisted of two randomized blocks with S-MM as border plants and controls for spontaneous infection. Four-week-old plants were used for whole-plant inoculation and single-leaf inoculation and different leaf samples were harvested from different plants. For the whole-plant inoculated plants, the second and third true leaves were collected and directly put into liquid N_2, the remaining plant was kept for macroscopic disease evaluation. Samples were collected at 0, 1, 2, 3, 5, 7 and 8 DPI (days post inoculation) for all the genotypes. For the single-leaf inoculated plant, the leaf opposite the inoculated leaf was harvested to investigate systemic induction of genes.

cDNA-AFLP and sequencing of interesting transcript-derived fragments

The cDNA-AFLP protocol was as described in Bachem et al. (1996) (Chapter 2). The differentially expressed TDFs (DE-TDF) were excised from PAGE gels using the Odyssey machine (LICOR, USA); excised bands were re-amplified, purified using G-50 column (Amersham Bioscience, USA), and sequenced with standard AFLP primers *Mse*I00 and *Eco*RI00 (Baseclear, the Netherlands).

Sequence analysis

The BLAST results were obtained against TIGR (http://www.tigr.org/tdb/tgi) tomato / *Arabidopsis* TC databases using BLASTN and TBLASTX. The sequenced DE-TDFs were mapped onto the tomato genome through blasting against Solanaceae genomics network (SGN) unigene and marker databases (http://www.sgn.cornell.edu/). The DE-TDFs, which are homologous to markers and unigenes with known locations on the genetic linkage map deposited in SGN, could be mapped on the corresponding locations.

Results

Disease evaluation

Disease symptoms were evaluated as described in Chapter 4, the disease indices and macro-HR incidence are indicated in Table 1.

Table 1 Disease indices, macro-HR incidences of S-MM and near isogenic lines with different quantitative (*R*-QTLs) and qualitative (*Ol-1*) resistance inoculated with *O. neolycopersici*

Genotypes	Resistance genes/QTLs	*R* gene or *R*-QTL location	Phenotype[#]	Disease index[&]	Macro-HR incidence[$]
S-MM (susceptible)	None	Not suitable	S	3	0
NIL-Ol-1[*]	*Ol-1*	Chr. 6	R	0.19	23/24
N1Qa (1 QTL)	QTL1	Chr. 6	R	0.81	17/24

Genotypes	Resistance genes/QTLs	*R* gene or *R*-QTL location	Phenotype[#]	Disease index[&]	Macro-HR incidence[$]
N1Qb (1 QTL)	QTL1	Chr. 6	IR	1.06	17/24
N2Qa (2 QTL)	QTL2 and QTL 3	Chr. 12	R	0.75	2/24
N2Qb (2 QTL)	QTL2 and QTL 3	Chr. 12	IR	1.67	2/24
N3Qa (3 QTL)	QTL1, QTL2, and QTL3	Chr. 6 & Chr. 12	R	0.04	7/24
N3Qb (3 QTL)	QTL1, QTL2, and QTL3	Chr. 6 & Chr. 12	R	0.04	19/24

* NIL: near isogenic line with Moneymaker genetic background; * S: susceptible (disease index≥2); IR: intermediately resistant (2>disease index>1); R: resistant (disease index≤1); [&] Disease index is the mean of the scores of 24 time-point × plant observations per genotype (scored at four time-points for each of the six investigated plants);

[$] Refers to the number of incidences of macro-HR detected out of the maximum 24 time-point × plant observations.

cDNA-AFLP analysis

The transcript profiles of S-MM, NIL-Ol-1, and the QTL-NILs were investigated using cDNA-AFLP. About 4,000 bands were displayed using 78 primer combinations, selected based on previous results (Chapter 2), while only 204 bands were differentially expressed (DE-TDFs) in the inoculated plants compared to mock-inoculated plants (Figure 1, class I - V). About 65% of these induced DE-TDFs were common for S-MM, NIL-Ol-1 and QTL-NILs, displaying up-regulation patterns (Figure 1, class I - III). More than half of these common DE-TDFs were also associated with the non-inoculated systemic leaf of single-leaf inoculated S-MM plants (Figure 1, class III). Unexpectedly, the mock-inoculated sample of NIL-Ol-1 at eight dpi strongly expresses Class III DE-TDFs. In fact, some leaves of this plant showed symptoms caused by either abiotic or biotic stress, but leaves without visible symptoms were harvested for cDNA-AFLP. Approximately 29% of the induced DE-TDFs were only up-regulated in resistant genotypes (Figure 1, class IV). These class-IV DE-TDFs displayed the following four patterns: induced only in NIL-Ol-1 and/or one QTL-NIL (class IV-1); induced in N1Q and N3Q, both containing the chromosome 6 *R*-QTL (IV-2a) or in N3Q and N2Q, with the chromosome 12 *R*-QTLs in common (class IV-2b); induced in all QTL-NILs except N2Qb (class IV-3); and induced in NIL-Ol-1 and all QTL-NILs except N2Qb (class IV-4). N2Qb has the same *R*-QTL combination as N2Qa but had a much lower resistance level (Table 1). The remaining 5% of the induced DE-TDFs showed a transient expression pattern in S-MM, NIL-Ol-1 and QTL-NILs (Figure 1, class V). Additionally, seventy-two DE-TDFs displayed a differential expression level between genotypes but not between inoculated and mock-inoculated plants (Figure 1, class VI). These DE-TDFs may represent polymorphic sequences associated with the introgressed region surrounding the *R*-QTLs, or without this association. From the 4000 bands observed, 69 bands (1.75%) were “polymorphic” within a NIL, indicating that those NILs were not completely pure yet.

Although the DE-TDFs of class I – III (Figure 1) are associated with all the genotypes, differences in expression timing and level were detected. DE-TDFs of class-I have similar temporal courses in resistant genotypes compared to S-MM. The expression levels are generally reverse correlated to resistance levels, because NIL-Ol-1, N3Qa and N3Qb that

Figure 1 Different classes of the DE-TDFs displayed in cDNA-AFLP analyses of susceptible genotype (S-MM) and resistant near isogenic lines, which contain *Ol-1*, different *R*-QTL combinations in the S-MM genetic background. These classes are illustrated by representative DE-TDF images

Class	**Number of DE-TDF**	**Description of expression pattern**	[&] S-MM			NIL-Ol-1		N1Qa		N1Qb		N3Qa		N3Qb		N2Qa		N2Qb	
			[#] w	inoc.	s	w	inoc.	w	inoc.	w	inoc.	w	inoc.	w	inoc.	w	inoc.	w	inoc.
			[*] P	8	013578	8	P	801578	P	8013578	P	8013578	P	8013578	P	8013578	P	8013578	P	8013578
I	45(23%[@])	Induced in all genotypes																	
II	18 (9%)	Induced in all genotypes and much higher in some resistant genotypes																	
III	67(33%)	Induced in all genotypes and systemically induced																	
IV-1	17 (8%)	Induced only in NIL-Ol-1 and/ or one QTL-NIL																	
IV-2	10 (5%)	Induced in (NIL-Ol-1), N1Q and N3Q or only N1Q and N3Q (IV-2a), or in N3Q and N2Q (IV-2b)																	
IV-3	21(10%)	Induced in all QTL-NILs except N2Qb																	
IV-4	12(6%)	Induced in NIL-Ol-1 and all QTL-NILs except N2Qb																	
V	13(6%)	Transiently expressed in S-MM and all resistant genotypes																	
VI	72	Differentially expressed between genotypes but not between treatments																	

[@] Percentage of all the induced DE-TDFs; [&] refers to genotypes; [#] refers to treatments, w - mock inoculation, inoc. - inoculation, s-systemic (sample were harvested from non-inoculated leaf of single-leaf inoculated plant); * refers to time-points, P represents the pool of mock inoculated samples harvested at 0, 1 and 3 days post inoculation (in NIL-Ol-1, 0, 1 and 2 days post inoculation); 0, 1, 3, 5 7, 8 refers to the number of days post inoculation of harvested mock inoculated and inoculated samples

show a high level of resistance (disease index <0.5) (Table 1) displayed an obviously lower expression level compared to S-MM, and the other four QTL-NILs (Table 1) displayed similar or slightly lower expression levels compared to S-MM. As for the DE-TDFs of class-II, temporal patterns were like those of class-I DE-TDFs; these DE-TDFs displayed an obvious higher expression level in N2Qa and N2Qb containing two *R*-QTLs compared to S-MM. In contrast to those of class I & II, DE-TDFs of class-III were induced in all genotypes and systemic S-MM leaf sample, and showed a higher level and/or an earlier timing in resistant genotypes compared to S-MM. These DE-TDFs showed lower expression levels in N3Qa compared to N3Qb.

The numbers of DE-TDFs of class I, II and III are 45, 18 and 67 respectively (Figure 1). Therefore about 14% (18/130) of these common DE-TDFs revealed by the selected 78 primer combinations showed the same timing but a higher expression level in N2Qa & b containing the two *R*-QTLs on chromosome 12 in comparison to S-MM. About half of these common DE-TDFs showed earlier timing/higher expression level in resistant genotypes, compared to S-MM, and were systemically induced in S-MM (class III).

Sequence information

Interesting DE-TDFs of expression class I-V and of class VI that were associated with the introgressed *R* gene or *R*-QTLs, and several "polymorphic" bands were selected for sequencing. A total of 140 bands were excised, re-amplified, purified and sent for sequencing, resulting in 68 good-quality sequences. From 72 bands no good-quality sequences were obtained, since the bands were a mixture of fragments. The BLAST results were obtained against TIGR (http://www.tigr.org/tdb/tgi/plant.shtml) tomato/ *Arabidopsis* TC databases. Based on the BLAST results, the DE-TDFs were classified into several functional groups (the standard employed for this is the same as that in Chapter 2) (Table 2). Fifteen of the 55 TDFs showed homology to plant ESTs that are

Table 2 Functional groups of 68 sequenced DE-TDFs based on the BLAST results against TIGR TC database

	Blast results of DE-TDF sequences	**Group**	**Number**
Function-informati	Known defense responses (secondary metabolite synthesis, cell	A	15
ve (with functional	wall associated, oxidative burst, etc.)		
information from	Signal transduction (GTP-binding proteins, kinases, etc.)	B	6
plant EST	Regulation (transcription factors, heat shock proteins, etc.)	C	13
databases)	Ubiquination pathway and protein synthesis related	D	3
	Photosynthesis, photorespiration and respiration	E	5
	Other*	F	1
No functional	Pathogen derived[#]	G	0
information from	Unknown**	H	12
plant EST	No (good) hits[$]	I	13
databases			
Total			68

* Genes that encode proteins with functions not associated with defense before; ** Genes that encode proteins of unknown functions; # Good-match found in fungal databases but not in plant databases; $ No homologous match in databases or hit with e value > 5.0e-2

directly involved in defense responses (Group A). For the remaining DE-TDFs, a division was made into transcripts involved in signaling (six DE-TDFs in Group B) and regulation (13 DE-TDFs in Group C) and into transcripts with housekeeping functions, like protein synthesis and degradation (three DE-TDFs in Group D) and energy metabolism (five DE-TDFs in Group E). Thirteen sequenced DE-TDFs had no match or no good match (e value > 5e-2) in the databases (Group I). Among the 55 sequenced DE-TDFs with hits in databases, none were likely to have a pathogen origin (Group G), and 13 DE-TDFs were homologous to sequences with unknown (Group H) or known functions not associated with defense before (Group F)

In Table 3, the BLAST results, functional classification, expression patterns and timing of DE-TDFs are presented. The sequenced common DE-TDFs induced in both susceptible and resistant genotypes, showing similar timing pattern between resistant and susceptible lines (Class I), were involved in housekeeping, regulation and known defense (Table 3, No. 1-4). Similar to the results of Chapter 2, the sequenced common DE-TDFs induced in both susceptible and resistant lines, displaying earlier timing in resistant genotypes compared to S-MM (Class III), are generally involved in defense related activities (Group A; Table 3, No. 5-13). Four sequenced common DE-TDFs of Class III were homologous to protein kinase, Cytochrome P450 and transcription factors (Table 3, No.14-17). Transcripts of the sequenced DE-TDFs of class-IV-1 & 2 were mainly associated with regulation of transcription and translation (Table 3, No. 18-25). Transcripts with class-IV-3 & 4 expression pattern were involved in protein synthesis. The sequenced transiently expressed DE-TDFs (Class V) and the constitutively expressed TDF, with different expression level between susceptible and resistant genotypes (Class VI), were homologous to genes with functions of signaling or regulation (Table 3, No.30-36). In addition, we also sequenced several interesting “polymorphic” TDFs, which may still be associated with resistance. These “polymorphic” TDFs (Table 3, No.38-42) are homologous to transcripts involved in signaling, ubiquintination pathway and photosynthesis. In Table 3, some TDFs have the same annotation (Table 3, No. 5, 6 & 7, No 18 & 34, and No. 30 & 36).

Table 3 The putative functions or defense pathways involved of the sequenced DE-TDFs of different classes (Figure 1), based on the BLAST results against TIGR tomato/*Arabidopsis* TC database (only the DE-TDFs with BLAST e value<5.0e-2 are listed).

No.	$PC-size	*Expression Class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
1	M18E41-260	I	-	5.3e-1/ 82%	A	>tomato|TC155487 weakly similar to UP|Q86GL5 (Q86GL5) Peroxiredoxin 3,
2	M12E58-290	I	-	8.9e-44/ 98%	A	>tomato|TC153678 similar to UP|CHIC_LYCES (Q05538) Basic 30 kDa endochitinase precursor
3	M21E53-310	I	-	4.2e-4/ 61%	C	>tomato|TC162485 weakly similar to UP|NUCL_HUMAN (P19338) Nucleolin (Protein C23)

No.	$PC-size	*Expression Class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
4	M15E34-170	I	-	8.9e-10/ 82%	D	>arab|BU634848 similar to SP|O59950|RS4_ 40S ribosomal protein S4 (S7).[Candida lipolytica] {Yarrowia lipolytica}
5	M13E51-460	III	+	1.3e-71/ 99%	A	>tomato|TC162242 homologue to UP|GTX1_SOLTU (P32111) Probable glutathione S-transferase (Pathogenesis-related protein 1)
6	M21E53-455	III	+	1.1e-64/ 99%	A	>tomato|TC162242 homologue to UP|GTX1_SOLTU (P32111) Probable glutathione S-transferase (Pathogenesis-related protein 1)
7	M21E49-455	III	+	3.9e-71/ 100%	A	>tomato|TC162242 homologue to UP|GTX1_SOLTU (P32111) Probable glutathione S-transferase (Pathogenesis-related protein 1)
8	M18E43-380	III	+	2.4e-53/ 92%	A	>tomato|TC153588 UP|PAL5_LYCES (P26600) Phenylalanine ammonia-lyase (PAL)
9	M14E42-429	III	+	3.1e-23/ 68%	A	>tomato|TC154996 similar to TIGR_Ath1|At3g51840.1 68416.m05685 short-chain acyl-CoA oxidase
10	M15E70-150	III	+	8.3e-12/ 93%	A	>tomato|TC162262 similar to UP|Q7XYY0 (Q7XYY0) AKIN gamma, partial (82%)
11	M21E57-312	III	+	1.0e-41/ 97%	A	>tomato|TC162547 weakly similar to TIGR_Osa1|9637.m02525 expressed protein, weakly similar to putative PrMC3
12	M20E37-365	III	+	4.4e-69/ 99%	A	>tomato|TC162154 UP|O04936 (O04936) Malate oxidoreductase, cytoplasmic
13	M13E66-330	III	+	2.3e-50/ 95%	A	>tomato|TC161990 similar to UP|Q6IV17 (Q6IV17) Protein disulfide isomerase
14	M14E67-135	III	+	8.4e-09/ 93%	B	>tomato|BI922654 homologue to GP|18087335|gb| serine/threonine protein kinase kkialre-like 1 {Homo sapiens}, partial (1%)
15	M12E42-265	III	+	2.5e-18/ 89%	A	>tomato|TC162024 similar to UP|C762_SOLME (P37122) Cytochrome P450 76A2 CYPLXXVIA2) (P-450EG7)
16	M14E42-355	III	+	3.3e-21/ 76%	C	>tomato|TC153580 homologue to UP|Q8S4L3 (Q8S4L3) MADS-box transcription factor

No.	$PC-size	*Expression Class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
17	M20E37-270	III	+	9.2e-38/ 96%	C	>tomato|TC155308 homologue to UP|Q94IK2 (Q94IK2) Storekeeper protein, partial, belong to DNA-binding proteins
18	M19E35-205	IV-1 (*Ol-1*)	+	1.8e-20/ 90%	C	>tomato|TC163744 similar to UP|Q9SEE9 (Q9SEE9) Arginine/serine-rich protein, a kind of RNA-binding protein contains domain of splicing factor
19	M16E68-255	IV-1 (*Ol-1*)	+	1.2e-37/ 98%	C	>tomato|TC164301 similar to UP|Q9SW44 (Q9SW44) RNA helicase (RH16), a kind of translation initiation factor kinase
20	M13E68-188	IV-1 (N 2Qa)	+	5.5e-18/ 99%	B	>tomato|TC164377 homologue to UP|Q08149 (Q08149) GTP-binding protein
21	M22E35-520	IV-1 (*Ol-1*)	+	1.3e-21/ 66%)	A	>arab|TC275227 UP|Q8H960 (Q8H960) Tobamovirus multiplication 2B
22	M13E40-235	IV-1 (N1 Q+*Ol-1*)	+	3.0e-20/ 98%	C	>tomato|TC162654 weakly similar to TIGR_Ath1|At5g09850.1 68418.m01139 transcription elongation factor
23	M13E40-220	IV-1 (N1 Q+*Ol-1*)	+	3.0e-20/ 98%	C	>tomato|TC162654 weakly similar to TIGR_Ath1|At5g09850.1 68418.m01139 transcription elongation factor
24	M12E42-225	IV-2 (N1 Q+N3Q)	+	1.0e-19/ 88%	C	>tomato|TC164052 weakly similar to TIGR_Ath1|At5g43960.1 68418.m05379 nuclear transport factor 2 (NTF2) family protein / RNA recognition motif (RRM)-containing protein
25	M21E57-280	IV-2 (N1 Q+N3Q +*Ol-1*)	+	6.1e-40/ 96%	C	>tomato|TC163311 homologue to UP|Q8LRL5 (Q8LRL5) Nam-like protein 10, a kind of transcription factor
26	M21E52-640	IV-3	+	1.4e-90/ 100%	B	>tomato|TC157608 similar to GB|AAA34745.1|171846|YSCLIPOIC lipoic acid synthase{Saccharomyces cerevisiae}, may function in cytokinin transport
27	M18E41-286	IV-3	+	1.9e-52/ 95%	E	>tomato|TC157238 similar to TIGR_Ath1|At1g70330.1 68414.m08091 equilibrative nucleoside transporter family 2 protein
28	M12E58-355	IV-4	+	3.2e-56/ 99%	E	>tomato|TC153698 homologue to gb|AF036493.1|AF036493 Tragopogon dubius large subunit 26S ribosomal RNA gene, partial sequence

No.	$PC-size	*Expression Class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
29	M15E76-390	IV-4	+	8.1e-68/ 99%	E	>tomato|TC162190 similar to UP|Q9M5M5 (Q9M5M5) 60S acidic ribosomal protein PO(Fragment)
30	M15E34-240	V	+	6.9e-12/ 84%	B	>tomato|TC163514 weakly similar to TIGR_Ath1|At3g47960.1 68416.m05229 proton-dependent oligopeptide transport (POT) family protein contains Pfam profile: PF00854 POT family
31	M14E42-465	VI	NI	1.4e-71/ 93%	C	>tomato|TC153824 UP|ENO_LYCES (P26300) Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), a bi-function transcription factor
32	M14E39-190	VI	NI	2.8e-21/ 93%	A	>tomato|TC160763 homologue to TIGR_Ath1|At1g78920.1 68414.m09201 vacuolar-type H+-translocating inorganic pyrophosphatase (AVPL1)
33	M12E34-275	VI	NI	2.0e-20/ 97%	C	>tomato|AI777576 similar to GP|6630539 putative RING zinc finger protein {Arabidopsis thaliana}
34	M19E37-205	VI	NI	1.2e-16/ 89%	C	>tomato|TC163744 similar to UP|Q9SEE9 (Q9SEE9) Arginine/serine-rich protein, a kind of RNA-binding protein contains domain of splicing factor
35	M16E75-135	VI	NI	5.9e-08/ 96%	B	>tomato|TC154636 weakly similar to TIGR_Ath1|At1g56720.1 68414.m06523 protein kinase family protein contains protein kinase domain, Pfam:PF00069
36	M15E34-215	VI	NI	7.9e-11/ 90%	B	>tomato|TC163514 weakly similar to TIGR_Ath1|At3g47960.1 68416.m05229 proton-dependent oligopeptide transport (POT) family protein contains Pfam profile: PF00854 POT family
37	M12E52-400	VI	NI	3.9e-57 / 92%	D	>tomato|TC153558 UP|Q39257 (Q39257) Ubiquitin
38	M21E49-240	P	NI	7.2e-27/ 91%	A	>tomato|TC155897 similar to UP|O48618 (O48618) Cytochome b5 (Fragment)
39	M15E34-130	P	NI	3.7e-4/ 90%	E	>tomato|TC155208 UP|Q8LKF6 (Q8LKF6) 5-formyltetrahydrofolate cycloligase
40	M21E49-480	P	NI	4.4e-80/ 96%	E	>tomato|TC161898 homologue to UP|Q8LSZ3 (Q8LSZ3) NADPH:protochlorophyllide oxidoreductase

No.	$PC-size	*Expression Class	**Earlier Timing?	e value/ Identity	#Group	Homology annotation&
41	M22E61-510	P	NI	3.0e-14/ 90%	D	>tomato|TC158503 similar to TIGR_Ath1|At2g33770.1 68415.m04141 ubiquitin-conjugating enzyme family protein
42	M15E76-122	P	NI	4.9e-2/ 94%	B	>tomato|TC168650 similar to TIGR_Ath1|At1g25390.1 68414.m03152 protein kinase family protein contains protein kinase domain, Pfam:PF00069

$ Primer combination and fragment size are listed; * Classes in this table are as those described in Figure 1; ** The "earlier timing" refers to whether the DE-TDF is earlier expression in resistant genotypes compared to S-MM, in this column, "+" means that the DE-TDFs showed earlier timing in resistant genotypes or specific to resistance genotypes, "-" means that resistant and susceptible genotypes have the same temporal pattern of the DE-TDF, "NI" means that the TDF is not induced one and irrelevant to the timing comparison between susceptible and resistant genotypes; #The functional groups are as those described in Table 2; & Homologies are the BLASTN results against TIGR tomato/*Arabidopsis* TC database.

Map position of DE-TDFs

Sequences of DE-TDFs were also blasted against all SGN unigene and marker databases (http://www.sgn.cornell.edu/) in order to obtain additional information about annotation and the map position of the DE-TDFs on the tomato genome. BLAST against TIGR and SGN databases resulted in similar annotations. Additionally, the information of map positions of 11 DE-TDFs on the tomato-EXPEN map series was available in SGN databases (Table 4). Two DE-TDFs (M14E72-210 and M14E72-213), which were specific to NIL-Ol-1 and N1Q & N3Q (containing chromosome 6 *R*-QTL in common) respectively and with only three nucleotides difference, represented different alleles of the same unigene SGN-U217783, developed by SGN. This unigene was assembled from 12 members of sequences including the EST marker cLET-6I13 (also named as SGN-C87964) that maps on chromosome 6 of the tomato-EXPEN 2000 map at the *Ol-1*/*R*-QTL1 region (Figure 2). With the same *in-silico* method, DE-TDFs M13E66-330 (Table 3, No. 13), with homology to a protein disulfide isomerase, were mapped on chromosome 6 at the *Ol-4* locus. The other DE-TDFs mapped to other chromosomal regions, not co-localizing with map positions of *Ol*-genes or *Ol*-QTLs.

Table 4 *In-silico* mapping of DE-TDFs: positions on tomato-EXPEN map series are based on the map positions of homologous sequences (BLAST with e value ≤ 2e-4). DE-TDFs with annotations are also listed in Table 3.

DE-TDF/No. in Table 3	Class	Annotation	Homologous marker/ e value	Chr. No.	Map position (cM)	Co-localization with *Ol*-loci
M14E72-210 / NA	IV-1	unknown	cLET-6-I13 (EST)/ 1e-46	6	36	*Ol-1*/*R*-QTL1
M14E72-213/ NA	IV-2	unknown	cLET-6-I13 (EST)/ 1e-46	6	36	*Ol-1*/*R*-QTL1
M13E66-330	III	Protein disulfide	T1082 (COS)/	6	3	*Ol-4*

DE-TDF/No. in Table 3	Class	Annotation	Homologous marker/ e value	Chr. No.	Map position (cM)	Co-localization with *Ol*-loci
/ No. 13		isomerase	1e-117			
M18E43-380/ No. 8	III	Phenylalanine ammonia-lyase	ct225-R (RFLP)/ 1e-132	9, 3 & 7	4, 34 & 39.3	-
M13E51-460/ No. 5	III	Probable glutathione S-transferase (GST)	T1703 (COS)/ 2e-4	6	51	-
M21E49-455/ No. 7	III	GST	T1703 (COS)/ 2e-4	6	51	-
M21E53-455/ No. 6	III	GST	T1702 (COS)/ 2e-4	6	51	-
M13E68-188/ No. 20	IV-1	GTP binding protein	T1665 (COS)/ 6e-9	2	49	-
M15E34-240/ No. 30	V	proton-dependent oligopeptide transport (POT) family protein	cLET-8-B23 (EST)/ 9e-32	5	10	-
M15E34-215/ No. 36	VI	POT	cLET-8-B23 (EST)/ 9e-32	5	10	-
M14E42-465/ No. 31	VI	Enolase	T0532 (COS)/ 1e-175	9	30	-

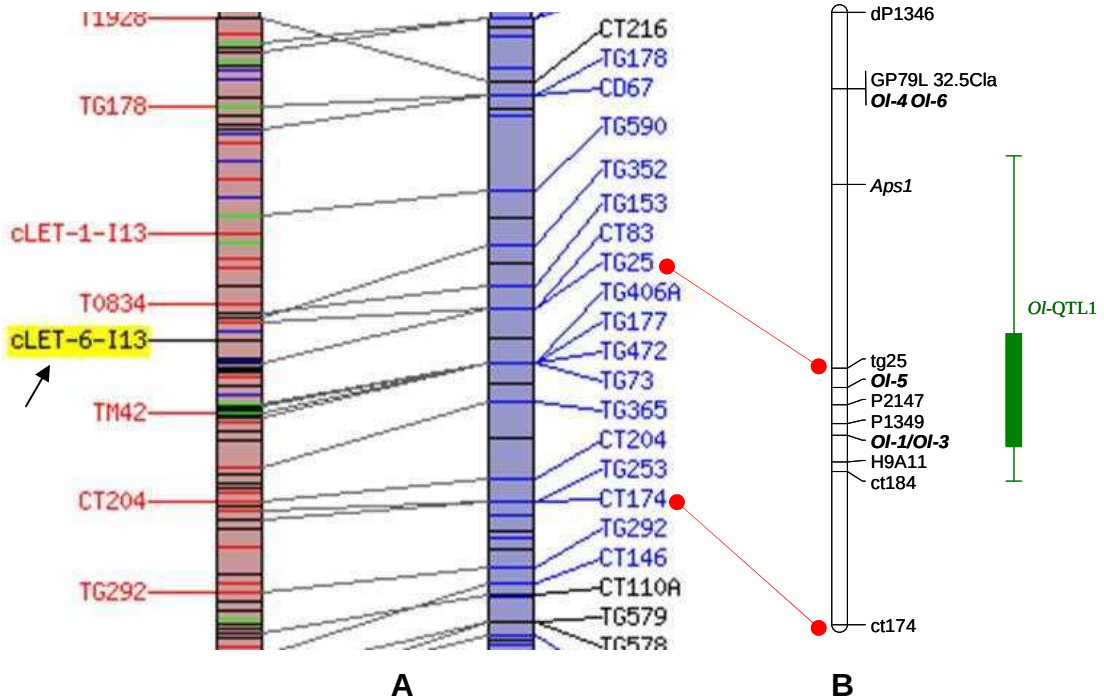

Figure 2 Determination of map position of two DE-TDFs (M14E72-210 and M14E72-213), homologous to cLET-6-I13, through comparative mapping using the SGN network

A: part of chromosome 6 of the Tomato-EXPEN 2000 map and the Tomato-EXPEN 1992 map, with bridging markers (SGN); B: *Ol*-QTL1 (*R*-QTL1) region of chromosome 6 of the tomato linkage map constructed by Bai et al (2004).

Discussion

During the plant - pathogen interaction, many host genes are activated; the clarification of the expression patterns of these genes will increase our understanding of the mechanisms of both compatible and incompatible interactions. Plants can defend themselves against pathogens using multiple systems including 1) basal defense, which limits the extent of disease caused by virulent pathogen races in the compatible interaction; 2) *R*-mediated resistance, which is triggered by *R* genes that allow the recognition of distinct races of biotrophic pathogens; 3) polygenic resistances mediated by several QTLs with yet unknown function and 4) systemic acquired resistance, which is induced by recognition of pathogens by either *R* genes or basal defense (Eulgem 2005). For the tomato – *O. neolycopersici* interaction, tomato interacts with the fungus by a susceptible or a monogenic- or polygenic resistance response, allowing us to investigate the differences between compatible and incompatible (monogenic and polygenic) interactions. Additionally, the near isogenic tomato lines with different combinations of *R*-QTLs offer the chance to study the resistance mechanisms governed by individual *R*-QTLs or different combinations of *R*-QTLs.

The tomato – *O. neolycopersici* compatible interaction is robust and suitable to monitor the reproducibility of cDNA-AFLP analysis

The 78 cDNA-AFLP primer combinations used to profile the transcriptomes of NIL-Ol-1, the QTL-NILs and S-MM during interactions with *O. neoloycopersici*, were selected based on the large-scale screening of primer combinations described in Chapter 2. In the compatible interaction both the expression pattern and timing of DE-TDFs are similar in the experiments of Chapter 2 and this Chapter. It suggests that the reproducibility of cDNA-AFLP analysis between experiments and biological replicates is high, and that expression patterns of compatible interactions are hardly influenced by environmental variation. This environmental variation is caused by several factors such as: soil composition, watering of plants, light conditions (age of lamps), quality and developmental stage of the conidial spores, which are harvested from S-MM plants, etc. In the incompatible interaction between *Ol-1* lines and *O. neolycopersici*, the same set of TDFs was differentially expressed in the two experiments (Chapter 2 and this Chapter), but the expression patterns were not the same. In Chapter 2, DE-TDFs in BC_1S_2 *Ol-1* lines showed a transient pattern, with an expression peak at seven days post inoculation, and down regulation at nine days post inoculation. In this Chapter, the expressions of DE-TDFs in NIL-Ol-1 constantly increased till the last time point, at eight dpi. We cannot exclude the possibility that expression would have decreased in later time-points, but this was not studied. The observed difference in timing can result from the analysis of different time-points, or can reflect influence of environmental variation as described above for the compatible interaction, or a biological difference as the genetic background of the *Ol-1* lines was not identical in both experiments (BC_1S_2 versus BC_3S_2 with S-MM as recurrent parent).

Our conclusion that the compatible interaction is robust is in contrast to the work of Tao et al. (2003), in which the transcriptomes of the *Arabidopsis - Pseudomonas syringae*

interaction was studied. These authors observed a considerable amount of variation between biological repeats in the compatible interaction compared to the more robust incompatible interaction using the GeneChip - AtGemone 1 Array. Hence, we conclude that different pathosystems and different methods may affect the robustness of the biological system.

The genetic background may affect the defense responses of *R*-QTLs

We demonstrated that tomato plants with the same *R*-QTL(s) in different genetic backgrounds do not respond to *O. neolycopersici* in an identical manner. Previous studies showed that HR is hardly involved in the quantitative resistance to *O. neolycopersici* in the wild tomato species *S. neorickii* (Huang et al., 2000 a&b). However, we recently found that HR played a major role in the resistance of NILs carrying the *R*-QTLs introgressed from *S. neorickii* (BC_2S_2 lines with about 87% of the genetic background from S-MM). The NILs N3Qa & N3Qb with the three major *R*-QTLs displayed an incidence of necrotic cells upon *O. neolycopersici* infection, comparable to that of NIL-Ol-1 (about 30%, Chapter 4). Plants of N3Qa and N3Qb, with per definition slightly different genetic backgrounds, displayed very different macro-HR incidence (Chapter 4), but the transcription profiles of these two tomato lines were very similar. Another two tomato lines, N2Qa and N2Qb with two *R*-QTLs on chromosome 12 displayed differences in both resistance level (Table 1) and in transcription profiles (Figure 1, class IV-3 & 4). It is remarkable, that only the DE-TDFs that are also present in the compatible interaction (Figure1, classes I, II, III and V) are also present in N2Qb, while all incompatible specific DE-TDFs of class IV are absent in this line, except for class IV-2-b. Results of both the disease tests and transcript profiles urge us to consider the possibility that N2Qb contains only *R*-QTL2 rather than *R*-QTL2 & 3, even though this is in conflict with molecular marker data and microscopic observations (Bai et al. 2003; Chapter 4), which showed that difference between N2Qa and N2Qb is small. Another explanation can be that the genetic backgrounds of N2Qa and N2Qb are different, and that N2Qb lacks some *S. neorickii* alleles that are needed or the expression of QTL 2 and 3. Further investigations on the genetic background and fine mapping of QTL 2 and QTL 3 will clarify this point.

Transcript profiles of basal defense, *R* gene mediated resistance and quantitative resistance responses largely overlap

The transcript profiles presented in this chapter confirmed our earlier conclusions (Chapter 2), that a major part of the genes that are differentially expressed upon *O. neolycopersici* inoculation are common for both compatible and slow HR (*Ol-1*) mediated incompatible interactions and the main difference of expression of these genes is timing. Our conclusion fits very well with those of other well-studied pathosystems, like *Arabidposis – Pseudomonas. syringae* and *Arabidoposis – Peronospora parasitca*, since also here differences between transcript profiles associated with *R* gene mediated resistance and basal defense are quantitative rather than qualitative (Tao et al., 2003; Eulgem 2005). In the present study we show for the first time that this quantitative variation in gene expression also holds for quantitative resistance. Meanwhile, the correlation of DE-TDF expression level and pattern (this Chapter) with resistance levels of

different NILs and the mosaic nature of infected tomato leaves (Chapter 4) allows us to predict the origins of the DE-TDFs as described below.

Genes involved in susceptibility or basal defense may be a reaction of compatible cells to the pathogen

About one third of the genes induced in both the compatible and monogenic- and polygenic incompatible interactions of tomato with *O. neolycopersici* displayed the same temporal pattern, while the expression levels of these DE-TDFs were generally higher in the more susceptible genotypes (Figure1, class I). We hypothesize that these DE-TDFs play a role in basal defense or susceptibility of the compatible interaction. Microscopic observations showed that the infected cells in both susceptible and resistant (*Ol-1* or different *R*-QTLs) tomato leaves react in an incompatible (HR or papillae) or a compatible (haustorium formed, no cell death) manner. Only the proportion of "incompatible" and "compatible" cells is different (Chapter 4). The higher proportion of compatible cells in the more susceptible lines may result in a higher expression level of basal defense genes and genes involved in susceptibility. BLAST results will help to identify the function of the DE-TDFs and hence to distinguish these two options. For example, DE-TDF M12E58-290 (Table 3, No. 2) of Class-I is homologous to an endochitinase precursor, suggesting a role involved in defense rather than in susceptibility. The higher expression level of Class-I TDFs in the susceptible line can also result from the higher number of interacting cells in susceptible genotypes compared to the resistant genotypes. However, the number of interaction sites per infection unit in N1Qb, a NIL containing the *R*-QTL1 on Chromosome 6, is about 20% higher then that of S-MM (Chapter 4).

About 14% of the common DE-TDFs belong to Class II and display the same temporal pattern in both susceptible and resistant genotypes, but at a higher expression level in N2Qa & b (containing *R*-QTL2 & 3 on Chromosome 12) compared to the susceptible genotype (S-MM). It will be interesting to find whether the Chromosome 12 *R*-QTLs are regulators of the expression of basal defense genes in N2Q plants. This may be one of the effects mediated by *R*-QTLs contributing to the quantitative resistance.

DE-TDFs, earlier and/or higher expressed in incompatible interactions compared to compatible interactions, are also induced systemically and may reflect basal defense genes that are faster induced in incompatible cells

The Class-III DE-TDFs, that are induced by *O. neolycopersici* in all genotypes studied, but at a higher expression level and/or at an earlier timing in resistant genotypes compared to S-MM, also are induced in systemic S-MM leaves. This implies that SAR may be induced in S-MM plants.

BLAST results of the Class-III DE-TDFs revealed homologies to several interesting genes. DE-TDF M18E43- 380 (Table 3, No 8) is homologous to phenylalanine ammonia-lyase (PAL), the key enzyme in the phenylpropanoid biosynthesis pathway, involved in the synthesis of salicylic acid (SA) (Gozzo, 2003). DE-TDFs M13E51-460, M21E53-455 and M21E49-455 (Table 3, N0. 5-7), are all homologous to glutathoine S-transferase (GST) that is induced during the oxidative burst and is associated with H_2O_2 production. It can serve as an indirect measure of reactive oxidative intermediates (ROIs) (Zeier et al., 2004).

The homologies of Class-III DE-TDFs to PAL and GST suggest that SA and ROIs are involved in basal defense, monogenic and *R*-QTL-mediated resistance of tomato to *O. neolycopersici.* These DE-TDFs were also induced systemically, that is in the non-inoculated leaf opposite the infected leaf of S-MM plants, which suggests the induction of Systemic Acquired Resistance (SAR). In a recent review, Eulgem (2005) described SAR, which can be induced by recognition of pathogens by either *R* genes or basal defense. Homologies of the Class-III DE TDFs to PAL and GST strengthen our conclusion that these DE-TDFs are associated with SAR, because SA and ROIs are among the most agreed signals of SAR (Durrant and Dong, 2004). Other DE-TDFs of Class III are homologous to transcription factors (Table 3, No. 16 & 17), protein kinases (Table 3, No.14) or genes with known function related to defense responses (Table 3, No. 9-13). That a number of these genes are likely to play a role in SAR as well is strengthened by the fact that the corresponding DE-TDFs are also expressed systemically in symptom less leaf samples of mock-inoculated NIL-Ol-1 at eight DPI, from which some leaves displayed symptoms caused by certain abiotic/biotic stresses. It will be interesting to test whether SAR is really induced upon infection of both susceptible and resistant (*Ol-1* or *R*-QTL mediated) tomato genotypes. This can be tested by removing spore inoculated leaves at different time-points after the inoculation, but before mycelium appears on infected leaves. These challenged plants and non- challenged plants can then be re-infected with *O. neolycopersici* spores, after which disease incidence will be monitored.

Class-III DE-TDFs are higher expressed in the more resistant genotypes, like in NIL-Ol-1 and N3Qa & b, which display a higher level of resistance compared to other resistant genotypes. This may indicate that these DE-TDFs are associated with the *R* gene or *R*-QTLs mediated resistance response from the infected “incompatible” cells rather than basal defense from the infected “compatible” cells. Several of the class-III DE-TDFs display a lower/later expression in N3Qa compared to N3Qb (while both N3Qa and N3Qb contain three *R*-QTLs and have a DI of 0). The macro-HR incidence of N3Qa is much lower than that of N3Qb (Table 1), but micro HR incidences at 89 hpi are similar in these two lines (Chapter 4). Thus more cells undergo HR in N3Qb, which result in a higher amplitude of class-III DE-TDFs. The faster or stronger HR responses and different HR phenotypes may be the result of different regulation of Class-III genes. However, no DE-TDFs were detected specific to one of these two N3Qa NILs.

Only a subset of induced genes differentiate defense responses to *O. neolycopersici* in NILs carrying *Ol-1* and/or individual *R*-QTL(s)

Generally, the same set of genes was induced or down regulated in all interactions studied here and most differences were in the fine-tuning of expression levels. Only a small percentage of the DE-TDFs identified in the large screening in Chapter 2 were specific to the incompatible interaction (about 3%). The primer combinations used in this chapter were a selection of the ones used in Chapter 2, with a bias towards Class IV DE-TDFs that are specific to incompatible interactions. Interestingly, a large percentage of these DE-TDFs (Class IV) are *Ol-1* or *R*-QTL specific. If N-2Qb is not considered, 12 DE-TDFs (20%) are induced in all resistant NILs (IV-4), 17 DE-TDFs (30%) are

specifically induced in NIL-Ol-1 (IV-1), 21 DE-TDFs (35%) are induced in all *R*-QTL NILs (IV-3), while 10 DE-TDFs (15%) are specific to *R*-QTL1 or *R*-QTL2 & 3 (IV-2), The IV-2 DE-TDFs are associated with N1Q and N3Q that have *R*-QTL1 on chromosome 6 in common or with N2Q and N3Q that have *R*-QTL2 & 3 on chromosome 12 in common. Our hypothesis is that these DE-TDFs represent the genes that, together with timing differences in the common set of induced genes, determine the different resistant phenotypes. Two of these IV-2 DE-TDFs M12E42-225 (Table 3, No. 24) and M21E57-280 (Table 3, No. 25) are homologous to nuclear transport factor 2 (NTF2) and Nam-like protein 10 respectively, which both involve the regulation of transcription. Sequencing of the other 8 DE-TDFs from this class is needed to further support our hypothesis.

Pyramiding of *R*-QTLs to *O. neolycopersici* into a single tomato line leads to a high-level resistance comparable to *R* gene (*Ol-1*) mediated resistance, and new necrotic cell types are associated with this resistance (Chapter 4). The disease tests indicate that *R*-QTL1 and *R*-QTL2 & 3 are additive to each other, since the pyramiding of these *R*-QTLs into a single tomato line leads to a much higher level of resistance, while a largely overlapping set of transcripts is activated by these *R*-QTL(s) individually and the combined *R*-QTLs. Interactions between the different *R*-QTLs may also be involved, since both new phenotypes appear (HR cell types) and a few *R*-QTL specific genes are induced.

Transcripts specific to resistant genotypes are mainly involved in signaling, and transcriptional and translational regulation

Class IV TDFs involved in fine-tuning defense pathways?

Several sequenced up-regulated DE-TDFs specific to different resistant genotypes (Table 3, No18- 25) are predicted to play a role in regulation of signal transduction, transcription and translation based on the BLAST results. For example, DE-TDF M13E68-188 (Table 3, No. 20) is homologous to a GTP-binding protein indicating a role in signal transduction; M13E40-235 and M13E40-220 (Table 3, No. 22 & 23) homologous to the transcription elongation factor, together with the two DE-TDFs M12E42-225 (Table 3, No. 22) and M21E57-280 (Table 3, No. 25) described above, all could be associated with regulation of transcription; DE-TDFs M19E35-205 and M16E68-255 (Table 3, No. 18 & 19) are homologous to transcripts involved in translational regulation. Since both the results described in this Chapter, and Chapters 2 and 3 indicate that transcript profiles in compatible and the incompatible *Ol-1* and *R*-QTL mediated interactions mainly differ quantitatively, it is not surprisingly that class-IV DE-TDFs, that are specific for the resistant responses, represent regulators of transcription and translation and signaling components, which fine-tune defense pathways. Similar results were obtained in microarray studies of the signal transduction network controlling plant responses to pathogens (Glazerbrook et al. 2003), and *Arabidopsis* responses to downy mildew infection (Eulgem et al. 2004).

Class VI TDFs are candidates for R genes/QTLs?

Several class-VI TDFs that are constitutively differently expressed between genotypes, but not differentially expressed upon fungal inoculation, are involved in

transcriptional/translational regulation and signaling according to the BLAST results. Examples are M14E42-465 (Table 3, No. 31), with homology to enolase, a bi-functional transcription factor, M12E34-275 (Table 3, No. 33), a putative RING Zn finger protein, M19E37-205 (Table 3, No. 34), a RNA binding protein, and M16E75-135 (Table 3, No. 35), a protein kinase family protein. TDFs with constitutively elevated expression levels in NIL-Ol-1 or QTL-NILs may represent genes that lead to a faster activation of defense pathways in incompatible interactions compared to the compatible interaction. Determination of the map position of these class-VI TDFs will clarify whether they map to the same position as *Ol-1* or the *R*-QTLs on chromosomes 6 and 12 of the tomato genome.

Map position of interesting DE-TDFs identify putative *R* gene/QTL candidates

More and more tomato genomic sequence information, EST sequences and linkage-mapping data are available, which allows the determination of the map-positions of sequenced DE-TDFs based on the BLAST results (http://www.sgn.cornell.edu). We have mapped 11 DE-TDFs by blasting DE-TDFs against SGN databases of unigenes, molecular markers and RFLP-COS markers. Remarkably, several DE-TDFs mapped on chromosome 6, co-localizing with the *Ol-1/R*-QTL1 interval on the long arm or the *Ol-4* locus on the short arm. Incompatible interaction specific Class-IV DE-TDFs M14E72-213 in NIL-Ol-1 and M14 E72-210 in N1Q and N3Q turned out to be two alleles, homologous to SGN unigene with unknown function, and mapped to the *Ol-1*/*R*-QTL1 locus. The fact that these DE-TDFs were alleles, already pointed to a map position in the common introgressed region, which is the *Ol-1* introgression from *S. habrochaites* in NIL-Ol-1 and the *R*-QTL1 introgression from *S. neorickii* in N1Q and N3Q. Further fine mapping and expression studies are needed to evaluate the value of the co-localization.

Class-III DE-TDF M13E66-330 (Table 3, No. 13), homologous to COS marker T1082 and annotated as a protein disulfide isomerase, was mapped to the *Ol-4/Ol*-6 locus. The *R* loci investigated in this study*, Ol-1* and *R*-QTL1, both map to the long arm of chromosome 6, while *Ol-4* maps to the short arm, thus this gene is not considered as candidate genes of the *R* gene/QTL. However, it is interesting that this locus encodes a gene that is induced in the defense response of both compatible and incompatible lines. Clarification of the expression pattern of this gene in *Ol-4/Ol*-6 lines may help to know whether they contribute to the fast-HR activation. The nine other DE-TDFs mapped to positions that are not linked to *O. neolycopersici* resistance loci. Chu et al (2004) have '*in-silico*' mapped 568 defense related ESTs to 588 loci on the rice linkage map based on sequence homology to the fully sequenced rice genome. The international tomato sequencing project is on going, and together with the high-density tomato linkage map and the ready accessible Solanaceae genomics network (SGN) it is plausible to *in-silico* map all DE-TDFs in the near future. The map positions of DE-TDFs, together with map position of the *R* genes and transcript profiles of test populations segregating for the *R* gene, will allow the identification of sets of genes that are co-regulated in defense responses.

In summary: We have demonstrated that the compatible interaction of tomato and *O.*

neolycopersici is robust and suitable to monitor the reproducibility of this pathosytem in cDNA-AFLP analysis. Generally, defense pathways involved in susceptible, monogenic- and polygenic resistance responses overlap. Genes involved in susceptibility or basal defense in compatible interaction showed similar expression timing in both compatible and incompatible interactions and may result from the compatible cells to powdery mildew in tomato. Transcripts of Class III that are differentially expressed in both the compatible and incompatible interactions of tomato and *O. neolycopersici* and are systemically induced, display earlier and/or higher expression in all incompatible interactions (monogenic and polygenic) compared to compatible interactions. SA and H_2O_2 may be important diffusive signals for both *Ol-1* and QTL mediated resistance in tomato NILs. The differentially expressed genes specific to resistant genotypes or individual *R*-QTLs, which occupy a small percentage of all DE-TDFs analyzed, are generally involved in transcriptional and translational regulation and signaling. It is likely that they fine-tune the activation of defense pathways in resistant genotypes through regulating transcription and translation. Pyramiding of *R*-QTLs into a single tomato line results in a high-level resistance, comparable to that mediated by *Ol-1,* and generally the same defense pathways are triggered by these combined *R*-QTLs compared to individual *R*-QTLs. One of the sequenced DE-TDFs with unknown function could be a good candidate for *Ol-1* and/or *R*-QTL1 because of its specific expression and co-localization with *Ol-1* and *R*-QTL1.

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Chapter 6

General discussion

In this thesis, the mechanisms of host susceptibility, monogenic- (dominant and recessive), and polygenic resistance responses during the interaction of tomato and *O. neolycopersici* have been investigated at the whole-plant, cellular and transcriptional level.

In the general introduction, the research progress on plant - biotrophic fungus interaction is reviewed and the different aspects are interrelated with the tomato - *O.neolycopersici* pathosystem: susceptibility and resistance; nonhost and host resistances; monogenic and polygenic resistance; recessive and dominant *R* genes. A large-scale cDNA-AFLP screening resulted in a general impression on the differences in pathogen-induced transcriptomes of susceptible, monogenic- (dominant) and polygenic resistant genotypes (Chapter 2). This was further elaborated in chapter 3, where the resistance responses, mediated by two monogenic dominant and one recessive *R* gene in near isogenic genetic backgrounds, were surveyed and compared with the susceptible response using cDNA-AFLP and RT-PCR with primers derived from genes involved in known defense pathways and components required for resistance or susceptibility (Chapter 3). Microscopic observations on fungal growth and host responses in susceptible plants, near isogenic lines containing a recessive or a dominant *R* gene, and different combinations of the resistance QTLs (*R*-QTL), allow us to propose cellular mechanisms underlying these host responses (Chapter 4). Furthermore, the pathogen-induced transcriptome changes of susceptible genotypes, these near isogenic lines containing monogenic *R* genes and different combinations of the three available *R*-QTLs from *S. neorickii* were monitored using cDNA-AFLP (Chapter 5).

In this chapter, we attempt to obtain a bird's eye view of the interaction of tomato and *O. neolycopersici* by cross-linking the different aspects of this interaction. Possible model plants suitable for studying this pathosystem will be discussed and future research based on the messages of this thesis will be proposed.

Host defense barriers are established at different infection stages in the *tomato – O. neolycopersici* interaction

Powdery mildew caused by the biotrophic fungus *O. (neo)lycopersici* has recently become a serious worldwide disease of tomato, especially in glasshouse cultivation (Jones et al., 2001). A compatible interaction between tomato and *O. neolycopersici* is the result of successful spore deposition, spore germination and germ tube development, appressorium formation, cell wall penetration and haustorium formation, colonization and sporulation (Niks and Rubiales, 2002; Chapter 4). During the incompatible interaction, resistant tomato plants can theoretically arrest the growth of the fungus at any of the infection stages. Based on the results of this thesis (Chapter 4) and our previous results (Bai et al., 2005), we propose that the tomato resistances investigated in this thesis, mediated by monogenic dominant *R* genes, a recessive *R* gene or *R*-QTLs, are associated with five different infection stages (Figure 1).

These infection stages include: 1) germination of fungal spores (*ol-2*), 2) penetration stage, with the attacked host cells staying alive (*ol2*); 3) penetration stage, with the attacked host cells subsequently activating a necrotic response, so called fast HR or single-cell HR (*Ol-4* and *R*-QTL); 4) post-primary-haustorial stage, when the secondary haustoria trigger programmed cell death in the attacked host cells, so called slow HR or multiple-cell HR (*Ol-1* and *R*-QTL); 5) post-secondary-haustorial stage, when penetrated-papillae, vesicle accumulation and structural changes in the extra-haustorial matrix appear, which may suppress the nutrient uptake of *O. neolycopersici* from tomato (*Ol-1* and *R*-QTL) (Chapter 4).

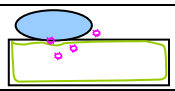
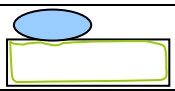
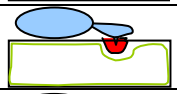
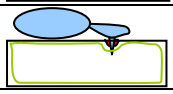
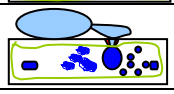
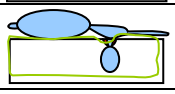
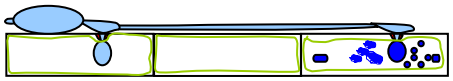
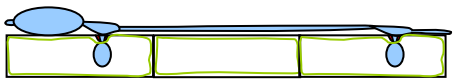
Stage	Gene/ QTL*	Incompatible interaction	Compatible interaction
I	*ol-2*		
II	*ol-2*		
III	*Ol-4*/ QTLs		
IV	*Ol-1*/ QTLs		
V	*Ol-1*/ QTLs		

Figure 1 Cartoon of the main interaction stages in compatible and incompatible interactions, at which tomato establishes defense barrier to hamper growth of the powdery mildew fungus (*O. neolycopersici*)

* Refers to the *R* genes or *R*-QTLs that mediate resistance associated with the corresponding stages

I. Germination of fungal spores

II. Penetration, papilla formation, while the attacked cell stays alive

III. Primary haustoria formed in cells that subsequently undergo an HR (so-called fast HR)

IV. Most primary haustoria are formed in cells that remain alive, while the later formed haustoria trigger necrotic responses in the attacked host cells (so-called slow HR)

V. Nutrient uptake through the haustoria is suppressed, correlated with penetrated papillae, vesicles around/in haustoria and changes in extra-haustorial matrix

Previous microscopic observations (Bai et al., 2005) revealed that the resistance in tomato to *O. neolycopersici* mediated by *Ol-3* and *Ol-5* is strongly associated with slow-HR, like *Ol-1*-based resistance, while resistance response mediated by *Ol-6* is associated with fast-HR like *Ol-4*-based resistance. The defense barriers established in tomato plants carrying *Ol-3* and *Ol-5* could be similar to those in *Ol-1*-mediated resistance. *Ol-4*- and *Ol-6*-based resistances could also involve similar defense barriers. Therefore, the studied tomato *R* genes (*Ol-1, ol-2, Ol-3, Ol-4, Ol-5,* and *Ol-6*) and *R*-QTLs are not affecting all the infection stages of the interaction. Screening tomato mutant libraries (EMS, ethyl methanesulfonate; Activation tagging and T-DNA insertion) may result in the discovery of host barriers in other infection stages, like germination tube and appressorium development.

Timing of resistance responses at cellular level determines the outcome of the tomato – *O. neolycopersici* interaction: Resistant or Susceptible

The resistance response triggered by *ol-2* mainly involves papillae formation and suppression of spore germination, but is not associated with HR. *Ol-1* and *Ol-4* mediated resistance responses are associated with slow HR and fast HR respectively (Bai et al., 2005; Chapter 4). We further demonstrated that fast-HR, that is micro-HR in cells with primary haustoria, is involved in *R*-QTL mediated resistance with a low incidence. By contrast, slow-HR, that is micro-HR in cells with secondary haustoria, is also involved in *R*-QTL-mediated resistance, but pyramiding of *R*-QTLs is correlated with an increased incidence of necrotic cells triggered by the pathogen (Chapter 4). The different individual *R*-QTL(s) mediate different micro-HR phenotypes, while the pyramiding of *R*-QTL(s) in one tomato line resulted in a "new" type of necrotic cells, which is associated with vesicles. The incidence of necrotic cells coincides with the resistance level in near isogenic lines containing different combinations of *R*-QTL(s); the more *R*-QTLs, the more necrotic cells, the higher the resistance (Chapter 4). The incidence of necrotic cells in near isogenic lines carrying all three *R*-QTLs is even somewhat higher than that in the near isogenic line (NIL) containing the *Ol-1* gene.

In the QTL-NILs, formation of penetrated-papillae, vesicle accumulation and structural changes in the extra-haustorial matrix are also observed, which may suppress the nutrient uptake by *O. neolycopersici* in the QTL-NILs (Chapter 4). Unexpectedly, some of the above-described responses were also observed in the susceptible genotype S-MM inoculated with *O. neolycopersici,* at later time-points (Chapter 4). For example, formation of penetrated papillae and structural changes in haustoria were detected in inoculated S-MM at 89 hours post inoculation (hpi), while these cellular responses appeared at 65 hpi in resistant genotypes. At 89 hpi, necrotic cells triggered by pathogen invasion were even detected in S-MM (Chapter 4). It appears that several cellular resistance responses are also employed by susceptible plants. However, these responses in susceptible plants are likely activated too slowly to stop the fungal growth, but may suppress fungal growth and represent the basal defense. In conclusion, timing of resistant responses at cellular level is crucial for the outcome of the interaction: susceptible or resistant. A critical time-point may exist for the interaction of tomato and *O. neolycopersici*. Among the *R* genes investigated in this thesis, the fast responses like formation of non-penetrated papillae mediated by *ol-2* and fast single-cell HR mediated by *Ol-4* lead to a high level of resistance.

Pathogen-induced transcript profiles of compatible and slow HR (*Ol-1*) mediated incompatible interactions of tomato and *O. neolycopersici* target overlapping gene sets and are controlled by quantitative mechanisms

Basal defense in susceptible genotypes restricts the extent of disease caused by virulent pathogen races (Eulgem, 2005). Our results suggest that the basal defense in susceptible genotype is a slow resistance response, which uses the same set of genes as the resistance response in *Ol-1* and *R*-QTL containing resistant genotypes during the interaction with *O. neolycopersici*. A faster activation of these defense genes responding to *O. neolycopersici* appears to result in a higher resistance level and earlier arrest of

pathogen growth in tomato, which coincides with the above-described cellular machinery of the interaction.

Thus, although the incompatible and compatible interactions result in qualitatively different macroscopic phenotypes, differences in pathogen-induced transcriptomes of compatible and incompatible interactions are quantitative rather than qualitative (Eulgem, 2005; Tao et al., 2003; Chapter 2). We indeed found that a large proportion of differentially expressed genes, activated in incompatible interactions (*Ol-1*-based and *R*-QTL-based), are triggered in compatible interaction as well in the tomato - *O. neolycopersici* pathosystem. About half of these commonly up-regulated genes displayed an earlier timing or a higher level of expression in incompatible interactions compared to compatible interactions (Chapter 2). In Chapter 5, we also profiled transcripts in non-inoculated systemic S-MM leaves, and found that most of these common transcripts with earlier timing or a higher level of expression are also induced systemically (Chapter 5). This quantitative and timely regulation of transcriptome coincides with microscopic observations, in which quantitative and time differences of cellular resistance responses affect the resistance at the whole plant level (Chapter 4).

We demonstrated that pyramiding of *R*-QTLs dramatically increases the resistance at the whole plant level and gives rise to a new type of necrotic cells (Chapter 4). Gene sets activated upon *O. neolycopersici* inoculation in tomato NILs with different combinations of *R*-QTLs predominantly overlap (Chapter 5); only a small subset of the induced genes are *Ol-1* or *R*-QTL specific. The genes may fine-tune the common employed defense pathways and be associated with the different incidence and types of necrotic cells in tomato lines carrying three *R*-QTLs.

Does *Ol-4* mediated resistance employ different mechanisms from *Ol-1* mediated resistance?

The resistances mediated by *Ol-4* and *Ol-1* both involve the hypersensitive response, however resistance mediated by *Ol-4* involves a fast, single cell HR and the resistance mediated by *Ol-1* involves a slow, multiple cell HR. Since HR is involved in both interactions, the expectation was that a similar set of genes would be induced with a different activation speed in the NIL-Ol-4 interaction compared to the NIL-Ol-1 interaction. However only less than one fourth of the mutually up-regulated DE-TDFs in S-MM (susceptible) and NIL-Ol-1 (slow-HR) are also associated with the *Ol-4*-mediated (fast-HR) resistance response (Chapter 3). This seeming contradiction can be brought about by three reasons: The first one is that fast-HR mediated resistance employs a different defense mechanism compared to slow-HR mediated resistance. The second one is that the defense response of *Ol-4*-mediated resistance is fast and restricted to the attacked cells only (Chapters 3 & 4), as a result the transcript amplitudes of many induced genes may be too low for detection by cDNA-AFLP analysis. Another explanation is that in the inoculated NIL-Ol-4 leaves virtually no compatible cells, which are cells that allow haustorium formation and do not undergo HR, exist. If the common up-regulated DE-TDFs with similar timing in compatible (S-MM) and incompatible (*Ol-1*) interactions represent the basal defense response or genes required for susceptibility, then these TDFs are not expressed in inoculated NIL-Ol-4.

The expression pattern of glutathione S-transferase (GST) (Chapter 3, table 3, No.18 & 19), an indirect measure of H_2O_2 production, indicates that H_2O_2 production is involved in HR based *Ol-4* and *Ol-1* mediated resistance responses, with an earlier expression in *Ol-4* mediated resistance, indicating the existence of a common defense mechanism between these two responses but differing in time course. This is consistent with the cellular mechanisms in the interaction of barley carrying different *R* genes with the barley powdery mildew fungus (e.g. Hückelhoven et al., 1999).

We also demonstrated that fast-HR associated resistance could employ different defense mechanisms compared to slow-HR associated resistance. The results suggested that the lipoxygenase pathway is involved in *Ol-4* mediated resistance, but not in *Ol-1* mediated resistance and basal defense of S-MM (Chapter 3). Both the SA and JA pathways are not involved in *Ol-4* mediated resistance, and probably the ethylene pathway is associated with the early expression of *PR1* and *GluB* (Chapter 3). In comparison to *Ol-4* mediated resistance, both SA and ethylene may play a role in the *Ol-1* mediated resistance response (Chapter 3).

In general, *Ol-4* mediated resistance may employ different defense mechanisms, which subsequently regulate the activation speed of defense mechanisms that are common between *Ol-4* and *Ol-1* mediated resistance.

Resistance mediated by the recessive *ol-2* gene employs different mechanisms from that mediated by dominant *R* genes or *R*-QTLs

The *ol-2*-based resistance is associated with papilla formation and suppression of spore germination, thus employs very different mechanisms from HR-associated resistances mediated by dominant *R* genes (Bai et al., 2005) or *R*-QTLs (Chapter 4). Expression profiles of several genes reflecting the well-described defense pathways (JA, ethylene and SA) were monitored. *GluB* and *ETR1* are not induced in the *ol-2*-mediated resistance response, while *leCOI1* and *Pin2*, show similar expression patterns in *ol-2*-mediated resistant responses and in susceptible interactions (Chapter 3), indicating that JA, SA and ethylene pathways are not involved in *ol-2* resistance. Therefore, we assume that the Ol-2 protein is a compatibility factor necessary for the successful establishment of compatibility between tomato and *O. neolycopersici*, or that *ol-2*-mediated resistance employs another defense pathway.

LoxD, which is involved in the octadecanoid defense signaling pathway and oxidative peroxidation of membranes, is induced in the *ol-2*-mediated resistance response, coinciding with the timing of papilla formation (Chapter 3). This lipoxygenase (LOX) is an isoform of LOX likely other than the isoform that is an enzyme in JA biosynthesis (Chapter 3). This indicates that lipoxygenase (LOX) plays a role in *ol-2*-mediated resistance response via oxidative peroxidation, traumatin or divenyl esters synthesis rather than the synthesis of JA.

A number of DE-TDFs specific to *ol-2*-mediated resistance response are homologous to signaling components, including an elicitor receptor kinase (Chapter 3, table 3, No.23), suggesting that a novel defense pathway is activated. Additionally a number of DE-TDFs, which were expressed earlier or higher in *ol-2*-mediated resistance response, are homologous to transcripts involved in regulation of transcription. These transcripts may

contribute to the fast formation of non-penetration papillae in incompatible cells in *ol-2*-mediated resistance responses compared to the penetrated papillae in other genotypes. Despite all differences, still about 40% of common genes of compatible (S-MM) and incompatible (*Ol-1* and *R*-QTLs) interaction are involved in the *ol-2*-mediated resistance response (Chapter 3). The fact that about 20% of the attacked cells in the tomato *ol-2* line display a compatible phenotype, allowing successful establishment of haustoria (Chapter 4), may partly account for the expression of these common induced genes in *ol-2*-mediated resistance response.

Genes required for *R*-gene-mediated resistance: implications for the tomato – *O. neolycopersici* interaction

The “gene for gene” model suits many pathosystems, however, direct interaction between AVR and R proteins is only proven in a few cases, such as the tomato Pto kinase with AvrPto from *Pseudomonas syringae* pv tomato, the rice Pi-ta with rice blast AVR-Pita, and the *Arabidopsis* RRS1-R with the corresponding Avr protein PopP2 of *Ralstonia solanacearum* (Tang et al., 1996; Jia et al, 2000; Deslandes et al., 2003). More and more research results suggest that the interaction between AVR and R proteins is not direct and a "third" party is involved (Dangl and Jones, 2001; De wit, 2002; Rooney et al., 2005). Therefore, it is very important not only to study the resistance genes themselves but also these so-called "third" parties in order to better understand the plant defense response. Many plant - pathogen systems do fit this model and the supplementary “guard” hypothesis (Dangl and Jones, 2001). The *Arabidopsis* gene product RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for *RPM1*-mediated resistance in *Arabidopsis* (Mackey et al., 2002; Coaker et al., 2005; Day et al., 2005 and Kim et al., 2005). For the well-studied pathosystem - tomato and *Cladosporium fulvum*, several fungal avirulence genes and corresponding tomato *Cf* genes have been cloned and are further investigated. *Rcr1* and *Rcr*2, are required in tomato for full *Cf-9*-dependent resistance to *Cladosporium fulvum* carrying *Avr9* (Hammond-Kosack et al., 1994). Likewise, Rcr3 protease is required for *Cf-2*-mediated resistance and binds to Avr2, but is not required for *Cf-5*-mediated resistance (Dixon et al., 2000; Kruger J et al., 2002; Rooney et al., 2005). In another well-investigated pathosystem, barley and *Blumeria graminis* f. sp. *hordei* (*Bgh*), dozens of resistance gene loci render the plant resistant against different *Bgh* isolates (Schulze-Lefert and Vogel, 2000). These genes govern fungal arrest at different stages of the interaction: at the penetration stage while the attacked cells stay alive (*mlo*); at the penetration stage in cells that subsequently undergo a single-cell HR (*Mlg*); or after fungal penetration by a subsequent multi-cell HR (*Mla12*) (Hückelhoven et al., 2001). *Rar1* and *Rar2* are required for the resistances mediated by several *Mla*, *Mih* and *Mlk* resistance genes, but not for *Mla-1*, *Mla-7*, *Mlp* and *Mlg*. *Ror1* and *Ror2* are necessary for the broad-spectrum resistance governed by *mlo* (Freialdenhoven et al, 1994 and 1996) (Figure 1).

In the tomato - *Oidium* interaction, *ol-2* mediated resistance to *O. neolycopersici* is associated with papilla formation, which is similar to the *mlo* dependent resistance against *Bgh*. *Ol-4* mediated resistance to *O. neolycopersici*, phenotypically similar to *Mlg* mediated resistance against *Bgh*, triggers a single-cell HR upon fungal penetration.

Additionally, *Ol-1* mediated resistance to the tomato powdery mildew fungus is associated with multi-cell HR, similar to *Mlal2* dependent resistance to the barley powdery mildew fungus (Bai et al., 2005; Chapter 4). We expect that in the tomato – *O. neolycopersici* pathosystem genes required for *R*-gene-mediated resistance should also exist and play roles like *RIN4, Rcr1, Rcr2, Rcr3 Rar1, Rar2, Ror1* and *Ror2*.

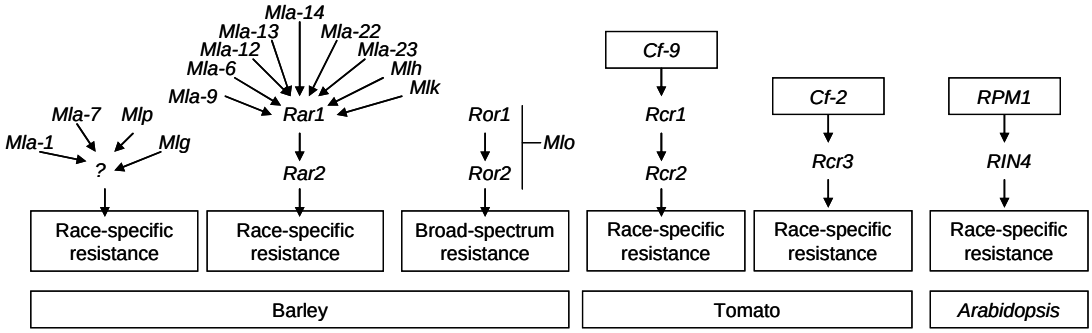


Figure 1 Pathways leading to resistance in barley, tomato and *Arabidopsis*, in which genes required for *R*-gene-mediated resistance are identified or expected. (Adapted according to Schulze-Lefert and Vogel, 2000; Hammond-Kosack et al., 1994; Dixon et al., 2000; Mackey et al., 2002)

In the present study, expression of three sequenced DE-TDFs homologous to heat shock proteins (HSPs) were induced in susceptible and resistant tomato plants after inoculation (Chapter 2). In plants, HSPs (HSP90 and HSP70 family) act as molecular chaperones of among others R proteins (reviewed by Schulze-Lefert P, 2004). We propose that HSPs are involved in the complex of Ol and AvrOl proteins during the interaction of tomato and *O. neolycopersici*. Further functional analyses, for example RNA interference, of the DE-TDFs obtained in this thesis may lead to the discovery of genes required for different tomato resistances to *O. neolycopersici*.

An efficient way to identify genes required for resistance is mutagenesis. *Rcr1* and *Rcr*2, which were discovered by screening an EMS treated population, are required in tomato for full *Cf-9*-dependent resistance to *C. fulvum* (Hammond-Kosack et al., 1994). *Rcr3*, a tomato gene required specifically by *Cf-2*, was also identified through screening an EMS treated population (Dixon et al., 2000). Through investigation of EMS treated barley populations, *Ror1, Ror2, Rar1* and *Rar2* have been identified (Freialdenhoven et al, 1994 and 1996). The isolation of T-DNA insertion allele *rin4* enabled the characterization of the functionality of *RIN4* in *Arabidopsis* (Mackey et al., 2002). We have well-defined NILs carrying different tomato *R* genes and *R*-QTLs, which are confirmed by linked molecular markers and disease tests. It is plausible to screen for susceptible mutants carrying *R* genes or *R*-QTLs in mutant libraries that can be derived from these resistant NILs. These susceptible mutants can lead to the discovery of the genes required for tomato resistances to *O. neolycopersici*.

It is more difficult to obtain randomized mutants in crops than in model plants that often have smaller genomes and are easier to manipulate. The work on model plants may result in prior knowledge for studies in crops. The fact that *ROR2* (basal penetration resistance to *Bgh* in barley) and *PEN1* (non-host resistance to *Bgh* in *Arabidopsis*) are

functionally homologous synataxin family members indicates a specialized resistance function conserved between *Arabidopsis* and barley (Collins et al., 2003). It increases our belief to use a model plant to study the tomato – *O. neolycopersici* interaction.

Possible model plants to study the tomato - *O. neolycopersici* interaction

Arabidopsis was reported to be susceptible to *O. neolycopersici* Oxford (Xiao et al., 2003), however our previous results indicated that *Arabidopsis* is resistant to the Dutch isolate of the tomato powdery mildew fungus. This resistance was considered as non-host resistance (Huang et al., 2000). New resistance tests with *Arabidopsis* [both Col0 and *pen1.1* mutant plants (Assaad et al., 2004) were tested] showed however that *Arabidopsis* is susceptible to the *O. neolycopersici* isolate from Wageningen. The *Arabidopsis* plants displayed obvious disease symptoms after inoculation with a spore suspension of *O. neolycopersici* (Figure 2-A), and back inoculation of tomato with infected *Arabidopsis* leaves using the print-inoculation method, showed that these spores were still virulent on tomato (Figure 2-B). These results make it possible to use *Arabidopsis* as a model plant to study the interaction with *O. neolycopersici*. The variety of genetic resources and well-defined mutant libraries, make it plausible to find suitable genotypes, like resistant accessions, to study the defense pathways against this pathogen. The ease of obtaining transgenic plants through flower dipping, the large number of mutant lines, rich germplasm resources and the sequenced genome of *Arabidopsis* ensure acceleration of the understanding of the interaction mechanisms with this pathogen from the plant side. Therefore, *Arabidopsis,* as a host of *O. neolycopersici* Wageningen, can be a useful model plant to decipher the resistance and susceptibility to this pathogen in tomato. The first step in this research will be the identification of resistant genotypes by screening germplasm and different mutant libraries, for example, the publicly available genome-wide T-DNA insertion lines maintained in ABRC at Ohio State University (Alonso et al., 2003).

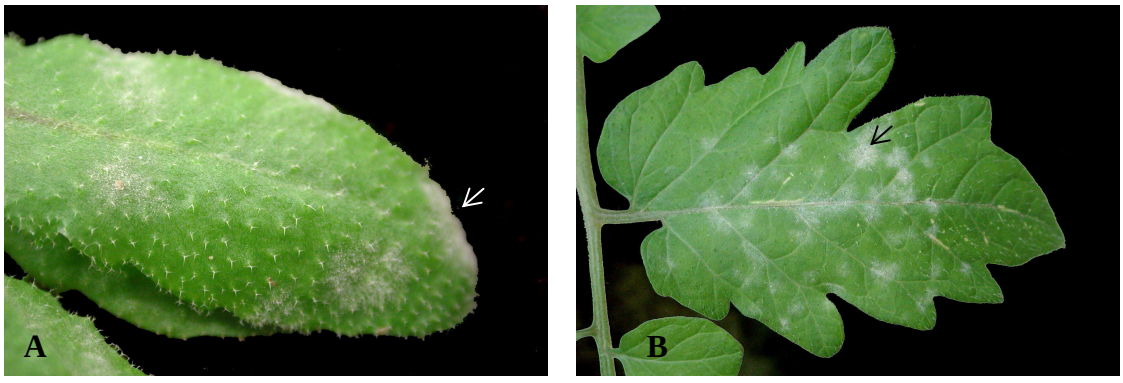


Figure 2 Pictures that illustrate *Arabidopsis* is a host of *O. neolycopersici*

A: The disease symptoms on an *Arabidopsis* leaf at 20 days post inoculation with a spore suspension of *O. neolycopersici;* B: The disease symptoms on a tomato leaflet at 14 days after the print-inoculation with *O. neolycopersici* infected *Arabidopsis* leaves. Arrows indicate the visible fungal colonies of *O. neolycopersici* on the infected *Arabidopsis* and tomato (S-MM) leaves

Tobacco is another nice model species to investigate the mechanisms of resistant and susceptible responses to *O. neolycopersici* in tomato. Firstly, tobacco is an alterative

host of this fungus (Huang et al., 2000a); secondly, tobacco and tomato are from the same family – Solanaceace; and thirdly, virus-induced gene silencing (VIGS) and transformation methods are feasible and well developed in tobacco. In addition, susceptible and resistant tobacco species to *O. neolycopersici* have been identified (Niks, laboratory of plant breeding WU, personal communication).

Perspective

The project described in this thesis is part of a joint PhD program between Wageningen University (WU) and the Chinese Academy of Agricultural Sciences (CAAS), which aims to educate the involved PhD students and stimulate future cooperation between the Chinese PhD students and Dutch scientists as well (Bonnema et al., in press). The results obtained in this thesis will form the basis of the future collaboration between the laboratory of plant breeding (WU) and our future lab in China. The planned future research on the same pathosystem with Chinese isolates is presented below.

Previous research (Bai PhD thesis, 2004) showed that tomato – *O. neolycopersici* pathosystem suits the “gene for gene” model. It will be very interesting to investigate the pathotypes of Chinese isolates of the tomato powdery mildew fungus in the future, since the observed tomato powdery mildew in China is most likely caused by a Chinese isolate of *O. (neo)lycopesici* (Baoju Li, institute of vegetables and flowers CAAS, personal communication). With China's entry into the world trade organization (WTO) and the increasing life standard of Chinese people, the Chinese tomato producers have to produce fungicide-free tomato fruit in order to increase their competition ability in the national and international tomato market. This requires powdery mildew resistance in Chinese tomato cultivations and urges to conduct research on the tomato interaction with Chinese isolates of *O. neolycopersici*. I propose that the research on tomato - *Oidium* interaction in China will be carried out efficiently as following: Firstly, the Chinese isolate(s) of *O. (neo)lycopersici* will be characterized molecularly and microscopically. Secondly, the Chinese isolate(s) will be tested on the near isogenic lines developed in our laboratory and on a collection of wild tomato accessions. Thirdly, available resistant resources of tomato will be used directly in breeding programs and for research. Further investigations on the interaction mechanisms between Chinese isolate(s) of powdery mildew fungus and tomato will be carried out based on the results of this thesis. Eventually it will give clues to durable resistance breeding for worldwide tomato production.

The accumulation of different QTLs results in a high-level resistance (Chapter 4), we are hypothesizing that pyramiding of *R* genes and QTLs will result in a durable and high-level resistance, by combining the advantages of *R* genes and QTLs. In the *tomato – O. neolycopersici* pathosystem, linked markers to both the *Ol* genes and 3 QTLs are available, that can facilitate pyramiding of *R* genes and QTLs. We are also wondering whether pyramiding *ol-2* and dominant genes into one single tomato line could lead to high level of resistance with durability.

Arabidopsis and tobacco will be used as model plants to study the tomato and *O. neolycopersici* interaction, and serve as carriers for the functional analyses of the interesting differentially expressed TDFs detected in this thesis. The fact that *O. neolycopersici* mainly infects the leaf epidermal cells of tomato may facilitate the

application of transient expression assays in tomato (Schweizer et al., 2000) for functional analyses of the interesting DE-TDFs revealed in this thesis.

A number of the DE-TDFs are candidates for *R* genes or *R*-QTLs according to their expression pattern, BLAST results and co-localization with the *R* loci by *in-silico* mapping studies (Chapter 5, table 4). A number of DE-TDFs, that may also represent candidate genes, could not be mapped *in silico*. These DE-TDFs will be mapped using available segregating populations in the WU Laboratory of Plant Breeding. The further functional analyses of these candidates will help to determine whether they represent the *R* genes or QTLs.

As the study of plant-pathogen interactions always involves signal and substance exchanges between two living organisms (Schulze-Lefert and Vogel, 2000), investigation from the plant side only is not enough to understand the interaction mechanism. Since several *Ol* genes have been defined and geographic studies indicate that tomato – *O. neolycopersici* pathosystem suits the “gene for gene“ model, corresponding functional *AvrOl* genes from *O. neolycopersici* are expected. A functional cloning strategy (Takken et al., 2000) may be employed to isolate *AvrOl* genes from the pathogen. Tomato powdery mildew is an obligate biotrophic fungus that cannot complete its life cycle without a living host, making this fungus difficult to transform. Fortunately, the first stable transformation of the barley powdery mildew fungus, also an obligate biotropic fungus, was described and this method was claimed to be suitable for any obligate biotrophic fungus (Chaure et al., 2000). It is exciting that Arabi and Jawhar (2002) first developed the technique for the *in vitro* cultivation of this fungus. This *in planta* transformation system and the *in vitro* cultivation technique, which enables the molecular manipulation of *Avr* genes and other fungal genes in any obligate biotrophic fungus, could shed light on the fungal responses in the tomato – *O. neolycopersici* interaction, which could be a model pathosystem for the future.

Acknowledgments

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Summary

Tomato powdery mildew (*Oidium neolycopersici)* is a worldwide fungal disease of tomato (*Solanum lycopersicum*). The disease causes large damage in European tomato production, especially in the glasshouse production. Because the disease is relatively new, only a few resistant commercial cultivars have been developed. The common way to control this disease is by applying fungicides. A better understanding of this disease is needed to avoid the losses in tomato production and reduce fungicide spraying. Breeders and scientists screened wild accessions for resistance to powdery mildew and identified several sources of resistance. So far, five dominant, one recessive resistance gene and three major resistance QTLs (*R*-QTLs) have been discovered and mapped or fine-mapped on the tomato genome.

We have investigated the fungus-induced transcriptome by differential gene expression profiling using cDNA-AFLP of mock- and inoculated susceptible, monogenic and polygenic resistant genotypes (Chapter 2). Our results showed that in the tomato - *O. neolycopersici* interaction, twice as many genes are induced in the compatible interaction as in the incompatible interactions. Genes involved in basal defense of the compatible interaction and *R*-gene/QTL mediated resistance response in the incompatible interactions largely overlap. About 40% of these “common” genes display earlier expression in the incompatible interactions compared to the compatible interaction, while the remaining genes show a similar temporal pattern of expression in both interactions. Transcripts of the sequenced differentially expressed TDFs (DE-TDFs) that are earlier up-regulated in the incompatible interactions compared to the compatible interaction or that are resistance specific, predominantly execute putative roles in plant defense and signal transduction. By contrast, transcripts of the sequenced DE-TDFs showing similar temporal patterns in compatible and incompatible interactions are often associated with housekeeping functions and regulation (Chapter 2). Therefore, we propose that the host plants employ similar components of the defense pathways during both compatible and incompatible interactions of tomato and *O. neolycopersici*, and that the timing difference in the expression of these components contributes to the final outcome of the interactions (Chapter 2).

A set of near or nearly isogenic lines (NILs) carrying the dominant *R* genes *Ol-1, Ol-3, Ol-4, Ol-5* and *Ol-6*, the recessive gene *ol-2*, and different combinations of *R*-QTLs were previously developed. These lines have been used for microscopic analysis of the infection process (Chapter 4) and for the study of the fungus-induced transcriptome (Chapter 3 and Chapter 5). Microscopic observations revealed that the recessive gene *ol-2*, dominant *Ol* genes and different combinations of *R*-QTLs confer resistance to *O. neolycopersici* at different infection stages of the interaction. These include but are not restricted to: 1) germination of fungal spores (*ol-2*), 2) penetration stage, with the attacked host cells staying alive (*ol2*); 3) penetration stage, with the attacked host cells subsequently activating a necrotic response, so called fast HR or single-cell HR (*Ol-4* and *R*-QTL); 4) post-primary-haustorial stage, when the secondary haustoria trigger programmed cell death in the attacked host cells, so called slow HR or multiple-cell HR (*Ol-1* and *R*-QTL); 5)

post-secondary-haustorial stage, when penetrated papillae, vesicle accumulation and structural changes in the extra-haustorial matrix appear, which may suppress the nutrient uptake of *O. neolycopersici* from tomato (*Ol-1* and *R*-QTL) (Chapter 4). Microscopic investigations of infected leaves of NILs carrying different *R* genes/QTLs demonstrated that HR is involved in *R*-QTL mediated resistance responses and NILs with different individual *R*-QTL(s) display different types of necrotic cells upon the fungal inoculation. Penetrated papillae, vesicle accumulation and changes in the extra-haustorial matrix are also associated with *R*-QTL-mediated resistance responses, but are not specific to individual *R*-QTL(s). The pyramiding of *R*-QTL(s) into a single tomato line results in a high-level resistance comparable to *Ol-1*-mediated monogenic dominant resistance, mainly because of a high incidence of a novel type of necrotic cells (Chapter 4). Our results suggest as well that the genetic background can influence the *R*-QTL mediated resistance, since we found that different tomato lines with the same combination of *R*-QTLs display different resistance phenotypes. For instance the macroscopic lesion size and incidence on inoculated leaflets are different in N3Qa and N3Qb, which both have all three *R*-QTLs (Chapter 4). A smaller lesion is associated with a faster reaction, thus less costly for the plant. Therefore, multi-*R*-QTL tomato plants with suitable genetic backgrounds could be selected in breeding programs through evaluation of the macroscopic lesion size after inoculation. Furthermore, we assume that *Ol* genes may be added to cultivars with all three *R*-QTLs in a suitable background to create an additional insurance for high-level durable resistance to *O. neolycopersici*.

Tomato lines containing the recessive *R* gene *ol-2*, the dominant *R* genes *Ol-1* and *Ol-4* mediate resistance responses involving papilla formation, slow HR and fast HR respectively. The resistance responses to powdery mildew (*O. neolycopersici)* controlled by these genes were investigated using cDNA-AFLP and reverse transcription PCR (RT-PCR). The results indicate that the transcript profiles of these responses are different. Generally, the transcript profiles of *Ol-1* mediated resistant and susceptible responses are similar. A large part of the common up-regulated genes in both the *Ol-1* mediated resistance and the compatible interaction (S-MM) was not induced in the *Ol-4* mediated resistance response and not or later up regulated in the *ol-2* mediated resistance response. Sequence information of a small number of differential expressed transcript derived fragments (DE-TDF) specific to *Ol-4* and *ol-2* mediated resistance responses further suggests that these responses are different from the *Ol-1* mediated resistance response. The RT-PCR analyses indicated that the *ol-2* mediated resistance involving papilla formation is independent of SA, JA and ethylene pathways. Therefore, we suggest that the Ol-2 protein is a compatibility factor necessary for the successful establishment of compatibility between tomato and *O. neolycopersici*, or that *ol-2*-mediated resistance employs another defense pathway. The *Ol-4* mediated resistance response is associated with the ethylene pathway but not JA and SA. An isoform of lipoxygenase (LOX), likely other than 13 LOX, plays a role in both *Ol-4* (fast HR) and *ol-2* (papilla formation) mediated resistance responses. *Ol-1* mediated resistance appears to be associated with both SA and ethylene pathways (Chapter 3).

We also analyzed the pathogen-induced transcript profiles of tomato lines containing different combinations of *R*-QTLs to *O. neolycopersici*, and compared those with the

profiles of the S-MM and a NIL carrying the dominant *R* gene *Ol-1*, using cDNA-AFLP. About 4,000 bands were displayed with 78 selected primer combinations. In total, 204 DE-TDFs were induced upon *O*. *neolycopersici* inoculation and 72 DE-TDFs displayed a differential expression level between NILs that was not dependent on inoculation. Transcripts that show similar timing in both compatible and incompatible interactions were associated with basal defense or establishment of compatibility, probably the result of the response of successfully attacked cells. Transcripts that display earlier/higher expression in the incompatible interactions compared to the compatible interaction showed a systemic induction as well. Salicylic acid (SA) and H_2O_2 might be important diffusive signals for both monogenic and polygenic resistance in these tomato NILs. The small fraction of differentially expressed genes specific to (partially) resistant genotypes may fine-tune the activation of defense pathways in resistant genotypes through regulating transcription and translation. Pyramiding of *R*-QTLs into a single tomato line leads to a high-level resistance comparable to that mediated by *Ol-1,* and generally the same defense pathways are triggered by these combined *R*-QTLs compared to individual *R*-QTLs. We propose that pyramiding of *R*-QTLs only alters the defense pathways quantitatively rather than qualitatively. The map locations of eleven sequenced DE-TDFs were *in-silico* determined. One of them could be a good candidate for *Ol-1* or *R*-QTL1 because of its specific expression to and co-localization with *Ol-1* and *R*-QTL1.

The combined results of Chapters 2-5 are discussed in relation to relevant literature. We demonstrated that tomato defense barriers to *O. neolycopersici* are correlated with different infection stages during the interaction. Conservation and quantitative nature of pathogen-induced transcriptomes of compatible and slow HR (*Ol-1*/*R*-QTL) mediated incompatible interactions of tomato and *O. neolycopersici* are proposed. Additionally, transcriptome changes during the resistance mechanisms mediated by fast HR (*Ol-4*) and by papilla formation (*ol-2*) suggest different resistance mechanisms that are proposed. Genes required for the resistance mediated by *R* genes/QTLs in tomato are hypothesized. The experiment that proves that *Arabidopsis* is a host of *O. neolycopersici* is presented and *Arabidopsis* and tobacco as model plants for studies on the interaction of tomato and *O. neolycopersici* are discussed. Future work is suggested according to the messages of the thesis.

中文摘要

单基因和多基因控制的番茄白粉病抗性的细胞学和转录组学分析

李成伟

番茄白粉病 (*Oidium neolycopersici)* 是一个世界范围内传播的真菌病害，对番茄(*Solanum lycopersicum*) 的生产特别是温室生产造成了很大的危害。由于这种病害是从上个世纪 80 年代才开始爆发，市场上现在只能找到为数不多的几个抗病品种，因此现在防治这种病害的主要方法是施用杀虫剂，这种方法不仅增加了番茄生产投入成本也造成了环境污染。抗性材料的筛选和机理研究可以为持久抗性品种的培育提供育种亲本和理论依据，进而减少杀虫剂的使用有利于降低成本和保护环境。迄今，有 5 个显性，1 个隐性基因和 3 个主效抗性数量性状位点（*R*-QTL）被报道并定位或者精准定位到番茄的遗传图谱上。本论文在表现型，细胞和转录水平上分析了显性，隐性基因和主效 *R*-QTL 控制的抗性机理。

研究采用了 cDNA-AFLP 的方法，以非接种为对照分析了病菌诱导的感病，显性单基因和多基因抗性遗传材料的基因表达差异（第二章）。结果表明在番茄和白粉病菌亲和互作中番茄的上调表达基因数是非亲和互作中的 2 倍。抗性基因和 *R*-QTL 介导的对白粉病菌的抗性反应和感病反应在很大程度上诱导了相同的基因，大约 40%的这些基因在抗性基因和/或 *R*-QTL 介导的抗性反应中表达较早，而其它的基因表达表现相似的时序性。通过序列分析发现，抗性反应特异性或者抗性反应中表达较早的差异表达的 cDNA-AFLP 片段 （differentially expressed transcript derived fragment, DE-TDF）主要来自植物防御反应和信号转导相关基因，而具有相似时序性的 DE-TDF 则来自调节和管家基因。因而我们认为抗病和感病反应可能诱导了相似的基因，而这些基因表达的时序性差异对番茄和白粉病菌互作的最终结果至关重要。

为了更好地研究不同基因或 QTL 控制的抗性反应机理，在细胞和转录水平上分析了以感病材料（Moneymaker, MM）为遗传背景的含有显性抗性基因 *Ol-1, Ol-3, Ol-5, Ol-4, Ol-5* 和 *Ol-6*, 隐性抗性基因 *ol-2*, 或者不同 QTL 组合的近等基

因系（near isogenic line, NIL）对白粉病菌的抗性反应（第三章和第五章）。显微镜分析结果显示这些基因和 QTL 控制的抗性反应可以表现在白粉病菌和番茄互作的不同阶段。这些抗性表现为：1）白粉病菌孢子萌发受到抑制（*ol-2*）；2）白粉病菌侵入番茄细胞阶段番茄细胞形成非穿透性乳突（non-penetration papilla）阻止了白粉病菌的进一步生长，番茄细胞没有发生程序性坏死（*ol-2*）；3）白粉病菌侵入番茄细胞阶段受入侵番茄细胞发生程序性坏死，所谓的快速过敏反应（hypersensitive response, HR）（*Ol-4* and *R*-QTL）；4）白粉病菌的次生吸器（haustorium）引起受入侵番茄细胞发生程序性坏死，所谓的慢性过敏反应(*Ol-1* and *R*-QTL)；5）白粉病菌吸器形成后番茄细胞内形成的穿透性乳突（penetrated papilla），小液泡和吸器基质结构异常化抑制白粉病菌从番茄细胞吸取营养（*Ol-1* and *R*-QTL）。

HR 在 *R*-QTL 介导的抗性反应中起主导作用并且在 3 个 *R*-QTL 之间表现加性，而穿透性乳突，小液泡和吸器基质结构异常化也和抗性反应相关但不具有加性效应。含有不同的 *R*-QTL 的 NIL 表现不同类型的坏死细胞。*R*-QTL 的聚合可以导致和 *Ol-1* 介导的单基因控制抗性相当的高抗水平，而且这种效应产生主要是因为坏死细胞比率的升高（第四章）。此外，含有相同的 *R*-QTL 姊妹 NIL 的可以具有不同的抗性表现，例如，接种白粉病菌的含有 3 个 *R*-QTL 的两个姊妹 NIL 的叶片上表现不同大小的坏死斑。小的坏死斑意味着快速的反应和植物可以用比较小的代价完成抗病过程。因而，含有多个 *R*-QTL 并且表现较小坏死斑的番茄植株应该可以作为一个比较好的抗病亲本应用到抗病育种中。进而我们还预测 *Ol* 和 *R*-QTL 的聚合可以使番茄具有高效持久抗性。

含有隐性抗性基因 *ol-2*，显性抗性基因 *Ol-1* 和 *Ol-4* 的番茄品系的抗性反应分别和乳突形成，快速 HR 和慢性 HR 相关。cDNA-AFLP 和 RT-PCR（reverse transcription PCR）分析表明这 3 种抗性反应的基因表达谱有所不同。大部分的 *Ol-1* 介导的抗性和感病反应中都表达的基因没有在 *Ol-4* 介导的抗性反应中表达，在 *ol-2* 介导的抗性反应中没有或者比较晚表达。通过对为数不多的几个特异于 *Ol-4* 或 *ol-2* 的 DE-TDF 进行序列分析，进一步验证了表达谱的不同。RT-PCR 分析显示 *ol-2* 介导的抗性反应和已知的水杨酸（SA），茉莉酸（JA） 以及乙烯防御反应途径无关。因而，我们推测 Ol-2 蛋白是一个番茄白粉病菌亲和

互作所必须的感病因子，或者 *ol-2* 介导的抗性反应采用了另外一种新的防御反应途径。乙烯防御反应途径参与 *Ol-4* 介导的抗性反应，但是水杨酸和茉莉酸防御反应途径和 *Ol-4* 介导的抗性反应不相关。脂氧化酶（LOX）在 *Ol-4* 和 *ol-2* 介导的抗性反应起重要作用，同时这种脂氧化酶被证实不是 13 LOX 异构酶。另外，*Ol-1* 介导的抗性反应和水杨酸和乙烯防御反应途径相关，但是需要进一步实验验证（第三章）。

根据以上的实验结果，使用 78 个经过挑选的引物组合对含有不同 *R*-QTL 组合和 *Ol-1* 的 NIL 以及感病材料 MM 进行了进一步的 cDNA-AFLP 分析。在显示的大约 4000 个条带中 204 个属于差异表达。其中 72 个 DE-TDF 只与基因型有关和处理不相关，132 个 DE-TDF 和处理相关。在亲和和非亲和互作中表现相同上调时序性的 DE-TDF 被认为来自于和基础防御反应或者亲和反应相关基因，这些基因的表达可能是被成功侵入的细胞反应的体现。那些在非亲和互作中表现较早表达时序性的基因被发现在感病植株中被系统性诱导。水杨酸和过氧化氢可能是这些单基因和多基因控制的番茄抗病材料的抗性反应中的重要信号分子。一小部分的特异差异表达的和调节转录和信号转导相关的基因可能负责着防御反应途径的精细调节。虽然 *R*-QTL 的聚合导致了抗病性水平的提高，但是没有采用不同于单独 *R*-QTL 诱导的防御反应途径。因此，我们认为 *R*-QTL 聚合仅从量上而不是从质上导致防御反应途径的改变。我们还通过电子（*in-silico*）作图进一步确定了 11 个 DE-TDF 在番茄遗传图谱上的位置。其中一个位于 *Ol-1* 和 *R*-QTL1 的区域，被确定为 *Ol-1* 或者 *R*-QTL1 的候选基因。

通过综合分析以上的结果并且和相关文献资料比较，我们得到以下结论：番茄白粉病抗性可以表现在番茄和白粉病菌互作的不同阶段。病菌诱导的番茄转录组变化在亲和互作和慢性 HR（*Ol-1* 和 *R*-QTL）引起的非亲和互作间具有保守性和数量性差异的特点。此外，快速 HR（*Ol-4*）和乳突形成（*ol-2*）介导的非亲和互作中番茄转录组变化表现出质的差异。

另外，拟南芥被证实是白粉病菌的寄主，而且论文还讨论了番茄白粉病抗性需要基因（genes required for resistance）和研究番茄和白粉病菌互作的模式植物以及对后续工作的构想。

Samenvatting

De schimmelziekte “echte meeldauw”, veroorzaakt door *Oidium neolycopersici,* is wereldwijd een van de belangrijkste ziekten in tomaat (*Solanum lycopersicum*). In de kasteelt in Europa veroorzaakt deze ziekte aanzienlijke verliezen en omdat de ziekte relatief nieuw is, is er nog maar een beperkt aantal resistente cultivars op de markt. Momenteel wordt deze ziekte vooral met chemische bestrijdingsmiddelen bestreden. Meer kennis over deze ziekte is nodig om verdere verliezen in de tomatenteelt te voorkomen en het gebruik van fungiciden te beperken. In veel wilde Solanum accessies is resistentie tegen echte meeldauw gevonden. Tot nu toe zijn er vijf dominante resistentiegenen, een recessief resistentiegen en drie kwantitatieve resistentiegenen (resistentie-QTLs) geïdentificeerd en gelokaliseerd op het tomatengenoom.

In dit proefschrift is de interactie van tomaat en echte meeldauw bestudeerd op gen-expressie niveau. Wanneer een tomatengenotype vatbaar is voor echte meeldauw is de interactie compatibel en wanneer een tomatengenotype resistent is spreken we van een incompatibele interactie. Met behulp van cDNA-AFLP (een RNA fingerprinting techniek) is het transcriptoom (de tot expressie komende genen) zichtbaar gemaakt, in zowel vatbare als monogeen en polygeen resistente tomatengenotypes, na inoculatie met echte meeldauwsporen. Dit wordt vergeleken met het transcriptoom na inoculatie met water (Hoofdstuk 2). De resultaten toonden aan dat in de tomaat - echte meeldauw interactie tweemaal zoveel genen geïnduceerd worden in een compatibele interactie vergeleken met de incompatibele interactie. Genen die betrokken zijn bij de basisresistentie in een compatibele interactie en bij de resistentie response van *R*-gen of resistentie QTL in de incompatibele interacties overlappen grotendeels. Het verschil is dat ongeveer 40% van deze gemeenschappelijke genen in de incompatibele interacties eerder tot expressie komt. Deze eerder tot expressie komende genen spelen voornamelijk een rol in de meer algemene afweer- en signaaltransductie, net als de genen welke specifiek zijn voor de incompatibele interactie. De resterende genen, die in zowel compatibele en incompatibele interacties op vrijwel hetzelfde moment tot expressie komen zijn daarentegen vaak geassocieerd bij de regulatie en ‘huishoud’ functies van de cel (Hoofdstuk 2). Hieruit werd geconcludeerd dat planten vergelijkbare componenten van een verdedigings signaal transductie netwerk gebruiken in zowel compatibele als incompatibele interacties tussen tomaat en *O. neolycopersici*, maar dat verschillen in timing van de betrokken genen bijdragen tot het uiteindelijke resultaat van de interactie: een resistente of een vatbare plant (Hoofdstuk 2).

In een voorgaand project is een set van bijna isogene lijnen (Nearly Isogenic Lines; NILs) ontwikkeld met daarin één van de dominante resistentiegenen *Ol-1, Ol-3, Ol-4, Ol-5*, *Ol-6*, het recessieve gen *ol-2*, of met verschillende combinaties van de kwantitatieve genen (resistentie QTLs). Deze lijnen zijn gebruikt voor de microscopische analyses van het infectieproces (Hoofdstuk 4) en om het door de schimmel geïnduceerde transcriptoom van de plant te bestuderen (Hoofdstuk 3 en Hoofdstuk 5). De microscopische studies toonden aan dat de resistenties tegen echte meeldauw in de verschillende bijna isogene lijnen zich openbaren in verschillende infectiestadia. Globaal

zijn dit de infectiestadia: 1) kieming van de schimmelsporen (*ol-2*), 2) penetratiestadium, waarbij de aangevallen cellen blijven leven (*ol-2*), 3) penetratiestadium, waarbij de aangevallen cellen doodgaan, de zogenoemde snelle overgevoeligheidsreactie (hypersensitieve response of HR) (*Ol-4* en resistentie-QTLs); 4) post-primair-haustoriaal stadium, wanneer secundaire haustoria geprogrammeerde celdood induceren in de aangevallen cellen, de zogenoemde langzame HR of multipele-cel HR (*Ol-1* en resistentie-QTL); 5) post-secondair-haustoriaal stadium, wanneer verschijnselen als gepenetreerde papillen, ophoping van vesikels en structurele veranderingen in de extra-haustoriale matrix verschijnen, die de opname van nutriënten uit tomaat, nodig voor de groei van *O. neolycopersici*, waarschijnlijk bemoeilijken (*Ol-1* en resistentie-QTLs) (Hoofdstuk 4). Microscopische observaties van geïnfecteerde bladeren, afkomstig van bijna isogene lijnen met verschillende resistentiegenen of resistentie QTLs, toonden dat de overgevoeligheidsreactie een belangrijke rol speelt in de kwantitatieve resistentie en dat verschillende necrotische celtypes worden geïnduceerd in schimmel geinfecteerde NILs waarin verschillende combinaties van resistentie QTLs aanwezig waren. De resistentie in tomatenlijnen met resistentie QTLs gaat verder gepaard met gepenetreerde papillen, ophoping van blaasjes en veranderingen in de extra-haustoriale matrix. Deze symptomen zijn echter niet uniek voor NILs met bepaalde resistentie-QTLs. Tomatenlijnen met een combinatie van alle drie resistentie-QTLs hebben een zeer hoog resistentieniveau, welke vergelijkbaar is met de resistentie veroorzaakt door het monogeen dominante resistentiegen *Ol-1*. Het veelvuldig voorkomen van cellen met een nieuw type necrose in deze multipele-resistentie-QTL NILs is hier waarschijnlijk debet aan (Hoofdstuk 4). De in dit proefschrift beschreven resultaten duiden er op dat de genetische achtergrond van een plant ook invloed heeft op de expressie van de kwantitatieve resistentie. Dit omdat de twee onafhankelijk ontwikkelde NILs met beide alle drie de resistentie-QTLs zich verschillend gedragen. Een voorbeeld hiervan was de grootte en frequentie van de macroscopisch zichtbare necrotische laesies. (Hoofdstuk 4). Een kleinere laesie gaat samen met een snellere afweerreactie, wat energetisch voordelig is voor de plant, en het is dus raadzaam in veredelingsprogrammas op basis van de grootte van de necrotische laesies een optimale genetische achtergrond te zoeken. Het combineren van *Ol* genen met de drie bekende resistentie-QTLs in tomatenlijnen met een optimale genetische achtergrond geeft de grootste kans hoogwaardige, duurzame resistentie tegen echte meeldauw te creëren.

In hoofdstuk drie is de resistentie in bijna isogene tomatenlijnen met het recessieve resistentie gen *ol-2* of één van de dominante *R* genen (*Ol-1* of *Ol-4*), onderzocht. De resistentie in deze lijnen gaat gepaard met papilvorming (*ol-2*), een langzame overgevoeligheidsreactie (*Ol-1*) en een snelle, single cel overgevoeligheidsreactie (*Ol-4*). De afweerreactie tegen echte meeldauw die door deze genen wordt gereguleerd is onderzocht op gen-expressie niveau met behulp van cDNA-AFLP en RT-PCR (*reverse transcription* PCR) van een set bekende, bij resistentie betrokken, genen. Uit de resutaten blijkt dat de genexpressie profielen van deze drie typen resistentie ook verschillend is. Over het algemeen zijn de genexpressie profielen van *Ol-1* gereguleerde resistentie en van de vatbare reactie vergelijkbaar. Een groot deel van de genen die zowel betrokken zijn bij *Ol-1* gereguleerde resistentie en bij de vatbare interactie in het ras Moneymaker

wordt niet geïnduceerd tijdens de *Ol-4* gereguleerde resistentie en niet of later geïnduceerd in geïnoculeerde *ol-2* lijnen. De basenpaarvolgorde van een klein deel van de onder invloed van *Ol-4* en *ol-2* specifieke, differentieel tot expressie komende genen suggereert dat de afweer reacties in deze lijnen substantieel verschillen van die in tomatenlijnen met *Ol-1*. RT-PCR met een selectie van genen welke indicatief zijn voor resistentie signaal transductie routes, toonde aan dat de resistentie in NILs met *ol-2,* die gepaard gaat met pappilvorming, onafhankelijk is van de salicylzuur (SA), jasmijnzuur (JA) en ethyleen signaal transductie routes. Gebaseerd op deze genexpressieprofielen concluderen we dat het Ol-2 eiwit een compatibiliteitscomponent is die onmisbaar is voor het succesvol tot stand komen van compatibiliteit tussen tomaat en *O. neolycopersici*, of dat deze resistentie een tot nu toe onbekend afweermechanisme gebruikt. Het lijkt erop dat de *Ol-4* gereguleerde resistentie afhankelijk is van de ethyleen route maar niet gebruik maakt van de salicylzuur (SA) en jasmijnzuur (JA) routes. Een isoform van lipoxygenase (LOX), anders dan de welbestudeerde 13 LOX, is betrokken bij de *Ol-4* (snelle HR) en *ol-2* (papilvorming) gereguleerde resistentie. De resistentie in NILs met *Ol-1* is geassocieerd met zowel de salicylzuur als de ethyleen routes (Hoofdstuk 3).

Met behulp van cDNA-AFLP hebben we ook de door de schimmel geïnduceerde genexpressie profielen van tomaten NILs met verschillende combinaties van resistentie QTLs verkregen en deze vergeleken met de profielen van geïnfecteerde vatbare Moneymaker en een NIL met het dominante *Ol-1* gen (Hoofdstuk 5). Met 78 AFLP primer combinaties werden ongeveer 4.000 banden (TDFs) gegenereerd. Van deze 4.000 TDFs kwamen er 204 differentïeel tot expressie na *O*. *neolycopersici* inoculatie, terwijl 72 DE-TDFs een differentieel expressie patroon hadden tussen de verschillende genotypes, onafhankelijk van *O. neolycopersici* inoculatie. De genen waarvan de TDFs gelijktijdig tot expressie kwamen in geinoculeerde vatbare Moneymaker en in de resistente lijnen (resistentie-QTL-NILs) waren over het algemeen betrokken bij de basis afweer of met de initiatie van compatibiliteit, wat waarschijnlijk het resultaat is van de reactie van succesvol geinfecteerde cellen in resistente lijnen. Genen met een vroegere of een hogere expressie in de incompatibele interactie vergeleken met de compatibele interactie komen vaak ook systemisch tot expressie. Salicylzuur en H_2O_2 zijn mogelijk belangrijke (vluchtige) signaalmoleculen voor zowel de monogene als de polygene resistentie in deze tomaten lijnen. De kleine fractie van differentieel tot expressie komende genen specifiek voor (partieel) resistente genotypen speelt wellicht een rol in de ‘fine-tuning’, via de regulatie van transcriptie en translatie, van de aktivatie van de verdedigingsroutes. Het stapelen van resistentie-QTLs in een enkele tomatenlijn of genotype leidt tot een hoog niveau van resistentie vergelijkbaar met het niveau dat verkregen wordt door het resistentiegen *Ol-1*. In het algemeen lijkt een enkele QTL dezelfde (weliswaar kwantitatief zwakkere) verdedigingsroutes aan te zetten als de gecombineerde resistentie-QTLs samen. Via *in silico* analyse konden elf gesequeneerde differentieel tot expressie komende TDF’s genetisch gekarteerd worden. Een van deze TDF’s is een mogelijk kandidaat gen voor *Ol-1* of resistentie-QTL1 vanwege zijn co-lokalisatie met *Ol-1* en resistentie-QTL1.

In hoofdstuk 6 worden alle resultaten van de hoofdstukken 2 tot en met 5 behandeld in relatie tot de relevante literatuur. We hebben aangetoond dat er een relatie bestaat tussen de verschillende afweer barrières en de verschillende infectie stadia van de *O.*

neolycopersici-tomaat interactie. We veronderstellen dat de door de pathogeen geïnduceerde transcriptomen in de compatibele en de incompatibele interacties (langzame HR (*Ol*-1) en resistentie-QTLs) geconserveerd en kwantitatief onderscheidbaar zijn. Tegelijkertijd kan gesteld worden dat de veranderingen in genexpressie tijdens de snelle HR (veroorzaakt door *Ol-4*) en tijdens de papilvorming (door *ol-2*) door andere resistentie mechanismen of routes moeten worden veroorzaakt. De verschillen in fenotypische respons van de NILs met dezelfde resistentie-QTLs worden bediscussieerd en er wordt gespeculeerd over de mogelijke identiteit van de genen die nodig zijn voor de resistentie veroorzaakt door *R* genen en resistentie-QTLs in tomaat. Een experiment dat bewijst dat *Arabidopsis thaliana* een gastheer is voor *O. neolycopersici* wordt gepresenteerd en er wordt gespeculeerd over de rol van *Arabidopsis* en tabak als model planten voor verdere studies betreffende de interactie van planten met *O. neolycopersici*.

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Chengwei Li
Wageningen, the Netherlands
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Curriculum vitae

Chengwei Li was born on 22 March 1972 in Minquan County, Henan province, China. He studied in Sun Lu junior middle school from 1983 to 1986, and in Minquan senior middle school from 1986 to 1989 before he entered the university. From 1989 to 1993 he did his undergraduate study and got his BSc degree in Henan Normal University. Afterwards he worked as a teacher in Shangqiu Teachers College for two years. In the year 1995 he began his study in Zhongshan (Sun Yet-San) University, which is located in Guang Dong province far from his hometown. He obtained the MSc degree in 1998, and went back to Shangqiu Teachers College working as a teacher for two years.

He commenced his PhD program in the Chinese Academy of Agricultural Sciences (CAAS) in 2000, which concerned investigations of the core collection of wheat germplasm using molecular markers. In 2001, he got the chance to participate in the Joint PhD program between CAAS and Wageningen University (WU). Thereafter, he changed his PhD research topic to “Study of the qualitative and quantitative resistance responses to powdery mildew in tomato by genetical genomics”. From October 2001 to October 2005, he shuttled between CAAS in China and WU in the Netherlands to accomplish his PhD study. He will defend his PhD thesis on 3 October 2005 in Wageningen to obtain his PhD degrees from both WU and CAAS. Hereafter he will do research on plant-pathogen interactions and teach students in Shangqiu Teachers College, China.

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3. **Chengwei Li**, Yuling Bai, Pim Lindhout, Xueyong Zhang and Guusje Bonnema. Study of the mechanisms underlying qualitative and quantitative resistance to powdery mildew in tomato by expression profiling. Abstracts of the 3rd Plant Genomics European Meetings (Plant GEMs) September 22-25 2004 Lyon France.
4. **Li CW**, Bai Y, Lindhout P, Zhang XY and Bonnema AB. Study of the mechanisms underlying qualitative and quantitative resistance to powdery mildew in tomato by genetical genomics. Abstracts of XI International Congress on Molecular Plant-Microbe Interactions, July 18-26 2003, St, Petersburg, Russia.
5. Yuling Bai, Ron van der Hulst, **Chengwei Li**, Caicheng Huang, Fien Meijer-Dekens, Guusje Bonnema and Pim Lindhout. Qualitative and quantitative resistance to tomato powdery mildew Oidium lycopersici studied by genetical genomics. Posters of Plant and Animal Genomie XI, January 11-12 2002, San Diego USA.
6. Yuling Bai, Caicheng Huang, Ron van der Hulst, Fien Meijer-Dekens1, **Chengwei Li**, Pim Lindhout and Guusje Bonnema. (2002) Qualitative and quantitative resistance to the tomato powdery mildew *Oidium lycopersici*. Abstract of First International Symposium on Crop Genomics and Genetic Improvement, Wuhan

China, September 21-28.

7. Li Zhuojie, Xiao Wang, Gao Manli and **Li Chengwei**. Study of using isoenzymes on the rapid purity measuring of hybrid watermelon seeds. In Programme & Abstracts of the Second International Conference on Seed Science and Technology (2nd ICCST). 12-16 May 1997. Guangzhou, China. PP. 105. China: the Journal of Sun Yet-San University.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Chengwei Li**
Date: **3 October 2005**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Study of the qualitative and quantitative resistance responses to Oidium lycopersici in tomato by genetical genomics	November 6, 2001
► **Writing a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Plant-pathogen relations (G200-216)	December, 2001
Bioinformation technology (BIT-1)(MIB-11306)	April, 2002
► **Laboratory use of isotopes**	
Safe handling with redioactive materials and sources	December, 2003
Subtotal Start-up Phase	*12.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD student day, Wageningen	November, 2001
► **EPS theme symposia**	
EPS theme 2 symposia	December, 2001
► **National meetings**	
NWO Lunteren days 2004	April 2004
NOW Lunteren days 2005	April 2005
► **Seminars (series), workshops and symposia**	
Auturm school 2003 (in CAAS)	November 2003
Flying seminar of EPS	June 24, 2004
Workshop durable resistance	February 5, 2002
► **Seminar plus**	
► **International symposia and conferences**	
3rd PlantGEM congress, Lyon, France	September 14-17, 2004
4th PlantGEM congress, Amsterdam, Netherlands	September 20-23,2005
1st Solanaceae Genome Workshop, Wageningen, Netherlands	September 19-21, 2004
► **Presentations**	
Poster presentation in MPMI 2003	July, 2003
Poster presentation in autum school (in CAAS) 2003	November, 2003
Oral presentation in NWO Lunteren days	April, 2004
Poster presentation in 3rd PlantGEM congress	September, 2004
► **IAB Interview**	
► **Excursion**	
Subtotal Scientific Exposure	*9.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD course**	
Nuclear acid (in graduate school of CAAS)	April, 2001
Molecular genetics (in graduate school of CAAS)	February, 2001
► **Journal club**	
Literature discussion in group meetings	2001-2005
► **Individual research training**	
How to cut out bands directly from LICOR gel using Odyssey, trained in Westburg (2 days)	February, 2005
Subtotal In-Depth Studies	*6.6 credits**

4) Personal development	*date*
► **Skill training courses**	
English (in graduate school of CAAS)	September 2000 till June 2001
How to write scientific proposal (in graduate school of CAAS)	March, 2001
Writing of scientific publication (in graduate school of CAAS)	February, 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.4**

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

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WU-CAAS joint PhD program, Asian Facility project AF01/CH/8
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Cover illustration:
Front cover - *Oidium neolycopersici* growing on an *Arabidopsis* leaf
Back cover – Infected leaves of different tomato lines, microscopic picture (1000 time magnification) of a fungal infection unit and cDNA-AFLP LICOR image

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Note:

Note: