Stellingen

1. De associatie van de overgevoeligheidsreaktie met alle vormen van resistentie tegen *Phytophthora infestans* impliceert dat resistentiegenen een belangrijke rol zullen spelen in het verkrijgen van duurzame resistentie.

   dit proefschrift

2. Het promoten van '*R*-gene free potatoes' door het 'International Potato Center' als uitgangsmateriaal voor resistentieveredeling getuigt van te weinig inzicht in de biologie van de aardappel - *P. infestans* interactie.

   CIP Program Report, 1995-1996
   dit proefschrift

3. The road to plant disease resistance will always be under reconstruction.

4. Nu onderkend wordt dat *Phytophthora* niet tot het schimmelrijk behoort, evolueert de 'nachtmerrie van de schimmelgeneticus' tot een 'droom voor de bioloog'; *Phytophthora* is nu immers het best bestudeerde genus binnen zijn Rijk.

   David Francis, Phytophthora Beyond Y2K Symposium, Wooster, 1999

5. *Phytophthora*-resistentie is milieudefensie.

   Dirk Budding

6. Definities zijn vooral nuttig in het beginstadium van biologisch onderzoek, maar in een meer gedetailleerde fase mogen ze een vrije manier van denken niet belemmeren.

7. Het feit dat de meeste mensen denken dat de champignon in de salade nauwer verwant is aan sla dan aan de kok, geeft aan dat mensen hun kennis over evolutie overschatten.

8. Een grote overeenkomst tussen de wetenschapper en de kunstenaar is verbeeldingskracht.

Stellingen behorende bij het proefschrift 'Molecular and cellular biology of resistance to *Phytophthora infestans* in *Solanum* species', door Vivianne G.A.A. Vleeshouwers, in het openbaar te verdedigen op 5 januari 2001, te Wageningen.

Molecular and cellular biology of resistance to *Phytophthora infestans* in *Solanum* species

Vivianne G. A. A. Vleeshouwers

Molecular and cellular biology of resistance to *Phytophthora infestans* in *Solanum* species

Vivianne G. A. A. Vleeshouwers

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 5 januari 2001
des namiddags te vier uur in de Aula.

On the cover: Resistance responses to *Phytophthora infestans* in a leaf of durably resistant potato cultivar Robijn.

Alice might have seen something even more wonderful if she had looked through a microscope instead of through a looking glass
(R. Hegner, 1938)

Contents

Chapter 1

General introduction

For potato breeding, novel sources of (durable) resistance to *Phytophthora infestans,* the causal agent of potato late blight, are required. At the starting point of this research, promising sources of resistance were available in wild *Solanum* species, yet the nature of this resistance was unknown. This thesis presents a study on *P. infestans* resistance in potato and wild *Solanum* species, with emphasis on the molecular and cellular biology of the plant-pathogen interaction. In this chapter, the players of the game will be introduced followed by a few remarks on the late blight disease and resistance. The scope of this thesis concludes the chapter.

Evolution and domestication of the potato (*Solanum*)

Solanum comprises an extremely large and diverse plant genus (D'Arcy, 1991), which includes many field crops such as potato (*S. tuberosum*), eggplant (*S. melongena*), pepino (*S. muricatum*), narangjilla (*S. quitoense*), and tomato (*Lycopersicon*). Although *Lycopersicon* has originally been classified as a separate genus (Hawkes, 1990), recent studies have included *Lycopersicon* in *Solanum* (Spooner *et al.,* 1993). The center of origin of *Solanum* is thought to be in Mexico, from which species migrated southwards and evolved into a separate gene pool in South America (Hawkes, 1990). Most tuber-bearing *Solanum* species used in this study evolved in the Andean mountain range. Also the cultivated potato *S. tuberosum* evolved in this area, probably from a complex of diploid ancient domesticated *Solanum* species. The Spanish invaders became familiar with the crop, and it was probably about 1570 that they shipped the first potatoes to Spain and Tenerife. From there, potatoes were spread around Europe, and reached North America in about 1621. By now, potato is widely grown around the world, and ranks the fourth starch crop in global food production.

The oomycete pathogen *Phytophthora infestans*

P. infestans belongs to the oomycetes, organisms that exhibit a filamentous growth habit and are often inaccurately referred to as fungi. Modern biochemical analyses as well as phylogenetic analyses based on sequences of ribosomal and mitochondrial genes suggest that oomycetes share little taxonomic affinity to filamentous fungi, but are more closely related to golden-brown algae and heterokont algae in the eukaryotic crown group of the Stramenophiles, Kingdom Protista (Kumar and Rzhetsky, 1996; Paquin *et al.,* 1997; van de Peer and de Wachter, 1997; Margulis and Schwartz, 1998) (Figure 1-1).
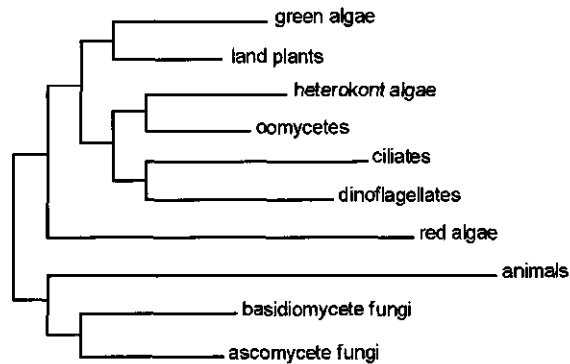
Figure 1-1

Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups (adapted from van de Peer and de Wachter, 1997). Note the position of the oomycetes compared with the other eukaryotic plant pathogens (filamentous fungi belonging to the basiodiomycetes and ascomycetes). Oomycetes appear as an independent group of plant pathogenic eukaryotes.

Obviously, oomycetes differ from true fungi in many aspects. For example, like plants and algae, the oomycete cell wall is composed of ß-glucans, whereas fungal cell walls mainly consist of chitin (Bartnicky-Garcia and Wang, 1983). In contrast to filamentous fungi, oomycetes are not able to synthesize sterols, but obtain them from their environment (Hendrix, 1970; Elliot, 1983). The oomycete life cycle includes a zoosporic phase, in which biflagellate swimming spores are released in an aquatic environment (Figure 1-2) (Fuller and Jaworski, 1987). The ultrastructure of the zoospore flagellum provides evidence for the classification of oomycetes with the heterokont algae (Barr, 1983)
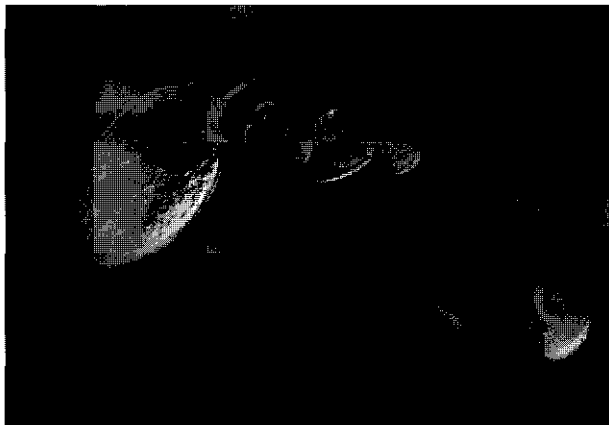
Figure 1-2

Release of *P. infestans* zoospores from a sporangiospore (Differential Interference Contrast, 1000x)

Late blight disease

Oomycetes are a diverse group of organisms, many of which are plant pathogens. The order Peronosporales includes about 60 species of the genus *Phytophthora* (Greek for 'Plant destroyer'), numerous genera of the biotrophic downy mildews, and more than 100 species of the genus *Pythium*. These pathogens cause devastating diseases on numerous crop and ornamental plants and these are notoriously difficult to manage. Economically important diseases include root and stem rot caused by *Phytophthora sojae*, which hampers soybean production in several continents, and black pod of cocoa caused by *Phytophthora palmivora*, a recurring threat to worldwide chocolate production.

The most devastating oomycete disease is potato late blight, which is caused by *Phytophthora infestans* (Figure 1-3). *P. infestans* (Mont.) de Bary is an heterothallic oomycete, which evolved in Toluca Valley, in the central highlands of Mexico. Before 1980, occurrence of both A1 and A2 mating types was limited to this region, whereas in the rest of the world, only the A1 mating type was present (Niederhauser, 1956; Gallegly and Galindo, 1958). After 1980, the old (A1) population has been replaced by a new (A1/A2) population, and a dramatic increase in genetic variation in *P. infestans* populations was observed and suggested to be caused by sexual reproduction (Fry *et al.*, 1992; Drenth *et al.*, 1994; Goodwin and Drenth, 1997).
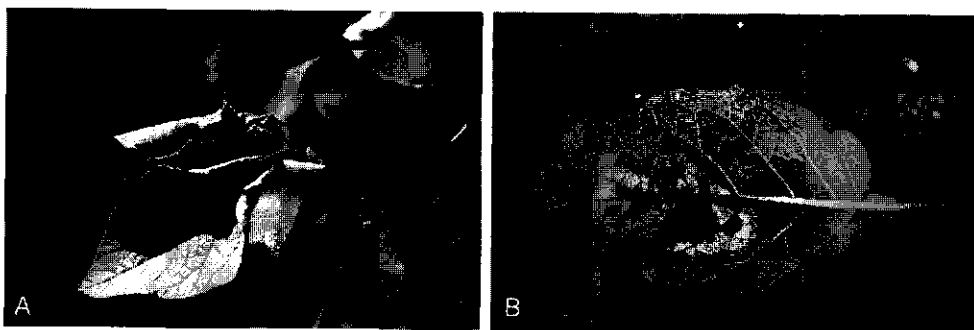
Figure 1-3

Late blight in a potato field. When *P. infestans* sporangiospores land on the foliage, they can either germinate directly or indirectly (via the formation of zoospores and cysts). Hyphal germtubes grow over the leaf surface, form an appressorium and penetrate the epidermis. After a biotrophic growth phase through the leaf mesophyll, sporangiophores emerge through the stomates and sporangiospores are produced for further dispersal of the disease. (**A**) Infected leaves get necrotic and turn black, rendering the 'blighted' look in the field. (**B**) Lower side of an infected leaf from a susceptible *S. microdontum* (BGRC 18570, clone 265). The sporulation zone is visible as white fluffy mycelium.

P. infestans mainly infects a selection of *Solanum* species, but also more disparate hosts have been reported (Turkensteen, 1973; Erwin and Ribiero, 1996). In the mid-19th century, *P. infestans* devastated the European potato fields resulting in a widespread famine in Ireland, and late blight still remains the most serious constraint to potato production (Fry and Goodwin,

1997). Worldwide losses due to late blight and control measures are estimated to cost around $3 billion annually. The use of chemicals targeted against *P. infestans* can provide some level of disease control. However, in the long term, the development of crops that possess durable genetic resistance, whether by classical breeding methods or by genetic engineering, provides the best prospect for effective, economical and environmentally sound control of the late blight disease.

Resistance

Types of resistance

Disease resistance processes in plants are diverse (Agrios, 1997). Resistance may occur at the subspecies or variety level (race- or cultivar-specific resistance) or at the species or genus level (nonhost resistance). In addition, resistance may be a quantitative phenotype (partial resistance) with a partial reduction in disease severity. An improved understanding of the molecular basis of the various types of disease resistance is essential to achieve durable resistance.

What do we know about resistance to oomycetes?

Since oomycetes include a unique group of eukaryotic plant pathogens, they evolved the ability to infect plants independently from true fungi (Kumar and Rzhetsky, 1996; Paquin *et al.*, 1997; van de Peer and de Wachter, 1997) (see Figure 1-1). This suggests that oomycetes may have distinct genetic and biochemical mechanisms for interacting with plants. For example, plant saponins target membrane sterols and are toxic to filamentous fungi, but not to oomycetes as these contain little or no sterols in their membranes (Osbourn, 1996b; Osbourn, 1996a). It can be argued that breeding for late blight resistance in potato has been so unsuccessful partly because knowledge of resistance to oomycetes is very limited. Therefore, to be able to develop late blight resistance, there should be more emphasis on studying the biology of the pathogen itself, and the interaction with its host.

Hypersensitive response

The hypersensitive response (HR) of plants is often associated with disease resistance (Dangl *et al.*, 1996). The HR generally occurs as a rapid, localized cell death, and is considered as a form of programmed cell death in plants (Mittler and Lam, 1996; Heath, 1998). The HR follows perception by the plant of pathogen signal molecules, elicitors, encoded by avirulence (*Avr*) genes (Staskawicz *et al.*, 1995). Specific receptors, encoded by *R* genes, interact directly or indirectly with elicitors, thereby initiating signal transduction pathways that lead to the HR and the expression of disease resistance response. One consequence of this model is that races of the pathogen that contain a mutation in their *Avr* gene(s) can arise and become virulent on

particular plant genotypes. The *R* genes from unrelated plant species share similar structural domains, suggesting conserved mechanisms of pathogen recognition and signaling of defense responses in the plant kingdom (Staskawicz *et al.*, 1995; Baker *et al.*, 1997).

Other resistance mechanisms

Various resistance mechanisms have been reported in addition to the HR. A general phenomenon occurring during defense against pathogen invasion is cell wall strengthening. As various plant pathogens attempt to feed on the plant by dismantling the cell walls, plants in turn deposit dense materials, such as callose or lignin, to hamper entrance of the cells (Hijwegen, 1963; Aist, 1976). In case of *P. infestans*, localized cell wall degradation at haustorial penetration sites is accompanied by accumulation of lignin-like material (Friend, 1973) and callose in papillae or collars (Wilson and Coffey, 1980; Cuypers and Hahlbrock, 1988; Gees and Hohl, 1988).

Resistance mechanisms of a more physiological nature include for example systemic acquired resistance (SAR), which can be induced upon pathogen attack, or can be constitutively activated at a certain level. SAR is associated with enhanced expression of pathogenesis-related (PR) proteins and salicylic acid (Ryals *et al.*, 1996), and has been described in various plants, including potato. Activation of SAR results in a systemic, broad-spectrum resistance to pathogens as diverse as viruses, bacteria, fungi and oomycetes (Ryals *et al.*, 1996). Also in the potato-*P. infestans* interaction, SAR has been reported (Doke *et al.*, 1987; Cohen *et al.*, 1991; Cohen *et al.*, 1993).

Sources of resistance

Genebanks collect and preserve germplasm to maintain genetic diversity for future generations. In present breeding programs, wild *Solanum* species are incorporated to introgress resistance against a broad spectrum of pathogens, including bacteria, viruses, nematodes, fungi and oomycetes (Ross, 1986).

To obtain late blight resistance, *R* genes have been introgressed from *S. demissum* into potato (Müller and Black, 1952). Since both *P. infestans* and *S. demissum* evolved in Mexico, it has been argued that these resistance genes co-evolved with the pathogen and may therefore be less durable than genes that evolved in other regions independent of the pathogen (Nelson, 1978; Colon, 1994). Late blight resistance was also found in various tuber-bearing *Solanum* accessions from South America (Colon and Budding, 1988; Wastie, 1991; Ruiz De Galarreta *et al.*, 1998; Micheletto *et al.*, 1999). Several wild *Solanum* species were identified as potential donors of resistance for breeding purposes (Hoekstra and Seidewitz, 1987; Colon and Budding, 1988; Colon, 1994) (http://www.plant.wageningen-ur.nl/cgn/potato). A selection from these plants is studied in detail for their resistance responses (Figure 1-4), and the results are described in this thesis.
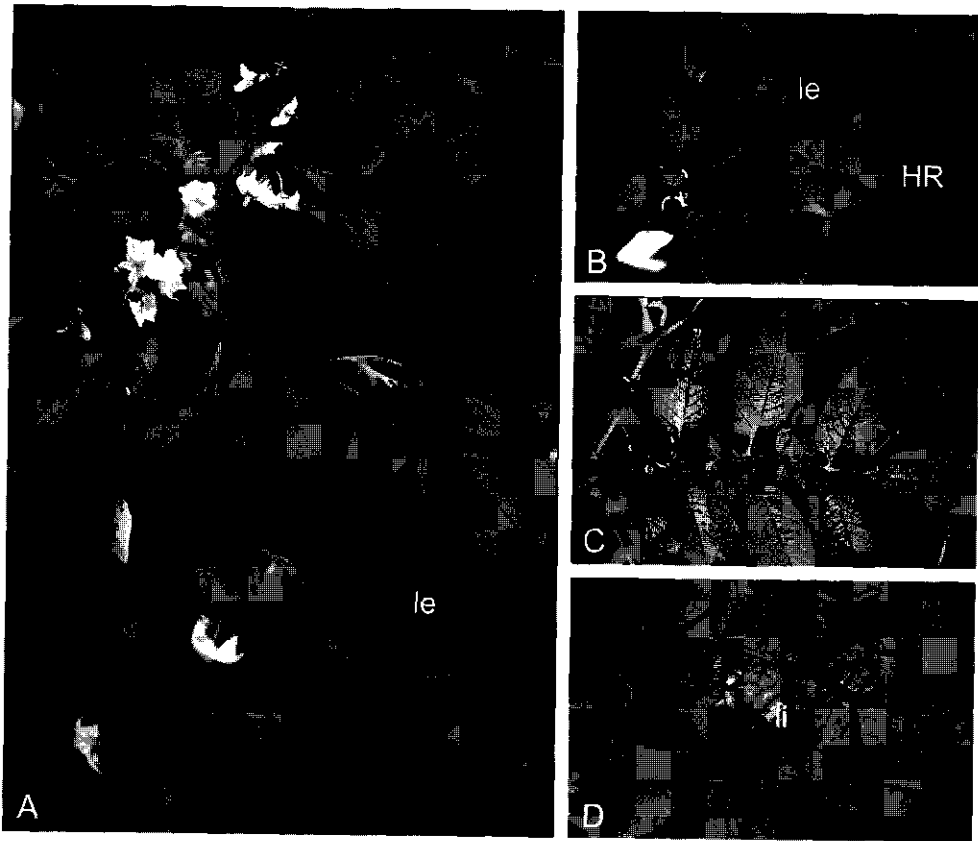
Figure 1-4

Wild and cultivated *Solanum* plants in the field. **(A)** *S. microdontum* BGRC 18570, clone 265, **(B)** *S. vernei* BGRC 24733, clone 530, **(C)** *S. berthaultii* BGRC 10063, clone 11, **(D)** *S. tuberosum* (potato cultivar). **(A, B, C)** Plants were spot-inoculated in a field experiment. The susceptible *S. microdontum* plant shows large expanding lesions on the inoculated leaves, the partially resistant *S. vernei* displays both slowly expanding lesions and arrested (HR) lesions, and the highly resistant *S. berthaultii* does not show any symptoms. **(D)** Occasionally, a lesion becomes bordered by a lignin-like zone and does not expand further. le, expanding lesion; HR, hypersensitive response; li, lignified border surrounding arrested lesion.

Scope of this thesis

A diverse set of *Solanum* plants with different types of resistance to *P. infestans* plays a central role in this thesis. The aim of this research was to study and compare the diverse resistances of the *Solanum* species to *P. infestans*. The rationale behind it was, that when defense mechanisms of durably resistant plants can be recognized, the knowledge obtained can be used to evaluate plant material for breeding programs.

To study the *Solanum* - *P. infestans* interaction at a cellular and molecular level, we first designed a laboratory assay with detached leaves (chapter 2). We compared this assay to the

practical field situation in which late blight resistance is to be used. After confirming that the resistance observed in the field was maintained in our laboratory assay, we performed a cytological survey on the resistance responses of the *Solanum* plants to various *P. infestans* isolates (chapter 3). In contrast to the classical opinion that durably resistant plants would not recruit the HR, we found that the HR was always associated with resistance, also in durably resistant plants. Interestingly, we noted differences in severity of the HR between the different plants: in fully resistant plants, the HR is extremely rapid and pathogen growth is aborted immediately, yet in partially resistant plants, hyphae occasionally escape from HR lesions. Those hyphae are able to establish a biotrophic interaction with the plant, which results in growing lesions. Variation in growth rates of those lesions between the plants suggested that defense mechanisms other than HR operated at different levels. One such mechanism is SAR, and in chapter 4 we used *PR* gene expression levels as molecular markers to measure SAR levels of the *Solanum* plants. In chapter 5 we returned to the HR-based resistance, and focussed on plant components of the molecular interaction, the *R* genes. We concentrated on one particular class of *R* genes, the *Pto* kinases, and generated a diversity of *Pto*-like sequences from *Solanum*. Analyses of the amino acid sequence characteristics revealed that the *Pto*-like sequences are highly conserved. We obtained a better understanding on the evolution of *Pto*-like genes, which appear to have evolved before *Solanum* species radiated. In the general discussion (chapter 6) we recapitulate the results, and discuss future perspectives of *P. infestans* resistance in potato.

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Chapter 2

A *laboratory assay for* Phytophthora infestans *resistance in various* Solanum *species reflects the field situation*

Vivianne G. A. A. Vleeshouwers, Willem van Dooijeweert, L. C. Paul Keizer,
Luc Sijpkes, Francine Govers, and Leontine T. Colon (1999)

Summary

Physiological and molecular research on resistance responses of *Solanum tuberosum* cultivars and partially resistant *Solanum* species to *Phytophthora infestans* requires a reliable resistance test that can be used in the laboratory. Laboratory tests performed on detached leaves and intact plants were compared with field tests for similarity of late blight reactions. Detached leaves from field-grown plants were as resistant as detached leaves from climate chamber-grown plants when challenged with *P. infestans*. However, detached leaves incubated in covered trays at high relative humidity were more susceptible than detached leaves kept in open trays or leaves on intact plants. The incubation conditions of detached leaves in covered trays rather than detachment itself appeared to affect the resistance expression. Detached leaves of some wild *Solanum* genotypes became partially infected, whereas intact plants were completely resistant when inoculated. Inoculation of leaves on intact plants, however, resulted in lower infection efficiencies. These limitations should be taken into account when choosing the appropriate inoculation method for specific purposes. For resistance screening, laboratory tests proved to be a good alternative for field tests. The ranking of resistance levels for twenty plant genotypes was similar under laboratory and field conditions.

Introduction

Partial resistance to *Phytophthora infestans,* the causal agent of late blight, occurs in several wild *Solanum* species and in some potato cultivars (Colon and Budding, 1988; Colon et al., 1995b). This quantitative type of resistance is influenced by changes in environmental factors (Umaerus, 1969); therefore, experimental conditions for testing levels of partial resistance should be chosen carefully. Although the field tests described by Fry (1978) and Colon and Budding (1988) closely resemble the natural conditions under which late blight resistance is important, field testing can only be performed once a year during the growing season. For large-scale resistance screenings in commercial breeding programs, the limitations of field tests may be accepted in contrast to more sophisticated, costly and time consuming laboratory tests. However, studies aimed to unravel resistance mechanisms at the physiological or molecular levels are best performed under controlled conditions in the laboratory. For these specific investigations, an experimental setup in which a high percentage of successful infections can be assured is a prerequisite.

Several methods have been described to assess foliar late blight resistance. In addition to field tests and whole plant greenhouse assays (Stewart et al., 1983), laboratory tests on detached leaves (Lapwood, 1961), leaflets (Malcolmson, 1969; Umaerus and Lihnell, 1976) or leaf discs (Hodgson, 1961) have been described. Resistance assessed in the field can be expressed as ADPC values (area under the disease progress curve) (Shaner and Finney, 1977), which are considered the best estimate of disease for multi-cycle pathogens like *P. infestans* (Fry, 1978). In laboratory tests, commonly used parameters for resistance

assessment are lesion growth rate (LGR), i.e. the rate of necrosis extension, lesion size (LS), which is correlated with LGR, infection efficiency (IE), i.e. the percentage of successful infections, latency period (LP) and spore density (SD) (Birhman and Singh, 1995). Less frequently used, but also suitable as a parameter, is *Phytophthora* biomass. Its quantification can be carried out either through serology-based tests such as ELISA (Harrison *et al.*, 1990; Beckman *et al.*, 1994) or through GUS assays using a transgenic *P. infestans* strain constitutively producing ß-glucuronidase (Kamoun *et al.*, 1998).

Several laboratory tests have been compared with field tests. Using ranking studies, the relative resistance levels of several cultivars in greenhouse or laboratory experiments appeared comparable to resistance levels in the field (Hodgson, 1962; Knutson, 1962; Stewart *et al.*, 1983; Dorrance and Inglis, 1997). LS, LP and SD measured in a laboratory assay on detached leaflets appeared well correlated with ADPC values obtained from a late blight field trial (Singh and Birhman, 1994) after multiple linear regression. In these studies, resistance data obtained in certain tests were simply compared with rough resistance scores obtained in completely different field tests. So far, the effect of conditions inherent to laboratory tests on the actual resistance levels has not been described. Recently, Dorrance and Inglis (1997) reported that a greenhouse test with intact plants corresponded better to ADPC values than a laboratory test with leaflets and leaf discs incubated on water agar. However, from a practical point of view, tests with detached leaves, leaflets or discs are more attractive. Additional studies are needed to verify whether resistance observed on detached leaf assays reflects the resistance found in field tests.

The aim of our study was 1) to design a reliable laboratory test for resistance assessment to *P. infestans*, and 2) to compare resistance data obtained in this test with resistance data obtained in field tests. To this end, well-characterized *Solanum* material with a broad range of resistances was used, and experimental conditions and methods were standardized.

Materials and methods

Plant material

The plant genotypes used in this study and their resistance characteristics are listed in Table 2-1. Material obtained from *in vitro* plantlets was used since the physiological age of tubers has been shown to have an effect on foliage resistance (Stewart *et al.*, 1983). In addition, *in vitro* propagation reduces the chance of virus contamination, and allows rapid multiplication of plant material. *In vitro* plantlets were grown in sterile glass tubes containing MS medium (Murashige and Skoog, 1962) supplemented with 15 g.l^{-1} sucrose and 15 g.l^{-1} mannitol at 23 C. For propagation, shoots were cut and transferred to fresh MS medium containing 30 g.l^{-1} sucrose. After one week of rooting, the *in vitro* plantlets were transferred into pots of sterilized soil and placed in a climate chamber. To allow a progressive adaptation to lower humidity, the plantlets were initially covered with small transparent containers that were removed the next day. The

plants were grown under controlled conditions with a 16h/8h day/night regime and 18/15°C, 65-80% relative humidity (RH); illumination was provided by 400 Watt Philips-HPIT lamps placed at 50 cm intervals at 150 cm above soil level. For the field tests, plants were grown for one month in the climate chamber, and subsequently transplanted in the field to grow for one additional month before inoculation.

Phytophthora infestans isolates, maintenance and inoculum preparation

P. infestans isolate 90128 (race 1.3.4.6.7.8.10.11) and IPO-0 (race 0, kindly supplied by Dr. L.J. Turkensteen, IPO-DLO, Wageningen, The Netherlands) were used throughout this study. Aliquots of sporangiospore suspensions in 15% dimethyl sulfoxide were preserved in liquid nitrogen. For each experiment, a fresh sample of sporangiospores was plated on rye agar medium supplemented with 20 $g.l^{-1}$ sucrose (Caten and Jinks, 1968) and incubated at 18°C in the dark. After a few days, a plug of mycelium was transferred to a fresh agar plate. One week later, when the plate was covered with mycelium, cold water (4°C) was added to the sporulating mycelium. The sporangiospore suspension was pipetted into a test tube and incubated at 4°C. After 1-2 hours, zoospores were separated from the sporangiospores by filtration through a 15 µm nylon mesh. The concentration was adjusted to 5×10^4 zoospores/ml for inoculation.

Inoculation: conditions and plant material

Eight to ten weeks old climate chamber or field-grown plants were spot-inoculated either on detached leaves or intact plants. The third to fifth fully developed leaves (counted from the top) were used. Five leaflets per compound leaf were inoculated (one spot per leaflet) by pipetting 10 µl droplets on the abaxial side.

Inoculations in the field were carried out just before nightfall. To obtain a high RH, necessary to get a good infection, the plants were thoroughly wetted by sprinkling during the afternoon prior to inoculation. During the entire experiment, the field was kept humid by regular overhead sprinkling. Climate chamber-grown plants were transferred to another climate chamber and incubated at a 16h/8h day/night photoperiod with fluorescent light (Philips TLD 36W/84o) tubes at 18/15°C and 95-98% RH for inoculation on intact plants. The next day the RH was brought back to 70%. For detached leaf inoculations, leaves were cut, placed in water-saturated florists foam (Oasis®) in a tray, and inoculated. The trays were wrapped in transparent plastic bags (covered trays) and incubated in the same climate chamber as the inoculated intact plants.

Table 2-1

Plant genotypes used in this study, known late blight responses (to other isolates) and their levels of resistance to *Phytophthora infestans* isolate 90128 as determined under laboratory conditions (experiment I), intermediate conditions (experiment II) and field conditions (experiment III). Resistance levels are expressed as the mean lesion growth rate (LGR, in mm day^{-1}). The infection efficiency (IE, in percentage) is presented in parentheses.

experiment		I[D]		II[C]		III[A]	
Solanum genotype	known resistance	LGR	(IE)	LGR	(IE)	LGR	(IE)
S. berthaultii-9 (BGRC 10063)	complete resistance[1]	0.0	(0)	0.0	(0)	0.0	(0)
S. berthaultii-11 (BGRC 10063)	high partial resistance[1]	2.7	(10)	0.0	(0)	0.0	(0)
S. arnezii x hondelmannii-63 (BGRC 27308)	partial resistance[3]	1.9	(95)	0.2	(7)	0.7	(73)
S. arnezii x hondelmannii-72 (BGRC 27308)	partial resistance[2]	3.4	(75)	2.2	(31)	1.5	(43)
S. circaeifolium ssp. *circaeifolium*-circ1 (BGRC 27058)	complete resistance[4]	0.0	(0)	0.0	(0)	0.0	(0)
S. microdontum-167 (BGRC 24981)	partial resistance[1]	3.3	(45)	1.9	(9)	0.8	(10)
S. microdontum-178 (BGRC 24981)	partial resistance[1]	2.8	(25)	nd	(nd)	0.0	(0)
S. microdontum var. *gigantophyllum*-265 (BGRC 18570)	susceptible[1]	5.4	(90)	3.6	(49)	2.2	(63)
S. sucrense-23 (BGRC 27370)	partial resistance[2]	4.5	(45)	0.0	(0)	0.0	(0)
S. sucrense-71 (BGRC 27370)	partial resistance[2]	4.1	(100)	0.6	(29)	1.0	(43)
S. vernei-530 (BGRC 24733)	partial resistance[1]	3.8	(80)	3.2	(36)	2.0	(93)
ABPT (30x33)-44[5]	partial resistance[3]	2.5	(80)	0.0	(0)	1.2	(55)
S. nigrum-SN18	nonhost resistance	0.0	(0)	0.0	(0)	0.0	(0)
S. nigrum-SN18 x potato cv. Désirée[6]	resistant[7]	nd	(nd)	0.0	(0)	0.0	(0)
Mirabilis jalapa	nonhost resistance	nd	(nd)	0.0	(0)	0.0	(0)
S. tuberosum cv. Bintje	susceptible	5.0	(100)	4.0	(47)	2.3	(53)
S. tuberosum cv. Ehud	susceptible, *R1* specific resistance	4.2	(100)	3.1	(56)	2.2	(63)
S. tuberosum cv. Estima	susceptible, *R10* specific resistance	4.8	(100)	2.5	(24)	1.2	(30)
S. tuberosum cv. Première	susceptible, *R10* specific resistance	3.5	(90)	2.8	(44)	1.3	(55)
S. tuberosum cv. Robijn	durable, partial resistance[8]	2.9	(100)	1.8	(47)	1.2	(58)
LSD (P<0.05)		1.7	-	0.6	-	0.6	-

[D] treatment D, Table 2-2, consisted of detached leaves, grown in climate chambers, tested in the laboratory
[C] treatment C, Table 2-2, consisted of intact plants, grown in climate chambers, tested in the laboratory
[A] treatment A, Table 2-2, consisted of intact plants, grown and tested in the field
[1] Colon *et al.* (1995)
[2] Colon and Budding (1988)
[3] Colon, personal communication
[4] Double-bridge hybrid of *S. acaule, S. bulbocastanum, S. phureja, S. tuberosum* (Hermsen and Ramanna, 1973)
[5] A sexual hybrid of *S. nigrum*-SN18 x potato cv. Désirée (Eijlander and Stiekema, 1994)
[6] Colon *et al.* (1993)

Estimation and analysis of lesion growth rate (LGR) and infection efficiency (IE)

Lesions were measured three times, usually at day three, four, and five after spot inoculation using an electronic caliper connected to a palmtop computer. The largest length and width (perpendicular to the length) of each lesion were measured, and the ellipse area ($A = 1/4 * \pi * length * width$) was calculated. The lesions were divided in two groups, i.e. 'no infection / arrested lesion' (no lesion, or lesion remaining within the size of the inoculum droplet, i.e. $A \leq 16$ mm^2), or 'growing lesion' (the area is larger than 16 mm^2 at least at one time point). The arrested lesions were regarded as unsuccessful infections where the pathogen had been stopped by a hypersensitive response (HR). Therefore, these lesions were not included in the estimation of LGR. From the 'growing lesions' group, the area of the ellipses was square root transformed, resulting in the radius of the lesions. The LGR was estimated by linear regression over time. The infection efficiency (IE) was calculated as the percentage of successful inoculations (i.e. percentage of growing lesions relative to the total number of inoculations) per plant. IE and LGR were estimated and analyzed with ANOVA using Genstat (Genstat 5 Committee, 1987).

Experimental design

As summarized schematically in Table 2-2, five different treatments can be distinguished. In this design, treatment A represents the field situation, treatment D represents the laboratory test as designed in this paper, treatment B and C are intermediates. Treatment E was included in order to analyze the effects of leaf detachment and environmental conditions separately.

Treatments A, B, C, and D were compared in a three factor experiment: within each growing condition (field or climate chamber) the resistance levels of two cultivars (Bintje and Robijn) were determined after inoculation with *P. infestans* isolate 90128 on either detached leaves or intact plants. The same inoculum suspension was used for the plants in the climate chamber and field. Through accurate labeling of the inoculation spots on designated leaves, potential confusion with outside *P. infestans* contaminants was excluded. The field and the climate chamber were each divided in two blocks, which were divided in three sub-blocks. The sub-blocks consisted of two Bintje and two Robijn plants, resulting in 48 plants per experiment. Two experiments were carried out at a one week interval, and the next summer these were replicated. The four experiments were combined for statistical analyses.

A two factor experiment was carried out on climate chamber-grown plants, which received treatment C, D, and E (Table 2-2) in four replications. Bintje and Robijn were inoculated with *P. infestans* isolate 90128. The results from duplicate experiments were combined for statistical analysis.

In a separate experiment, leaves from Bintje and Robijn were detached either one hour or one day prior to inoculation. The detached leaves were inoculated with *P. infestans* isolate 90128 and incubated in covered trays (treatment D, Table 2-2). Two plants were used per cultivar, per incubation period. LGRs were compared between different times of incubation prior to detachment. Three identical experiments were performed and combined for statistical analysis.

Table 2-2
Treatments used to test the effect of growing conditions, inoculation material and environment on resistance of plants to *P. infestans*. Experiments were performed at indicated test locations.

treatment	A	B	C	D	E
plants grown in	field	field	climate chamber	climate chamber	climate chamber
inoculation on	intact plants	detached leaves	intact plants	detached leaves	detached leaves
environment	open air	covered trays	open air	covered trays	open air
test location	field	laboratory	laboratory	laboratory	laboratory

Resistance tests with a set of Solanum genotypes

Average LGRs and IEs were determined of nineteen *Solanum* genotypes and on the nonhost *Mirabilis jalapa* (Table 2-1) under different experimental conditions (treatment A, C, D, Table 2-2) after inoculation with either *P. infestans* isolate IPO-0 or 90128. A randomized block design was applied, with 3 or 4 blocks, depending on the experiment. LGRs could not be determined for genotypes that were completely resistant (lesion size remains 0) or displaying a HR, such as *S. berthaultii*, *S. circaeifolium*, *S. nigrum*, *M. jalapa* (Table 2-1). Genotypes displaying no symptoms at all were considered more resistant than genotypes predominantly exhibiting HR. To include these highly resistant genotypes in the resistance rating, the average LS at day 6 was calculated.

Correlations between different experimental conditions

To test whether the resistance levels of *Solanum* genotypes were comparable under the different experimental conditions, separate experiments were compared to each other. Per individual experiment, the LGRs and LSs at day 6 from the plant genotypes were ranked in decreasing resistance, and Spearman's rank correlation test was applied to pairs of experiments.

Results

Comparisons between laboratory and field tests

When comparing the various experimental conditions (Table 2-2) late blight lesions extended always significantly ($P < 0.001$) more rapidly on Bintje than on Robijn (Table 2-3). During incubation, plants in the field encounter other environmental conditions than plants in the climate chamber. Thus, for intact plants, the effects of growing conditions and inoculation material (detached leaves vs. intact plants) are unavoidably interwoven with each other. Therefore, analysis of the effect of growing conditions was carried out with the LGRs estimated on detached leaves (treatment B vs. D, Table 2-2) from Bintje and Robijn. The effect of growing conditions on LGR was not significant ($P = 0.65$), but there was a significant interaction between growing conditions and cultivars ($P < 0.001$). These results suggest that plants grown in the climate chamber are as resistant as field-grown plants. The effect of inoculation on intact plants vs. detached leaves was analyzed for climate chamber-grown plants (treatment C vs. D), and a highly significant ($P < 0.001$) effect of the inoculation material was found, with an interaction between inoculation material and cultivar ($P = 0.029$).

The table of means from the total experiment is presented in Table 2-3. The LGR on intact plants was lower in the field (A) than on intact plants in the climate chamber (C). IEs showed a similar pattern as LGRs (data not shown). In general, climate chamber-grown plants appeared to have the same resistance level as field-grown plants, while detaching the leaves significantly reduced the expression of resistance. This suggests that either the environmental conditions in a covered tray, or leaf detachment, affects resistance expression.

Table 2-3

Effect of four different treatments (Table 2-2) on lesion growth rates (LGR, in mm day^{-1}) after inoculation with *P. infestans* isolate 90128, on potato cultivars Bintje and Robijn (n = 360 inoculation spots).

	Bintje		Robijn	
	intact plants	detached leaves	intact plants	detached leaves
field	1.88[A]	3.81[B]	0.63[A]	1.87[B]
climate chamber	2.87[C]	3.94[D]	0.75[C]	1.65[D]

LSD = 0.12 ($P < 0.05$)
[A] treatment A, Table 2-2, representing the field test
[B] treatment B, Table 2-2
[C] treatment C, Table 2-2
[D] treatment D, Table 2-2, representing the laboratory test

Effect of leaf detachment

To discriminate between the effects of environmental conditions and leaf detachment on LGR, three treatments (C, D, E) were compared for Bintje and Robijn. Incubation conditions ($P < 0.001$) and cultivars ($P < 0.001$) significantly influenced LGRs. Lesions extended significantly faster on Bintje than on Robijn in all treatments (Table 2-4). The LGRs on detached leaves from both cultivars were significantly higher in covered trays (D) compared to open trays (E). However, there was no significant difference between the LGRs on intact plants (C) and detached leaves in open air (E). These data suggest that the decreased resistance of detached leaves is caused by environmental conditions, rather than by leaf detachment.

For Bintje, high IEs were reached in all treatments (Table 2-4), contrasting with the situation on partially resistant Robijn, where a high IE was achieved only on detached leaves in covered trays (92%). IE was significantly lower on detached leaves in open trays (70%), and lowest on intact plants (52%).

Table 2-4

Effect of different incubation treatments (Table 2-2) on lesion growth rates (LGR, in mm day^{-1}) and infection efficiency (IE, in percentages) of _P. infestans_ isolate 90128 on potato cultivars Bintje and Robijn (n=120 inoculation spots).

	Bintje		Robijn	
	LGR (mm day^{-1})	IE (%)	LGR (mm day^{-1})	IE (%)
detached leaves in covered trays [D]	3.55	100	2.51	92
detached leaves in open trays [E]	2.92	95	1.40	70
intact plants [C]	3.19	93	1.41	52

LSD$_{LGR}$ = 0.54 (P<0.05)
LSD$_{IE}$ = 17 (P<0.05)
[C] treatment C, Table 2-2
[D] treatment D, Table 2-2
[E] treatment E, Table 2-2

To test whether incubation in the trays has an effect on resistance expression, Bintje and Robijn leaves were detached either one hour or 24 hours prior to inoculation. The effect of the incubation period was not significant ($P = 0.393$), the cultivar effect was highly significant ($P = 0.003$); and there was no interaction ($P = 0.979$) between incubation period and cultivar (Table 2-5). The same pattern was found for the IEs (data not shown). This supports the hypothesis, that leaf detachment _per se_ does not affect resistance expression.

Table 2-5

Lesion growth rates (LGR, in mm day^{-1}) of *P. infestans* isolate 90128 on leaves of cultivars Bintje and Robijn detached one hour and 24 hours prior to inoculation (n = 90 inoculation spots).

	Bintje	Robijn
detached one hour prior to inoculation[D]	3.35	2.25
detached 24 hours prior to inoculation	3.07	1.96

LSD = 0.90 (P < 0.05)
[D] treatment D, Table 2-2

Resistance assessment of Solanum genotypes

Resistance tests on a set of nineteen *Solanum* genotypes and the nonhost *M. jalapa* revealed that in general the LGR was the highest on detached leaves (D), intermediate on intact plants in the climate chamber (C), and the lowest in the field (A) (Table 2-1). Occasionally, some genotypes that were resistant when intact plants were inoculated became partially infected when detached leaves were used (*S. microdontum*-178, *S. berthaultii*-11, and *S. sucrense*-23). Although *S. sucrense* appeared exceptionally susceptible on detached leaves compared to intact plants, in general the differences between LGRs were usually proportional to each other. However, the frequency of successful infections on intact plants was very low, especially in the climate chamber (Table 2-1). Even on susceptible genotypes (e.g. Bintje, Ehud, and *S. microdontum*-265) of which detached leaves were completely infected, the IE on intact plants barely reached 50%.

To test whether different experimental conditions have an effect on the genotype ranking order for resistance, three resistance tests in which *P. infestans* isolate 90128 was used for inoculation (Table 2-6), and five tests in which isolate IPO-0 was used (Table 2-7) were compared. The experiments with isolate IPO-0 that were performed under the same standard laboratory conditions (treatment D, Table 2-7, experiment IV, V, VI, VII) were highly correlated with each other (average correlation coefficient 0.84 for LGR, 0.85 for LS, P < 0.001). When resistance data obtained from experiments with isolate 90128 under different growing conditions were compared, similarly high correlations were found (0.94 and 0.85, for LGR and LS respectively, Table 2-6). Therefore, no significant differences in resistance ranking could be found between plants that had been grown in the climate chamber or in the field.

Table 2-6

Spearman's rank correlations between three resistance experiments with *Solanum* genotypes performed under different experimental conditions. Values represent correlations calculated for lesion growth rates (LGR) and lesion size (LS) at day 6 after inoculation with *Phytophthora infestans* isolate 90128. The number of *Solanum* genotypes is shown between parentheses.

experiment	parameter	I[D]	II[C]
II[C]	LGR	0.76 (17)***	
	LS	0.90 (17)***	
III[A]	LGR	0.72 (18)***	0.94 (19)***
	LS	0.76 (18)***	0.85 (19)***

*** P < 0.001

[1] Resistance data from these experiments are presented in Table 2-1.

[A] treatment A, Table 2-2, representing the field test

[C] treatment C, Table 2-2

[D] treatment D, Table 2-2, representing the laboratory test

In experiments with isolate 90128, the correlation coefficients between resistance ratings obtained with different inoculation material (treatment C vs. D) were slightly lower (0.76 for LGR, 0.90 for LS, Table 2-6), but still highly significant (P < 0.001). In experiments with isolate IPO-0, the correlations (experiment VIII, with IV, V, VI, VII, Table 2-7) were clearly lower, i.e. on average 0.57 for LGR and 0.62 for LS. In general, the lower correlations indicate that there might be differences in the level of resistance expression between detached leaves and intact plant inoculation. This effect was also found between field and laboratory tests (treatment A vs. D) performed with isolate 90128, where Spearman's coefficients of rank correlation were 0.72 and 0.76 for LGRs and LS, respectively. Although the rank correlations between field and laboratory tests are still considerable and highly significant (P < 0.001), the decrease in correlation coefficients compared to repeated experiments (IV, V, VI, VII, Table 2-7) under the same standard conditions indicates that environmental conditions in a laboratory affect the resistance response. Since no effect of growing conditions has been found, these results suggest that the difference between the field situation and laboratory may be caused by inoculation of detached leaves instead of intact plants, confirming the results obtained with Bintje and Robijn.

Table 2-7

Spearman's rank correlations between five resistance experiments with *Solanum* genotypes performed under different experimental conditions. Values represent correlations calculated for lesion growth rates (LGR) and lesion size (LS) at day 6 after inoculation with *Phytophthora infestans* isolate IPO-0. The number of *Solanum* genotypes is shown between parentheses.

experiment	parameter	IV[D]	V[D]	VI[D]	VII[D]
V[D]	LGR	0.83 (17)***			
	LS	0.88 (17)***			
VI[D]	LGR	0.83 (17)***	0.85 (15)***		
	LS	0.87 (17)***	0.88 (15)***		
VII[D]	LGR	0.87 (17)***	0.78 (15)***	0.87 (18)***	
	LS	0.71 (17)***	0.68 (15)**	0.75 (18)***	
VIII[C]	LGR	0.57 (18)*	0.59 (16)*	0.46 (18) ns	0.67 (18)**
	LS	0.60 (18)**	0.77 (16)***	0.59 (18)**	0.51 (18)*

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

[C] treatment C, Table 2-2, consisted of intact plants, grown in climate chambers

[D] treatment D, Table 2-2, consisted of detached leaves from climate chamber-grown plants

Discussion

A reliable laboratory test is essential for studying plant-pathogen interactions at the physiological or molecular level. In this paper, we compared the suitability of a laboratory test for *P. infestans* resistance in *Solanum* species with a field test. Our data indicate that resistance expression is similar for field- and climate chamber-grown *Solanum* plants. Colon *et al.* (1995) compared field- and greenhouse-grown potato leaves and found that the latter appeared more resistant. They hypothesized that greenhouse-grown plants might exhibit induced resistance due to heat and drought stress. Leaves derived from climate chamber-grown plants, as described here, did not show differences in resistance expression compared to leaves derived from field-grown plants.

LGRs found on Bintje and Robijn plants that were inoculated and incubated in the field (A) were lower than those found on plants inoculated and incubated in the climate chamber (C) (Table 2-3). Since the effect of growing conditions was not significant, the lower LGR values in the field were probably due to differences in environmental conditions after inoculation. Although the average outside temperature and humidity during the field experiments were comparable to those in the climate chamber, many fluctuations occurred during the day in the field. In addition, the light conditions in the field and in the climate chamber are different, both qualitatively and quantitatively.

Resistance data for Bintje and Robijn revealed that detached leaves exhibited significantly less resistance than intact plants. In the detached leaf test, the leaves were

incubated at a RH that was usually higher than the RH used with intact plants. By incubating detached leaves in open trays, significant differences in LGR between treatments were no longer observed. This suggests that the environmental conditions in the trays, rather than detachment *per se*, have an effect on the expression of resistance. In addition, LGRs on freshly detached leaves and on leaves detached at 24 hours prior to inoculation were compared. If detachment would play a role, it is expected that during incubation in trays, resistance would either be (partly) lost or enhanced e.g. due to stress. No significant differences in LGR were found between leaves that had been incubating in trays for different time periods prior to inoculation, confirming the hypothesis that detachment *per se* does not affect resistance.

From the previous results, we conclude that the lower expression of resistance in the detached leaf tests is due to differences in environmental conditions. The constant highly favorable environment the pathogen finds in the closed trays apparently enhances infection by the zoospores, as visualized by a high IE. Once the requirements for successful infection are established, hyphae can feed on the plant cells and a high growth rate of the lesions can be measured. The quantitative nature of *P. infestans* resistance in potato is described as the competition between mycelium growth and HR of invaded cells (Umaerus, 1969). In the open air, the physical requirements for HR may be more optimal than in the closed trays. The fact that similar LGR values were found on uncovered detached leaves and intact plants suggests that the use of uncovered detached leaves may be a good alternative for intact plants. Unfortunately, considerably lower IEs were found on uncovered leaves, despite the use of humidifiers. For research in which a high amount of successful infections are favored, e.g. in cytological, molecular biological studies, the detached leaves test in closed trays can be recommended. However, when the IE is to be used as a parameter for resistance, e.g. in resistance testing of germplasm, a different methodology may be chosen, e.g. incubation of detached leaves in open trays, or intact plants in climate chamber or field.

Ranking for resistance of twenty plant genotypes gave significant correlations between experiments performed with inoculation of intact plants vs. detached leaves (Table 2-6 and Table 2-7). Although significant, these correlations were lower than those found between replications of identical experiments. This suggests that the type of inoculated plant material has an effect on resistance expression. Testing a diverse set of *Solanum* species provided a wide range of resistance levels and showed that individual species may respond differently under different experimental conditions. An example is *S. sucrense*, of which detached leaves are much more susceptible than intact plants. In other *Solanum* species, this difference was less pronounced.

Our conclusion that for late blight assessment laboratory tests are significantly correlated with field tests is in agreement with conclusions drawn by Hodgson (1962), who found that the relative resistance of eight potato cultivars in a laboratory test on leaf discs correlated with the resistance score in the field. In contrast, Stewart *et al.* (1983) did not find satisfactory correlations in resistance scores when they compared glasshouse and field tests for resistance to foliar blight. However, in their experiments two completely different tests were compared, and even the inoculation procedures were different. Knutson (1962) found that the relative resistance of the cultivars Pontiac, Sebago and Ostbote was consistent between

different tests, but obtained contradictory results with cultivar Cobbler, which appeared susceptible in the field but resistant in the laboratory. It is not clear whether Knutson (1962) used the same isolates in field and laboratory experiments, and hence, the occurrence of race specific resistance cannot be excluded.

From our studies we conclude that different growing conditions do not significantly affect the resistance levels to *P. infestans*, thus allowing late blight testing on *Solanum* plants grown in climate chambers. Although using detached leaves in resistance tests does not have a significant effect, incubating detached leaves in closed trays appears to decrease resistance expression. The ranking of resistance levels for a set of *Solanum* genotypes with different types and levels of resistance was generally consistent across different types of experiments, but occasionally discrepancies were noted for some resistant wild *Solanum* species. Therefore, a suitable experimental condition has to be chosen depending on the aim of an experiment. When the expression of resistance is to be examined on detached leaves, the reduced level of resistance should be weighed against the low infection frequency inherent to intact plant. Inoculation of intact plants is preferred, but in most cases the inoculation of detached leaves incubated in covered trays appears to be an adequate alternative.

Acknowledgments

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Chapter 3

The hypersensitive response is associated with host and nonhost resistance to Phytophthora infestans

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Summary

The interaction between *Phytophthora infestans* (Mont.) de Bary and *Solanum* was examined cytologically using a diverse set of wild *Solanum* species and potato (*S. tuberosum* L.) cultivars with various levels of resistance to late blight. In wild *Solanum* species, in potato cultivars carrying known resistance (*R*) genes and in nonhosts the major defense reaction appeared to be the hypersensitive response (HR). In fully resistant *Solanum* species and nonhosts, the HR was fast and occurred within 22 h. This resulted in the death of one to three cells. In partially resistant clones, the HR was induced between 16 and 46 h, and resulted in HR lesions consisting of five or more dead cells, from which hyphae were occasionally able to escape to establish a biotrophic interaction. These results demonstrate the quantitative nature resistance to *P. infestans*. The effectiveness of the HR in restricting growth of the pathogen differed considerably between clones and correlated with resistance levels. Other responses associated with the defense reaction were deposition of callose and extracellular globules containing phenolic compounds. These globules were deposited near cells showing the HR, and may function in cell wall strengthening.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is the most devastating disease of potato (*Solanum tuberosum*) world-wide. To limit chemical control, breeding potato to incorporate durable forms of genetic resistance is needed. The genus *Solanum* comprises an extensive gene pool, in which a broad spectrum of pathogen resistance has accumulated throughout evolution (Ross, 1986). In some wild *Solanum* species, resistance to *P. infestans* that may be of a durable nature has been identified (Colon and Budding, 1988). Also some old potato cultivars have a seemingly durable resistance (Wastie, 1991; Colon *et al.*, 1995b).

Resistance responses to pathogens are traditionally classified as race-specific, race-nonspecific, and nonhost resistance (Agrios, 1997). In this concept, race-specific resistance is based on the presence of major resistance genes (*R*), which are conserved among plant species. The *R* genes are thought to encode specific receptors that upon triggering by elicitors initiate signal transduction pathways leading to the hypersensitive response (HR; Hammond-Kosack and Jones, 1997). In the gene-for-gene model (Flor, 1971), the presence of both a plant *R* gene and a corresponding avirulence (*Avr*) gene from the pathogen results in resistance (incompatible interaction), whereas absence of either the *R* gene or the *Avr* gene results in disease (compatible interaction). In total eleven *R* genes to *P. infestans* have been introduced from *S. demissum* into potato (Müller and Black, 1952). In nature, numerous races of *P. infestans* have evolved that are able to infect plants containing these *R* genes (generally known as complex races). In contrast, a race 0 is defined as a race unable to infect plants containing any of the known *R* genes. However, it should be noted that it cannot be predicted

whether the interaction of a race 0 with plants containing unknown R genes will be compatible or incompatible. Cytological observations (Ferris, 1955; Hohl and Suter, 1976; Wilson and Coffey, 1980; Gees and Hohl, 1988) have indicated that the presence of R genes in potato cultivars results in incompatible interactions with avirulent P. infestans isolates: a rapid plant cell death response is induced upon penetration of the epidermal cell, and the pathogen is prevented from further growth, resulting in resistance. This accelerated localized plant cell death response is defined as the HR. In compatible interactions, the HR is not induced or is induced to a lesser extent and a biotrophic relation can be established, resulting in plant disease.

Race-nonspecific resistance may be due to intrinsic properties of the plant or may be induced by nonspecific elicitors produced by all races of the pathogen (Agrios, 1997). Deposition of structural compounds, which are thought to have a function in cell wall strengthening, can be seen as a nonspecific resistance mechanism. In compatible and incompatible P. infestans-potato interactions, wall appositions were found with accumulated callose (Wilson and Coffey, 1980; Cuypers and Hahlbrock, 1988; Gees and Hohl, 1988). Also lignification, which is considered as a general response to pathogen attack in several plant species, appears to play a role in resistance to P. infestans (Friend, 1973).

Nonhost resistance is defined as a full resistance at the species or genus level (Kamoun et al., 1999b). Although nonhost resistance to fungal pathogens such as rusts has been studied in detail (Heath, 1991), much less is known about nonhost resistance to oomycetes. Penetration of P. infestans in the nonhost parsley resulted in the HR (Schmelzer et al., 1995). Elicitins, proteins secreted abundantly by Phytophthora species, induce the HR in nonhost plant species of the genus Nicotiana (Kamoun et al., 1993). P. infestans transformants deficient for INF1 elicitin production have an expanded host range (Kamoun et al., 1998). Multiple pathogen elicitors may interact with R gene receptors from diverse plants and mediate nonhost resistance (Kamoun et al., 1999b).

In addition to the qualitative resistance observed in several R gene-containing cultivars, resistance to P. infestans in S. tuberosum and other Solanum species displays a quantitative nature. Several wild Solanum species have been shown to display different levels of partial resistance ranging from immunity to susceptibility (Wastie, 1991). So far, the molecular mechanisms that determine partial resistance have not been identified. Here we describe the resistance responses to P. infestans in a set of Solanum species displaying different types and levels of resistance. For comparison, we also examine cytologically the interaction of diverse nonhost species with P. infestans. Comparing infection processes and resistance responses of plants with different types and levels of resistance might lead to a better understanding of mechanisms involved in durable resistance.

Materials and methods

Plant material

The plant material used in this study is listed in Table 3-1. The *Solanum* plants and *M. jalapa* were propagated in vitro. Plants of the other species were obtained from seeds. Plants were grown in climate chambers under controlled conditions (Vleeshouwers *et al.*, 1999).

Phytophthora infestans isolates and inoculation

P. infestans isolates IPO-0 (race 0), Bonn Complex (race 1.3.4.7.10.11, both kindly provided by L.J. Turkensteen), 90128 (complex race 1.3.4.6.7.8.10.11), and *P. mirabilis* isolate CBS 136.86 (obtained from Centraal Bureau Schimmelcultures, Baarn, The Netherlands) were used in this study. Isolate Bonn Complex was continuously propagated on potato leaves. The isolates IPO-0 , 90128 and CBS 136.86 were preserved in liquid nitrogen, and for each experiment a fresh sample was plated on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968). Standardized procedures of inoculum preparation and spot inoculations were performed (Vleeshouwers *et al.*, 1999).

Resistance test

The resistance levels of the *Solanum* clones to IPO-0 were determined in a routine assay by spot inoculation on detached leaves. The fourth, fifth, and sixth day after inoculation the lesion diameters were measured, the ellipse areas (A = 1/4 * π * length * width) were calculated and divided in three groups, i.e. 'no symptoms' (A = 0), 'arrested lesion' (A $\leq$ 16 mm^2), or 'growing lesion' (A > 16 mm^2). The average infection efficiency (IE) and lesion growth rate (LGR) were estimated with ANOVA or REML using Genstat (Genstat 5 Committee, 1987), as described previously (Vleeshouwers *et al.*, 1999).

Table 3-1

Plant material used in this study: individual clones, the origin, and known *P. infestans* resistances

Plant species	family	clone	Origin	Resistance previously observed	reference
S. berthaultii	Solanaceae	9	BGRC[1] 10063	complete	Colon et al. (1995)
S. berthaultii	Solanaceae	11	BGRC 10063	partial	Colon et al. (1995)
S. arnezii x hondelmannii	Solanaceae	63	BGRC 27308	partial	Colon and Budding (1988)
S. arnezii x hondelmannii	Solanaceae	72	BGRC 27308	partial	Colon and Budding (1988)
S. circaeifolium ssp. circaeifolium	Solanaceae	circ1	BGRC 27058	complete	
S. microdontum	Solanaceae	167	BGRC 24981	partial	Colon et al. (1995)
S. microdontum	Solanaceae	178	BGRC 24981	partial	Colon et al. (1995)
S. microdontum var. gigantophyllum	Solanaceae	265	BGRC 18570	susceptible	Colon et al. (1995)
S. sucrense	Solanaceae	23	BGRC 27370	partial	Colon and Budding (1988)
S. sucrense	Solanaceae	71	BGRC 27370	partial	Colon and Budding (1988)
S. vernei	Solanaceae	530	BGRC 24733	partial	Colon et al. (1995)
ABPT[2]	Solanaceae	(30x33)-44	Wag. Univ.	partial	
S. tuberosum	Solanaceae	cv. Bintje	Plant Res. Int	susceptible	
S. tuberosum	Solanaceae	cv. Bildstar	Plant Res. Int	susceptible	
S. tuberosum	Solanaceae	cv. Ehud	Plant Res. Int	R1 (strong R gene)	
S. tuberosum	Solanaceae	cv. Estima	Plant Res. Int	R10 (weak R gene)	Turkensteen (1987)
S. tuberosum	Solanaceae	cv. Première	Plant Res. Int	R10 (weak R gene)	Turkensteen (1987)
S. tuberosum	Solanaceae	cv. Robijn	Plant Res. Int	durable, partial	Colon et al. (1995)
S. nigrum-SN18	Solanaceae	SN18	Plant Res. Int	nonhost	
S. nigrum x tuberosum hybrid[3]	Solanaceae	SN18 x Désirée	Plant Res. Int	complete	Colon et al. (1993)
Nicotiana rustica	Solanaceae	var. WAU	Wag. Univ.	nonhost	
Nicotiana tabacum	Solanaceae	cv. Xanthi	Wag. Univ.	nonhost	
Raphanus sativa	Cruciferae	cv. Daikon	Wag. Univ.	nonhost	
Arabidopsis thaliana	Brassicaceae	ecotype Colombia	Plant Res. Int	nonhost	
Mirabilis jalapa	Nyctaginaceae	unknown	Commercial	nonhost	

[1] Genetic Resource Centre at Braunschweig

[2] Double-bridge hybrid of S. acaule, S. bulbocastanum, S. phureja, S. tuberosum (Hermsen and Ramanna, 1973)

[3] A sexual hybrid of S. nigrum-SN18 x potato cv. Désirée (Eijlander and Stiekema, 1994)

Microscopy

Leaf discs were subjected to a trypan blue staining / clearing method (Heath, 1971; Wilson and Coffey, 1980) to study both plant and *P. infestans* structures. Callose staining on leaf discs was performed with aniline blue (Wilson and Coffey, 1980). The discs were examined in a Zeiss Axiophot (Zeiss) microscope equipped with a high pressure mercury vapor lamp (HBO 50 W). Fluorescence of aniline blue-stained tissue, and autofluorescence of trypan blue-stained tissue to identify the HR was observed with a G365 excitation filter, FT395 interference beam splitter and LP420 barrier filters. Photographs were taken on 35mm Kodak Ektachrome 160T (bright field) or 400 (fluorescence) films. Cryo scanning electron microscopy of nitrogen-frozen inoculated leaves was performed using an Oxford Instruments CT-1500 cryo transfer unit attached to a JEOL 6300F microscope.

Experimental set up

Standardized materials and methods were used to achieve reliable results between different inoculation experiments (Vleeshouwers *et al.*, 1999). The preparations for the microscopical survey on the *Solanum* clones were derived from four separate inoculations, the resistance assays were performed independently.

    A microscopical survey was conducted for seventeen *Solanum* clones inoculated with *P. infestans* isolates IPO-0 and 90128. Per stage, i.e. 22, 46 and 70 hours after inoculation (hai), three leaf discs of approximately 25 mm^2 were cut and subjected to the trypan blue or aniline blue staining procedure. Remaining leaflets were kept in the trays to check macroscopically for symptom development at later stages. The HR was quantified for the IPO-0 – *Solanum* interaction using the trypan blue-stained leaf discs. For each stage, one leaf disc was scanned completely. The number of epidermal and mesophyll cells responding with the HR was determined for each penetration site.

Results

Resistance levels.

The resistance levels of the *Solanum* clones ranged from complete resistance to full susceptibility to *P. infestans* isolate IPO-0 (Table 3-2). The completely resistant *S. nigrum*-SN18 predominantly showed HR lesions upon inoculation. In plants with high levels of partial resistance, such as *S. berthaultii*-11, a high percentage of unsuccessful (aborted) infections was found, but also a low percentage (16%) of slowly growing lesions (1.2 mm/day). Plants with lower levels of partial resistance, such as *S. arnezii x hondelmannii*-72, displayed higher IEs (86%) and LGRs (2.4 mm/day). Susceptible clones became fully infected and showed fast expanding lesions (up to 4.0 mm/day). In general, IE and LGR appeared to be correlated.

Table 3-2

Overview of resistance assessment and microscopical data of *Solanum* clones inoculated with *P. infestans* isolate IPO-0. Clones are ranked in order of decreasing resistance level, with the percentage of growing lesions as sorting parameter. Resistance data are presented as IE and LGR. Microscopical data at 22 and 46 hai are presented as the average number of (epidermal and mesophyll) HR cells for n penetration sites. The main observed responses are described briefly, confined HR lesions indicate a sharp confinement of heavily stained, collapsed HR cells between normal, symptomless cells.

Solanum clone	Resistance assessment			LGR (mm/day)	Microscopy[1]			
	IE[2] no sympt. (%)	arrested lesions (%)	growing lesions (%)		22 hai number of HR cells (n)	Main observations	46 hai number of HR cells (n)	Main observations
S. nigrum-SN18	4	96	0	0.0	2.2 (37)	HR (100%)	2.8 (43)	HR, infection aborted
S. berthaultii-9	81	16	3	1.0	2.3 (25)	HR (96%)	3.1 (25)	HR, infection aborted
S. circaeifolium ssp. circaeifolium -circ1	2	93	5	1.1	1.4 (5)	HR (100%)	5.4 (21)	HR, infection aborted
S. tuberosum - Ehud	1	84	15	1.4	2.8 (17)	HR (100%)	11.0 (33)	HR, not completely confined
S. berthaultii -11	66	18	16	1.2	1.3 (17)	HR (94%)	5.8 (56)	HR, not completely confined
ABPT(30x33)-44	6	77	17	1.8	5.1 (39)	HR (95%)	10.9 (49)	HR, not completely confined
S. vernei -530	42	31	27	1.4	3.7 (35)	HR (100%), biotrophy	7.2 (34)	confined & expanding HR lesions
S. microdontum-178	4	68	28	1.5	2.4 (68)	HR (100%)	3.1 (57)	HR, infection aborted
S. tuberosum - Robijn	13	50	37	1.4	1.6 (20)	HR (100%), 'slow HR'	13.4 (28)	confined & expanding HR lesions
S. sucrense-71	5	39	56	2.6	3.0 (38)	HR (79%), biotrophy	nd[3]	confined & expanding HR lesions
S. tuberosum - Première	13	29	58	2.8	2.3 (13)	HR (92%), biotrophy	5.0 (11)	confined & expanding HR lesions
S. arnezii x hondelmannii -63	15	20	65	1.9	1.6 (35)	HR (100%), biotrophy	3.7 (33)	confined & expanding HR lesions
S. arnezii x hondelmannii -72	9	5	86	2.4	0.6 (11)	HR (64%), biotrophy	1.4 (8)	confined & expanding HR lesions
S. tuberosum - Estima	1	11	88	3.4	2.8 (19)	HR (74%), biotrophy	11.7 (31)	confined & expanding HR lesions
S. sucrense-23	6	6	88	2.9	0.3 (92)	biotrophy, HR (16%)	nd[3]	expanding lesion
S. microdontum var. gigantophyllum-265	0	0	100	3.9	0.3 (20)	biotrophy, HR (30%)	nd[3]	expanding lesion
S. tuberosum – Bintje	0	0	100	4.0	0.6 (22)	biotrophy, HR (28%)	nd[3]	expanding lesion
LSD	21	21	16	0.9				

[1] Microscopical data are based on an average of two independent experiments for S. nigrum-SN18, Bintje, Ehud, Estima, Robijn (Table 3-3), and on one single experiment for the other clones.
[2] IE data are based on n=14 plants for all clones, except for both S. sucrense clones, where n=11. Calculations of the LSD (IE) is based on n=14.
[3] nd = not determined.

Potato cultivars carrying *R* genes from *S. demissum* respond differentially towards complex and race-0 isolates of *P. infestans*. Resistance levels of the various *Solanum* species to the complex *P. infestans* isolate 90128 have been reported previously (Vleeshouwers *et al.*, 1999). Compared to isolate IPO-0, isolate 90128 was more aggressive on most *Solanum* clones, but in general similar IEs and LGRs were found. This indicates that the resistance observed is not mediated by the eight *R* genes known from *S. demissum* that differ in the response to the two isolates. A differential response in IE and LGR between the two isolates was found for cultivar Ehud carrying the strong *R1* gene, but not for Estima and Premiere, both carrying the weak *R10* gene. *S. sucrense*-23 displayed a lower IE to *P. infestans* isolate 90128 than to IPO-0, but the LGRs appeared similar.

We also examined a number of plants that are considered nonhosts of *P. infestans*. As expected, *Arabidopsis*, tobacco, radish and *M. jalapa* were all fully resistant to *P. infestans* and macroscopic disease lesions were never observed.

Figure 3-1

Compatible interaction between susceptible potato cultivars and *P. infestans*. No plant response is visible during early stages of infection. **(A)** Penetration of an epidermal cell (22 hai with *P. infestans* isolate IPO-0 on potato cultivar Bintje; DIC). **(B)** Branching hyphae with haustoria in mesophyll cells (31 hai with *P. infestans* isolate Bonn Complex on potato cultivar Bildtstar; PH). c, cyst; a, appressorium; ha, haustorium; hy, hypha; iv, infection vesicle; bar = 35 µm.

Figure 3-2

Hypersensitive response as the major defense response in the *Solanum* - *P. infestans* interaction. Characteristics of HR cells are the granular structure of the cytoplasm (visible with DIC optics), thickened cell walls and autofluorescence (under UV illumination). The HR occurs in susceptible **(A - B)**, race-specific **(C - E)** and nonhost **(F - G)** interactions at 22 hai with *P. infestans* isolate IPO-0. **A, B** HR epidermal cell of potato cultivar Bintje with DIC **(A)** and UV **(B)**. **C - E** three HR epidermis cells **(C)** of potato cultivar Ehud (*R1*), and the adjacent HR mesophyll cell **(D)**, with DIC **(C, D)** and UV **(E)**. **F,G** three HR epidermis cells of *S. nigrum*-SN18, with DIC **(F)** and UV **(G)**. p, penetration site; hy, hypha; HR, HR responding cell; cw, thickened cell wall. Condensed nuclei are not in focus (not indicated). bar =30 µm.
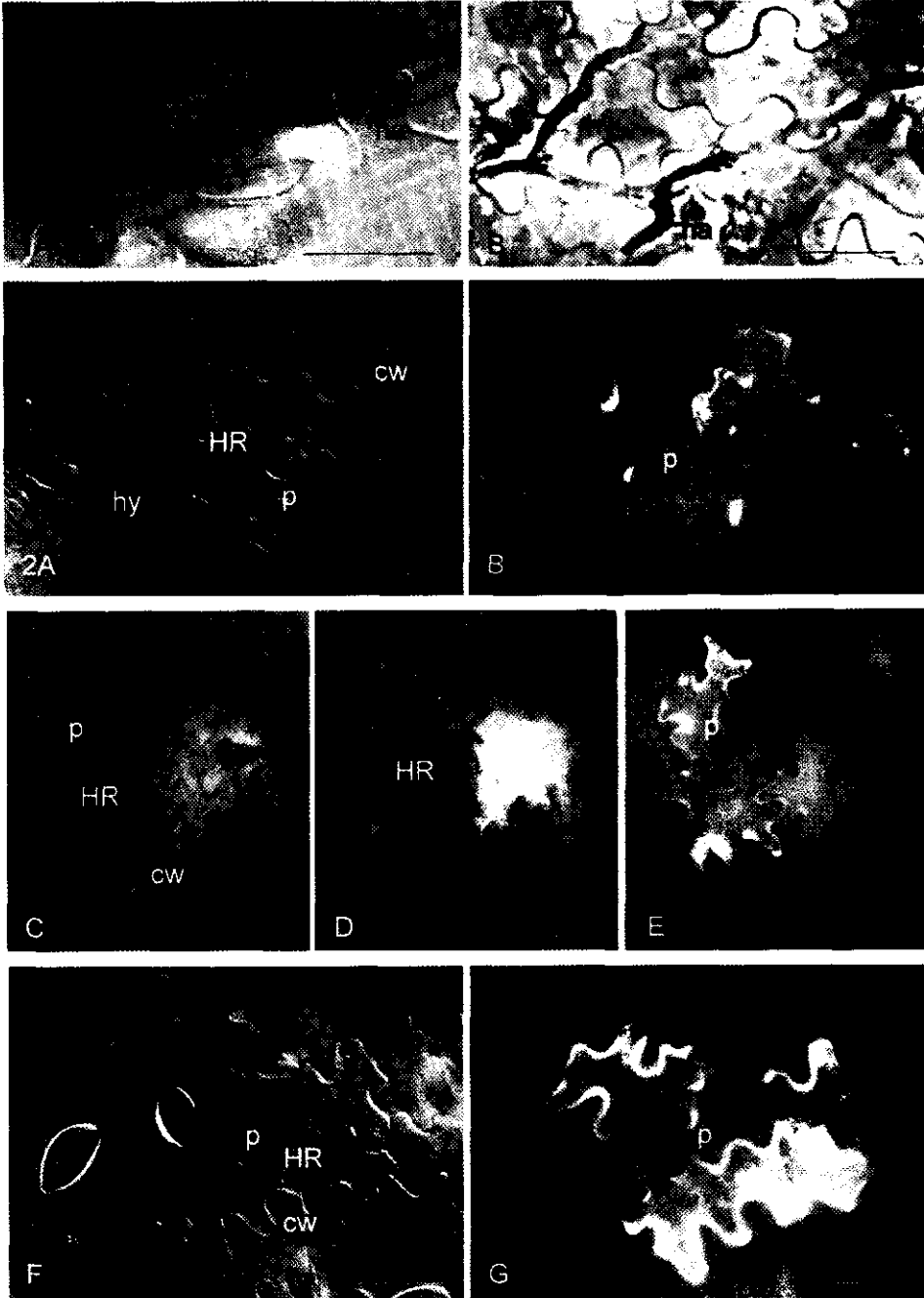
Microscopical overview: potato.

Inoculation of susceptible potato cultivar Bintje with *P. infestans* isolates IPO-0 and 90128, and Bildtstar with *P. infestans* isolate Bonn Complex resulted in a biotrophic interaction (Figure 3-1). After cyst germination and appressorium formation, penetration occurred and at 16 hai or later an intracellular infection vesicle was formed in the epidermal cell. Subsequently, hyphae grew into the intercellular space and ramified through the mesophyll. In this early biotrophic stage (22 hai), one to two haustoria per encountered parenchyma cell were formed. In later stages, expanding hyphae barely formed haustoria. At 46 hai, sporangiophores started to emerge through stomata, sporangia were formed and infected cells started to necrotize. These features are characteristic of compatible interactions as described before (Ferris, 1955; Wilson and Coffey, 1980; Gees and Hohl, 1988). However, not every attempt of *P. infestans* to colonize the leaf tissue was successful. Occasionally, penetrated epidermal cells showed the characteristics of the HR (granular and often brownish cytoplasm, thickened cell walls, autofluorescence under UV light, and condensed nuclei near the penetration site at 22 hai, Figure 3-2). The HR was mostly restricted to one epidermal cell (see below).

Cultivar Ehud (*R1*) showed a biotrophic interaction with *P. infestans* isolate 90128 (not shown), and displayed the HR when inoculated with isolate IPO-0, as expected (Figure 3-2). The cultivars Estima and Première carrying the weak *R10* gene also showed the HR upon inoculation with IPO-0. However, the induction of the HR started relatively late after penetration, hyphal growth often proceeded beyond the HR lesions, and a compatible interaction was established in most cases. This suggests that the HR in Estima and Première was less effective in pathogen containment than in Ehud. Upon inoculation of Estima and Première with isolate 90128, the HR was also induced, but appeared less effective than in the interaction with isolate IPO-0.

Potato cultivar Robijn, which displayed durable resistance in the field and does not posses any known *R* genes from *S. demissum*, displayed the HR upon inoculation with both *P. infestans* isolates IPO-0 and 90128. However, at 46 hai and following stages, hyphae escaped from the HR lesion (Figure 3-3) and established a biotrophic interaction with the mesophyll cells. These escaping hyphae bore one or two haustoria per encountered plant cell similarly to early biotrophic stages (22 hai) of a compatible interaction on susceptible plants. Sometimes a fully biotrophic interaction was observed after the escape, but often a trailing HR was noted, in which growing hyphae stayed ahead of HR responding cells. In contrast to the pathological necrosis observed in compatible interactions, the trailing HR was induced much faster.

Microscopical overview: wild Solanum species

The major defense response in the wild *Solanum* species with different resistance levels but no (known) *R* genes appeared to be the HR (Table 3-2, Figure 3-4 – Figure 3-7). The pathogen was able to penetrate the epidermis of all tested plants.

In all but one of the wild _Solanum_ clones, _P. infestans_ isolates IPO-0 and 90128 encountered similar resistance responses, but the invasion of 90128 appeared to proceed slightly faster. In _S. sucrense_-23, differential responses were observed between both _P. infestans_ isolates in two independent experiments. Upon inoculation with isolate 90128, the HR was observed in the vast majority of penetration sites at 22 hai. The trailing necrosis of some escaping hyphae was remarkably sharply confined between healthy plant cells. Together with the strong browning of responding cells this clearly demonstrated the characteristics of HR. At 46 hai, the majority of HR lesions had not expanded much further in size. However, at 70 hai, escaping hyphae were observed occasionally, and a trailing necrosis was noted. In the interaction of _S. sucrense_-23 with _P. infestans_ isolate IPO-0, a biotrophic interaction was established at 22 hai, resulting in necrotized tissue at 46 hai. Whether this is due to delayed HR or to a pathological necrosis could not be distinguished unambiguously.

Microscopical overview: nonhosts.

To study nonhost resistance responses, Bintje, _S. nigrum_ and _Mirabilis jalapa_ were inoculated with _P. infestans_ (IPO-0) and with _P. mirabilis_ (CBS 136.86), a close relative of _P. infestans_ (Möller _et al._, 1993) and a natural pathogen of _M. jalapa_. The compatible interactions Bintje - _P. infestans_ (Figure 3-1) and _M. jalapa_ - _P. mirabilis_ resulted in typical biotrophic growth of the pathogen. In contrast, inoculation of _M. jalapa_ and _S. nigrum_ (Figure 3-2) with _P. infestans_, and Bintje and _S. nigrum_ with _P. mirabilis_ resulted in a rapid HR. This response was also observed when the nonhost plants _Raphanus sativa_, _Nicotiana tabacum_, _N. rustica_ and _Arabidopsis thaliana_ (Figure 3-7) were inoculated with _P. infestans_ isolate IPO-0. In all these nonhosts, epidermal and occasionally mesophyll cells were always penetrated, but a rapid HR was associated with the cessation of colonization by the pathogen.

Penetration frequency

Differences in penetration frequency were found among the different _Solanum_ clones, but this parameter was not correlated with the IE or the LGR (Table 3-2).

The relation between the HR and the cessation of pathogen growth

Although all _Solanum_ clones reacted with a similar type of response to _P. infestans_ isolate IPO-0, major differences were observed in severity and timing of the HR between clones with different resistance levels (Table 3-2).
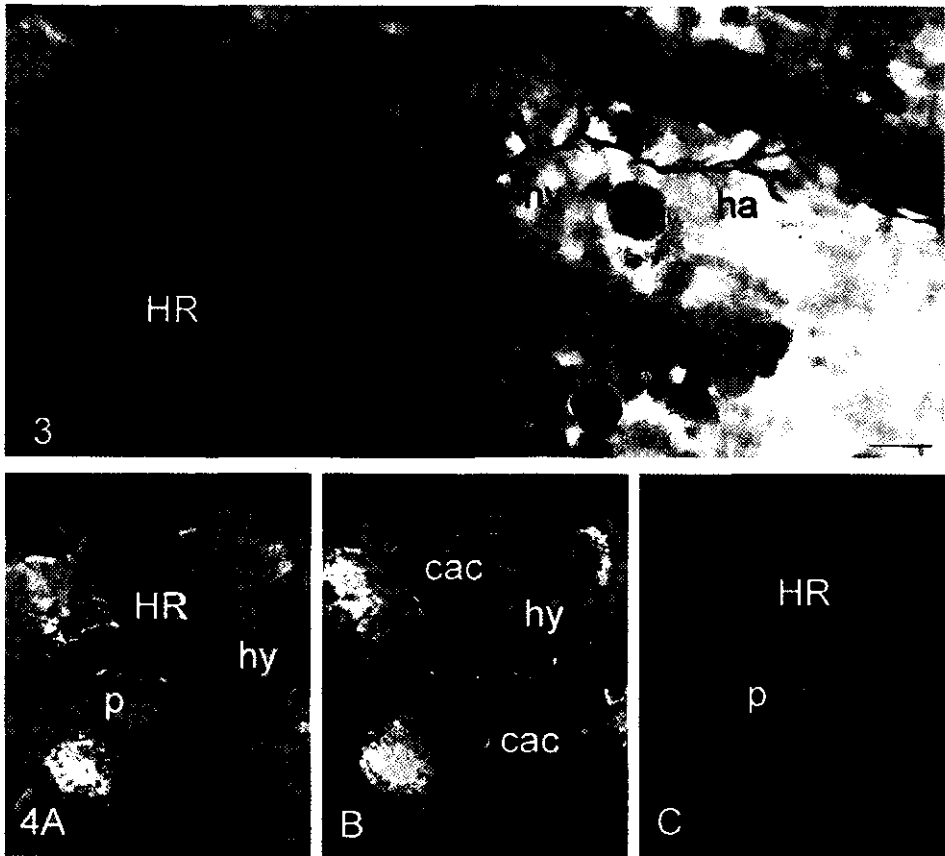
Figure 3-3

Lesion by HR with escaping hyphae in cultivar Robijn, 46 hai with *P. infestans* isolate IPO-0, (bright field) HR, HR lesion; hy, hypha; ha, haustorium; bar =40 μm

Figure 3-4

Increased trypan blue staining in HR-neighboring mesophyll cells of *S. berthaultii*-9 at 22 hai with *P. infestans* isolate IPO-0. **A** HR of one epidermal cell. Penetration occurred in the stomatal guard cell. **B** Typical increased trypan bleu staining and typical cytoplasmic structure in four adjacent mesophyll cells. **C** Autofluorescence of the HR epidermal cell. HR, HR cell; p, penetration site; hy, hypha; cac, cell showing an increased cytoplasmic activity; bar =20 μm.

In the nonhost *S. nigrum*-SN18, the HR was induced extremely fast. One to three cells displayed the HR at 22 hai (Figure 3-2). In most cases the response remained limited to these cells and *P. infestans* was not detected at 46 hai. In *S. berthaultii*-9 and *S. circaeifolium*-circ1, the HR was established at a slower rate but was finally completed at 46 hai.

Partially resistant *Solanum* clones such as *S. berthaultii*-11 and ABPT-44 exhibited a less effective HR as more cells displayed the HR before the pathogen was restricted. At 46 hai, it appeared that hyphae had grown out of the initially responding epidermal cells into mesophyll cells. These cells subsequently responded with the HR, resulting in increased sizes of HR lesions. In more susceptible clones, such as *S. amezii x hondelmannii*-72, the HR occurred later, hyphae escaped and growing disease lesions were formed.

In the susceptible clones *S. microdontum*-265 and *S. sucrense*-23 the HR was induced only occasionally. As in Bintje, no early plant response was visible. At 46 hai, the entire leaf disc was overgrown by hyphae, and extensive necrosis near the inoculation spot made further examinations impossible.

In a subset of clones that exhibit phenotypes ranging from resistance to susceptibility, i.e. *S. nigrum*-SN18, Ehud, Robijn, Estima and Bintje, the number of HR cells was quantified over time in two independent inoculation experiments (Table 3-2, Table 3-3). No increase in the number of cells exhibiting the HR was observed in the resistant *S. nigrum*-SN18 after 22 hai (Table 3-3). Concurrent with the decreasing resistance level in Ehud, Robijn, and Estima, more cells exhibited the HR at 46 hai compared to 22 hai, suggesting that the induction of the HR occurred slower and extended over a longer period. These results suggest a correlation between the effectiveness of the HR and the level of resistance to *P. infestans*.

Reversible response

Mesophyll cells adjacent to HR cells often showed increased trypan blue staining (Figure 3-4). Phenotypically, these cells looked completely different from dead cells. In contrast to dead mesophyll cells (Figure 3-3) they retained an organized structure, and cytoplasmic strands could be discerned. In some cases, these cells appeared to restore their normal appearance. This reversible response was noted in all *Solanum* clones, particularly in highly resistant clones. Although the specimens sampled at 46 hai were different from those sampled at 22 hai, these observations were made in several independently repeated experiments, and suggest true reversibility of early stages of the HR.

Table 3-3

Effectiveness of the HR in *S. nigrum*, Ehud, Robijn, Estima and Bintje, ranked according to decreasing resistance levels (see Table 3-2), after inoculation with *P. infestans* isolate IPO-0. HR cells were quantified by determining the average number of HR cells per penetration site at 22 and 46 hai. Indicated is the increase of the average number of HR cells (avg.), and standard errors (se) between 0 – 22 hai and 22 – 46 hai, calculated from two independent experiments (n=2).

clone	Increase in HR cells, 0-22 hai		Increase in HR cells, 22-46 hai	
	avg.	se	avg.	se
S. nigrum- SN18	2.2	0.5	0.6	0.2
Ehud	2.8	0.6	8.3	0.8
Robijn	1.6	0.2	11.8	4.1
Estima	2.8	0.4	9.0	1.9
Bintje	0.6	0.2	nd	nd

Hyphal growth in veins

After penetration and haustoria formation in epidermal cells that covered vascular tissue (rib cells), hyphae sometimes grew into the xylem vessels in the veins. This was observed in several clones, including the completely resistant *S. nigrum*-SN18 (Figure 3-7). Since xylem vessels are dead cells, the HR cannot serve as a resistance mechanism in this tissue. Occasionally, hyphae aborted in growth were noted near HR cells adjacent to veins, suggesting that the HR was induced as soon as the hyphae left the xylem vessel.

Callose deposition

Cells adjacent to HR cells usually showed callose deposited on cell walls, whereas HR cells usually did not show any callose staining (Figure 3-5). Lesions following HR were often found completely surrounded by callose depositions. In regions of penetration attempts and hyphal growth, callose deposition was also found in collars and papillae. Susceptible clones displayed a higher number of collars and papillae, inherent to *Phytophthora* growth throughout the tissue, whereas resistant clones mainly showed callose deposition around HR cells. Partially resistant clones showed an intermediate phenotype.

   S. nigrum x Désirée displayed callose deposition that was not associated with the inoculated area. This randomly deposited callose was noted at all time points after inoculation with *P. infestans* isolates IPO-0 and 90128, and also in non inoculated tissue. Phenotypically, this artificial hybrid also displayed spontaneous necrosis resulting in HR-like lesions. This

phenotype is reminiscent of the lesion mimic phenotype described in other plants, such as *Arabidopsis* (Greenberg *et al.*, 1994) and maize (Pryor, 1987).

Deposition of phenolic compounds

Particularly remarkable depositions were found as extracellular globules of considerable sizes. These were studied by SEM in frozen leaf discs and by LM in trypan blue stained leaf discs (Figure 3-6). The brown color of the globules seen under bright field and as autofluorescence under UV light indicates the presence of phenolic compounds. Failure of aniline blue staining shows that these globules are not related to the callose papillae described above. Moreover, in contrast to callose papillae, the globules were usually not associated with *Phytophthora* structures, but were often observed near HR lesions on spongy parenchyma cells and on epidermal cells.

The extracellular globules were observed at all studied stages in all studied plant species. They were deposited outside the plasma membrane on the surface of the cell walls. In *S. berthaultii,* the globules were mainly found deposited in the epidermis and the first cell layer of the mesophyll. In *S. arnezii x hondelmannii*, abundant deposition of globules was observed on the cell walls of the mesophyll around HR lesions and near adjacent veins (Figure 3-6). Occasionally, on walls of adjacent cells, a smear of phenolic compounds was noted. The differences in globule location between the *Solanum* clones is expected to be related to the position of the HR, since the HR remained predominantly limited to the epidermis in *S. berthaultii* and to the mesophyll in *S. amezii x hondelmannii.* In general, there was no indication for a correlation between the amount of globule formation and resistance levels of the different *Solanum* clones.

Occasionally, intracellular accumulation of phenolic material was observed, particularly in nonhost plants. In *S. nigrum,* one hypha was invading the veins without any visible plant response, whereas a neighboring hypha penetrating the midrib cell was coated with an autofluorescing substance (Figure 3-7). Similar features were found in *Arabidopsis*, where the primary and secondary hyphae in the HR epidermal cell were coated with autofluorescent material (Figure 3-7). In addition to this lignification-like response (Mauch-Mani and Slusarenko, 1996), the penetrated epidermal cell also showed the characteristics of the HR.
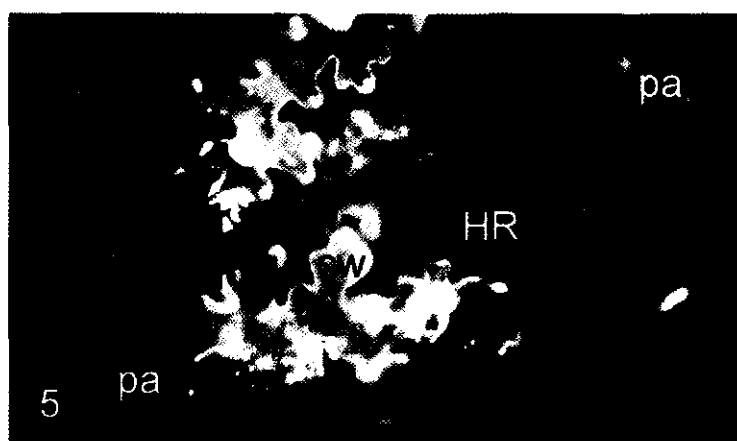
Figure 3-5

Callose depositions near an HR lesion in hybrid ABPT-44, 46 hai with *P. infestans* isolate IPO-0. Callose is deposited on cell walls of cells adjacent to HR cells, or in papillae. cw, callose deposition on cell wall; HR, HR lesion; pa, papillae; bar =25 μm

Discussion

Despite the severe late blight threat every growing season, only limited success has been achieved in potato breeding for resistance to *P. infestans*. Although a rich pool of resistance sources is available in wild *Solanum* species, little is known about the physiological and molecular basis of the various resistance phenotypes. As an initial step toward a comprehensive characterization of resistance to *P. infestans*, we surveyed the defense responses of potato cultivars, wild *Solanum* species and representative nonhost species. We found that the HR was the major defense response as it was associated with all forms of resistance in all tested plant species. Previously, responses in plants exhibiting race-specific and race-nonspecific resistance to *P. infestans* were reported to be similar (Wilson and Coffey, 1980; Gees and Hohl, 1988). Here, we show that both partial and nonhost resistance, which are considered to be durable in the field, are associated with the HR. In resistant interactions between *P. infestans* and both solanaceous (*Solanum species, N. tabacum, N. rustica*) and non solanaceous plants (*A. thaliana, R. sativa, M. jalapa*), the HR was always observed. Based on the current knowledge on the role of *R* genes in initiating signal transduction cascades leading to the HR (Baker *et al.*, 1997), recognition of pathogen elicitors by plant receptors followed by rapid plant cell death may take place in all types of resistant interactions (Kamoun *et al.*, 1999b). Throughout evolution, durable resistance may have developed in nonhost plant species as a response to elicitors from *Phytophthora* (Heath, 1991).

A particularly remarkable feature is the ambiguous response of many plants to *P. infestans* penetration, even within the same inoculation spot. In many cases a mosaic of responses was observed within the same inoculation spot. Some sites are infected, whereas others are resistant. The excellent description of compatible and incompatible interactions in potato to *Phytophthora* by Cuypers and Hahlbrock (1988) can be extended. Our results indicate that the difference between compatibility and incompatibility is quantitative rather than qualitative. In some interactions, the HR appeared to be ineffective, since hyphae were able to escape and establish growing lesions. In highly resistant clones, all cells responded with a rapid HR upon penetration, whereas in fully susceptible clones, only a low percentage of cells displayed the HR. The observation that the HR is often restricted to the epidermis, suggests that the pathogen can be blocked by physical barriers, or that cell death can be induced rapidly in epidermal cells.

The timing of HR induction differed remarkably between clones and quantification of the number of HR responding cells suggested a correlation between resistance level and HR effectiveness.

Are R genes involved in all types of resistance to oomycetes?

The prominent HR in *Solanum* species may indicate the presence of an arsenal of *R* genes (as defined in the introduction) targeted against *P. infestans*. The differential cytological responses between isolate IPO-0 and 90128, in addition to the significantly lower IEs of isolate 90128 compared to IPO-0 in resistance tests (Table 3-2; Vleeshouwers *et al.*, 1999) may be explained by the presence of one particular partial *R* gene in *S. sucrense-23* that discriminates between these two strains. Major genes for *P. infestans* resistance have also been found in *S. berthaultii*, (Ewing *et al.*, 2000), *S. microdontum* (Sandbrink *et al.*, 2000) and *S. stoloniferum* (Schick *et al.*, 1958). At the moment, it cannot be ruled out that some *Solanum* species possess *R* genes targeted against all known strains and races of the pathogen.

The classical (*R*) gene – for - (*Avr*) gene model can be used to explain the quantitative character of partial resistance. The observation that the HR correlates with partial resistance suggests the involvement of possibly "weak" *R* gene - *Avr* gene interactions. For example, potato cultivars carrying the "weak" *S. demissum R10* gene show altered levels of resistance and allow some pathogen colonization under certain conditions. Likewise, transformation of homologues of the *Cf* and *Xa21 R* genes into susceptible plants conferred partial resistance to, respectively, *Cladosporium fulvum* in tomato (Laugé *et al.*, 1998) and *Xanthomonas oryzae* pv *oryzae* in rice (Wang *et al.*, 1998). Similarly, pathogen *Avr* gene products may encode "weak" ligands. For example, the expression of *Avr* genes with reduced elicitor activity in engineered potato virus X derivatives resulted in lower resistance responses in tobacco (Kamoun *et al.*, 1999a). These data support a potential role for *R* genes in partial resistance and illustrate the ambiguity of known vs. unknown *R* genes in potato breeding. The current bias of potato breeders for selecting cultivars without classical *R* genes to achieve durable resistance appears rather irrational. To achieve durable resistance, the classical ideas are either to introduce *R* genes that recognize a vital avirulence factor, or to stack different *R* genes. However, in

addition, the introduction of nonhost resistance genes into potato may provide a novel perspective (Kamoun *et al.*, 1999b).

The differential HR effectiveness between *R1* and *R10* cultivars may suggest the existence of differential thresholds between *R1* and *R10* to activate the HR. A dosage effect in receptors or *R* genes may increase sensitivity in pathogen perception and subsequent resistance. Duplex (tetraploid) potato plants, containing two *R* genes of the same kind (e.g. *R1R1r1r1*) were found more resistant to *P. infestans* than simplex (*R1r1r1r1*) plants (Ferris, 1955). However, in the latter study the presence of other unknown *R* genes could not be excluded. The theory of differential thresholds activating different genes has also been suggested for resistance-related genes such as in barley *mlo* (Buschges *et al.*, 1997) and for *Arabidopsis lsd1* (Dietrich *et al.*, 1997), from which mutants exhibit a lowered sensitivity threshold for triggering the HR.

Programmed cell death

During the HR a conserved programmed cell death (pcd) mechanism is activated (Mittler and Lam, 1996; Heath, 1998), and in various plants, several cytological phenomena display characteristics of apoptosis (Ryerson and Heath, 1996), whereas others resemble necrosis (Bestwick *et al.*, 1995). Also anti-cell death programs are thought to be active in plants (Dietrich *et al.*, 1997), and HR neighboring cells, which transiently seem to be metabolically active in the pathosystem described in the present study, may express such anti-cell death program. The question whether the trailing necrosis is either a form of pcd or a pathological necrosis cannot unambiguously be answered. However, the sharp confinement and morphology of cells adjacent to escaping hyphae, suggests a rapid activation of a pcd mechanism. In addition, the timing of the trailing necrosis in partially resistant clones is considerably faster than the pathological necrosis in susceptible clones. Thus, despite the lack of ultimate evidence, we hypothesize that pcd is activated during the HR in the *P. infestans* - *Solanum* interaction.

Resistance response after penetration

Penetration frequency differed among the *Solanum* species, but did not correlate with resistance level. This is in line with the similar penetration frequencies found on several potato cultivars differing in general resistance levels (Gees and Hohl, 1988). Variation in morphological structure of epidermal layers or cuticle may explain the differences in penetration frequency, and this may be innate to certain species or clones. The ability of *P. infestans* to penetrate cells of any plant species, including nonsolanaceous plants as shown here and previously (Hori, 1935), indicates that the major resistance responses are effected post penetration.

Callose

Callose deposition was observed in all *Solanum* clones upon infection by *P. infestans*. Patterns of callose deposition appeared mainly associated with the condition of the tissue due to the HR. Although Hohl and Stössel (1976) reported differential responses in callose deposition in tubers between susceptible and resistant cultivars, we favor the view that callose deposition in leaves is a general response (Aist, 1976). In addition, the overall deposition of callose which was not associated to *P. infestans* infection in *S. nigrum* x Désirée, suggests that callose deposition may be a general stress response.

Phenolic compounds

Extracellular globules, most likely containing phenolic compounds, were observed near HR responding tissue in all *Solanum* clones. Similar structures have been described in tomato as a response to *Cladosporium fulvum* infection (Lazarovits and Higgins, 1976). Lesions bordered with a lignin-like material have been observed in potatoes infected with *P. infestans* (Figure 1-4D). In soybean, treatment with a *Phytophthora sojae* elicitor resulted in increased peroxidase activity concurrent with accumulation of phenolic polymers, including lignin-like polymers (Graham and Graham, 1991). Autofluorescing compounds were also found to accumulate on intracellular *P. infestans* hyphae in fully resistant *S. nigrum* and *Arabidopsis*, where recognition may have occurred faster. Lignification on hyphae has been shown previously for the oomycetes *Peronospora parasitica* in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996), and *Bremia lactucae* in lettuce (Bennett *et al.*, 1996).

In conclusion

A fine balance between induction of defense responses and growth of the pathogen seems to determine resistance or susceptibility, and illustrates the quantitative nature of resistance to *P. infestans* in *Solanum* species. The cellular interaction phenotype of resistant *Solanum* species and nonhosts with *P. infestans* is the HR, as observed in many pathosystems. Since the HR is usually associated with gene-for-gene interactions involving pathogen recognition conferred by *R* genes, resistance in *Solanum* species and nonhosts may involve the same *R* gene specific recognition events. Partially resistant clones display a less effective HR, which is possibly caused by an inadequate or delayed recognition of elicitors by weak *R* genes. Other responses such as callose deposition, accumulation of phenolic compounds, and other biochemical changes that are not detected at a cytological level can also influence the balance in favoring either cell death or inhibiting pathogen growth.
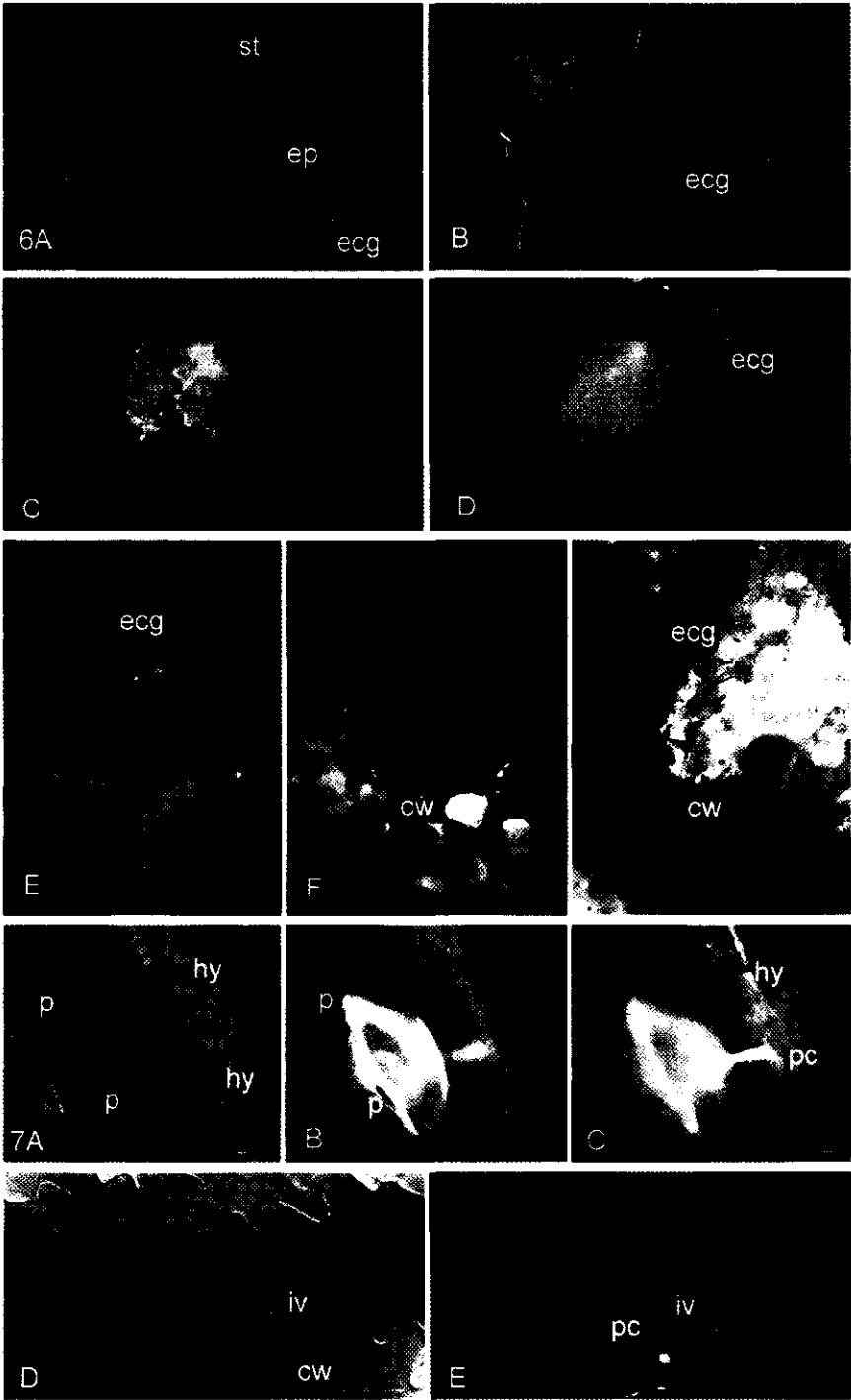
Figure 3-6

Extracellular globules observed by SEM as deposited on epidermis (A) or mesophyll cells (B) near hypersensitively responding cells in potato cultivar Robijn 70 hai with *P. infestans* isolate IPO-0. C HR in the epidermis, and D (same spot) extracellular globules in the mesophyll in *S. arnezii x hondelmannii*-63 46 hai with IPO-0, UV. E Extracellular globules in the mesophyll in *S. arnezii x hondelmannii*-63 with IPO-0 at 70 hai, UV. In F (UV, same spot, lower focus) autofluorescing cell walls are visible. G shows the same spot at bright field. Both the globules and the thickened cells walls have a brown color. Together with the autofluorescence under UV light, this indicates the accumulation of phenolic compounds. cw, thickened cell wall; ecg, extracellular globule; ep, epidermal cell; HR, HR cell; st, stomatal guard cell; bar =10 µm (A-B) resp. 50 µm (C-G).

Figure 3-7

Accumulation of phenolic compounds on *Phytophthora* structures. A – C penetration of a midrib cell in *S. nigrum*, 46 hai with *P. infestans* isolate IPO-0. Two hyphae have penetrated (A, DIC) via the anticlinal cell walls (B) and the cell walls adjacent to the intercellular space show autofluorescence (B, C). One hyphae appears to be able to grow into the vein, whereas the other hypha is enwrapped with phenolic compounds (C). D, E show an (HR) epidermis cell of *A. thaliana* 46 hai with *P. infestans* isolate IPO-0. Accumulation of phenolic compounds as a plug adjacent to the intracellular infection vesicle (D, DIC) is visible showing autofluorescence under UV illumination (E). cw, cell wall; hy, hypha; iv, infection vesicle; p, penetration site; pc, phenolic compounds; bar =15 µm.

Acknowledgements

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Does basal PR *gene expression in* Solanum *species contribute to nonspecific resistance to* Phytophthora infestans*?*

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Summary

Systemic acquired resistance (SAR) occurs in many plant species, including potato. SAR can be induced by various signals, but also basal levels of SAR may vary between plants. In *Arabidopsis* mutants, basal SAR levels positively correlate with pathogen resistance. Here we test whether in thirteen wild *Solanum* clones and five potato cultivars, basal expression levels of SAR marker genes correlate with resistance to *Phytophthora infestans*. Most of the examined *Solanum* plants displayed significant and variable levels of race/isolate-nonspecific, partial resistance to five *P. infestans* isolates of diverse origin. Constitutive mRNAs levels of the pathogenesis-related genes *PR-1*, *PR-2*, and *PR-5* in non-infected leaves varied between the *Solanum* clones. However, no correlation between basal *PR* mRNA levels and resistance was observed at the genus level. In contrast, significant correlation was found at the species level in *S. arnezii x hondelmannii*, *S. microdontum*, *S. sucrense* and *S. tuberosum*. In *S. tuberosum* cultivars, the levels of *PR* gene expression were the highest in resistant Robijn, intermediate in partially resistant Première, Estima and Ehud, and the lowest in susceptible Bintje. These results suggest that constitutive expression of *PR* genes may contribute to nonspecific resistance to *P. infestans* in *Solanum*. Therefore, *PR* mRNAs could serve as molecular markers in potato breeding programs.

Introduction

Resistance of plants to pathogens can be attributed to the action of various resistance mechanisms, each functioning at a certain level and specificity. One such mechanism is systemic acquired resistance (SAR), which generally follows a localized unsuccessful pathogen attack, involves an enhanced state of resistance to a broad spectrum of pathogens and is associated with an increased expression of genes encoding pathogenesis-related (PR) proteins (Ryals *et al.*, 1996). In other cases, pathogen attack is hampered by another type of resistance, called induced systemic resistance (ISR), which is not associated with increased PR gene expression (Pieterse and van Loon, 1999).

SAR has been described in several plant species, including potato, but has best been documented in *Arabidopsis thaliana*. Several *Arabidopsis* mutants altered in SAR have been identified. For example, mutants in the *npr1* gene (*non*-expresser of *PR* genes) fail to respond to SAR inducing treatments, and are susceptible to the bacterial pathogen *Pseudomonas syringae* in contrast to wildtype plants (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). Overexpression of *npr1* led to enhanced resistance to *P. syringae* and the oomycete *Peronospora parasitica* in a dosage-dependent fashion since levels of *npr1* mRNA, NPR1 protein, *PR-1* mRNA and resistance were positively correlated in the transformants (Cao *et al.*, 1998). The *cpr* (constitutive expressers of *PR* genes) (Bowling *et al.*, 1994) and *dnd1* (defense with *no* cell death) mutants (Yu *et al.*, 1998) exhibit constitutive SAR related phenotypes

including elevated mRNA levels for genes encoding PR proteins. The analyses of these *Arabidopsis* mutants indicate that subtle mutations may affect basal SAR levels, and that higher resistance levels can be reached by manipulating basal levels of SAR.

In solanaceous plants, SAR has also been reported. In potato, SAR could be induced by treatment with hyphal wall components, unsaturated fatty acids and jasmonic acid, resulting in enhanced resistance to the late blight oomycete pathogen *Phytophthora infestans* (Mont.) de Bary (Doke *et al.*, 1987; Cohen *et al.*, 1991; Cohen *et al.*, 1993). In engineered potato plants containing a transgene encoding a bacterio-opsin proton pump from *Halobacterium halobium*, the expression of several *PR* genes was increased and resistance to *P. infestans* was enhanced (Abad *et al.*, 1997). Naturally occurring constitutive SAR was found in hybrids of tobacco and in certain tomato lines in which enhanced resistance to various pathogens was associated with constitutive *PR* gene expression (Ahl Goy *et al.*, 1992; Yalpani *et al.*, 1993; Lawrence *et al.*, 1996).

In addition to realization of full-fledged SAR, the effects of overexpression of single *PR* genes on resistance have been described. Overexpression of *PR-1* in tobacco increased resistance to *Phytophthora parasitica* and *Peronospora tabacina* (Alexander *et al.*, 1993), and overexpression of *PR-5* (osmotin) slightly enhanced resistance to *P. infestans* in potato (Liu *et al.*, 1994; Pereira *et al.*, 1998), R. Li and A. Pereira, pers. comm.] and *Solanum commersonii* (Zhu *et al.*, 1996). PR-1-like proteins are conserved within the plant, fungal, vertebrate and invertebrate kingdoms. In animals these proteins function as venoms, allergens, or are implicated otherwise in defense (van Loon and van Strien, 1999). The purified PR-1 protein from tomato and tobacco inhibited germination of *P. infestans* zoospores *in vitro* and lesion growth *in vivo* (Niderman *et al.*, 1995). *PR-2* and *PR-3* encode glucanases and chitinases respectively, and these enzymes may play a role in cell wall degradation. However, oomycetes lack chitin in their cell wall and are not expected to be affected by chitinases. Actin-binding studies suggested that a basic chitinase and an osmotin-like protein might be involved in cytoplasmic aggregation, an important event in potato's cellular defense to *P. infestans* (Takemoto *et al.*, 1997). In addition, PR-5 proteins play a role in osmotic stress, freezing tolerance, permeabilization of fungal and oomycetal plasma membranes and pathogen resistance (Woloshuk *et al.*, 1991; Liu *et al.*, 1994; Abad *et al.*, 1996; Zhu *et al.*, 1996).

P. infestans is a major pathogen of potato and tomato. In recent years, the severity of this disease has increased dramatically, and a more profound insight in the mechanisms of resistance to *P. infestans* is needed to develop novel control strategies. In the *P. infestans* – potato interaction, the most commonly studied type of resistance is race-specific resistance, which is governed by single dominant resistant genes (*R* genes). Unfortunately, race-specific resistance is only effective against certain strains of the pathogen, and is easily overcome by rapid evolution of the pathogen resulting in a lack of durability in the field. In contrast, race-nonspecific resistance is effective against all known strains or races of the pathogen. It is thought to be based on multiple genes, may be durable, and is generally of a partial nature. Several wild *Solanum* species possess varying levels of partial resistance to *P. infestans* (Colon and Budding, 1988; Vleeshouwers *et al.*, 1999), and in old potato cultivars, such as cv. Robijn, partial resistance appeared to be durable (Colon *et al.*, 1995). In a previous study, we

cytologically analyzed *Solanum* species inoculated with *P. infestans*, and found that defense responses were always associated with the hypersensitive response (HR), a programmed cell death defense response of plants. In partially resistant clones, hyphal escape occurred and growing lesions were established (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000). The growth rate of these lesions varied between different *Solanum* clones, indicating that defense mechanisms other than the HR operate at different levels in the different clones.

Even though a causal link between the accumulation of PR proteins and SAR has not always been established, a correlation between the timing of *PR* gene expression and the onset and duration of SAR is evident from many studies (Ryals *et al.*, 1996). Therefore, measuring expression levels of *PR* genes is an appropriate method for determining levels of SAR (Ryals *et al.*, 1996; Maleck and Dietrich, 1999). In this study, we determined the variation in basal mRNA levels of SAR marker genes (*PR-1*, *PR-2*, and *PR-5*) in *Solanum* plants, and tested whether resistance to *P. infestans* in *Solanum* species is associated with high basal levels of SAR.

Material and methods

Phytophthora infestans

Phytophthora infestans isolates of different origins were used (Table 4-1). The propagation of the isolates and the preparation of inoculum was performed following standard procedures (Vleeshouwers *et al.*, 1999).

Plant material

The plant material used in this study is listed in Table 4-2. The origin of the plant material and the *in vitro* propagation was described previously (Vleeshouwers *et al.*, 1999). Plants were grown under controlled conditions in climate chambers with a 16h/8h day/night regime at 18/15°C and HPIT (Philips) illumination. The condition of different batches of plants used for the various resistance tests and for the *PR* gene expression analyses was comparable (Vleeshouwers *et al.*, 1999).

Table 4-1

Phytophthora infestans isolates used in this study.

Isolate	clonal lineage	race	origin	Host of origin	year	mating type	provided by
IPO-0	US-1	0	Unknown	Unknown	1987	A1	Lo Turkensteen, Plant Research Int., Wageningen, The Netherlands
90128	Unknown	1.3.4.6.7.8.10.11	Geldrop, the Netherlands	Potato	1990	A2	Francine Govers, Wageningen University, Wageningen, The Netherlands
Mex580	Unknown	Complex	Toluca Valley, Mexico	S. *demissum*	Unknown	A1	Bill Fry, Cornell University, Ithaca, USA
BIN-16	US-6	Complex	Northwestern USA	Unknown	1992	A1	Bill Fry, Cornell University, Ithaca, USA
ME93-2A	US-8	Complex	Maine, USA	Potato	1993	A2	Bill Fry, Cornell University, Ithaca, USA

Resistance assessment

The resistance levels of 18 *Solanum* clones to six *P. infestans* isolates were determined using a routine resistance assay (Vleeshouwers *et al.*, 1999). Detached leaves were spot-inoculated (10 μl) with a zoospore suspension of 50.000 spores/ml, and incubated at high humidity in the dark. On the fourth, fifth, and sixth day after inoculation, the largest length and width (perpendicular on the length) of the lesions were measured. The ellipse area (A = 1/4 * π * length * width) was calculated, and the lesions were divided in two groups, i.e. 'no growing lesion' (A ≤ 16 mm²), or 'growing lesion' (A > 16 mm²). The infection efficiencies (IE) were calculated as the percentage of growing lesions. The areas of the 'growing lesion' group were square root transformed, and the average lesion growth rate (LGR) was estimated by linear regression on time. The mean LGRs were analyzed with REML using Genstat (Genstat 5 Committee, 1987).

Southern and northern blot analysis

DNA was isolated from leaves of *Solanum* plants and digested with *Eco*RV (Shure *et al.*, 1983). The DNA was electrophorized, transferred to Hybond-N⁺, and the Southern blot was hybridized with various probes. For expression analyses, leaf material (3rd, 4th and 5th fully developed leaf) from healthy, uninoculated plants was harvested and immediately frozen in liquid nitrogen. Two independent RNA isolation (Verwoerd *et al.*, 1989) series were carried out. For each RNA sample 15 μg was loaded, electrophorized and transferred to Hybond-N⁺. The northern blot was hybridized concurrently with the Southern blot with probes representing the *PR-1*, *PR-2*, *PR-5* and tubulin gene at 65, 60, 60, and 65°C respectively, and the blots were washed at 1x, 0.5x, 0.5x, 1x SSC stringency respectively.

     Messenger RNA levels were determined from the northern blots using a Fujix Bio-Imaging analyzer (BAS 2000). The signals were quantified in photo-stimulated luminescence (PSL) per mm². To correct for slight differences in loading the signals for *PR* gene expression were normalized to the constitutively expressed tubulin signal.

DNA probes

PR gene members previously described to be correlated to resistance were selected. As DNA templates for probe synthesis the following fragments were used: a 400 bp *Eco*RI/*Kpn*I fragment of *StPR1-1*, a *PR-1* cDNA clone from potato (van 't Klooster *et al.*, 1999), a 1300 bp *Eco*RI/*Xho*I fragment of an acidic glucanase cDNA clone from tomato (van Kan *et al.*, 1992), a PCR fragment amplified on tobacco genomic DNA for *PR-5* (Melchers *et al.*, 1993), and a 1800 bp *Eco*RI/*Xho*I fragment from cDNA clone pFB19 encoding tubulin from potato.

Table 4-2

Resistance levels of Solanum clones to P. infestans isolates IPO-0, 90128, Mex580, BIN-16, and ME93-2A. The resistances are expressed as mean lesion growth rate (LGR, in mm day^{-1}) and infection efficiency (IE, in percentage), and are based on two experiments for IPO-0, Mex580, BIN16, and one experiment for 90128, and ME93-2A.

Solanum	clone	IPO-0		90128		Mex580		BIN-16		ME93-2A	
		LGR	IE	LGR	IE	LGR	IE	LGR	IE	LGR	IE
Solanum berthaultii	ber-9	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
S. berthaultii	ber-11	1.6	25	2.4	10	1.9	18	0.0	0	0.0	0
S. arnezii x hondelmannii	axh-63	2.0	60	1.9	95	2.2	88	1.6	30	1.8	25
S. arnezii x hondelmannii	axh-72	2.9	88	3.3	75	2.6	65	2.3	10	1.8	5
S. circaeifolium ssp. circaeifolium	crc-circ1	0.0	0	0.0	0	0.7	3	0.0	0	0.6	25
S. microdontum	mcd-167	0.0	0	3.2	45	1.8	5	1.5	3	0.0	0
S. microdontum	mcd-178	1.3	18	2.7	25	1.1	8	2.4	15	1.4	15
S. microdontum var. gigantophyllum	mcd-265	4.6	100	5.4	90	4.1	95	3.2	100	2.0	65
S. sucrense	scr-23	3.4	88	5.0	45	3.0	85	4.1	15	3.1	15
S. sucrense	scr-71	3.5	43	4.1	100	2.2	55	2.1	30	2.9	20
S. vernei	vrn-530	1.4	28	3.8	80	2.9	53	4.5	18	1.0	10
ABPT (30x33) hybrid	ABPT-44	2.3	13	2.6	80	2.0	10	1.7	23	3.4	25
S. nigrum	ngr-SN18	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
S. tuberosum	Bintje	4.1	100	5.0	75	3.7	98	2.7	85	4.0	35
S. tuberosum	Ehud (R1)	0.0	0	4.2	100	3.7	100	3.0	83	3.3	35
S. tuberosum	Estima (R10)	3.6	80	4.8	100	3.5	33	2.8	53	0.8	10
S. tuberosum	Première (R10)	3.1	45	3.5	90	3.8	33	3.3	28	4.0	15
S. tuberosum	Robijn	0.9	18	2.9	100	1.7	50	1.6	25	2.0	40

LSD$_{LGR}$ = 1.3
LSD$_{IE}$ = 24

Results and discussion

Specific and nonspecific resistance in Solanum

To test the correlation between high levels of SAR and resistance to *P. infestans*, we first carefully determined the resistance levels of a set of 18 *Solanum* plants to five isolates from different clonal lineages, races, geographical origins, hosts of origin, years of isolation and mating types (Table 4-1). Various types of resistance were noted as illustrated by the mean LGRs and IEs data (Table 4-2).

Statistical analyses revealed a highly significant interaction ($P < 0.001$) between *Solanum* clones and *P. infestans* isolates, indicating that race-specific resistance occurs. In potato cv. Ehud (*R1*), race-specific resistance was evident since inoculation with race 0 strain IPO-0 resulted in complete resistance (IE = 0, LGR = 0), whereas inoculation with isolate 90128 (virulent on *R1* plants), resulted in high IEs and LGRs (Table 4-2). In Estima and Première (*R10*), the race-specific response was less pronounced, as a considerable percentage of growing lesions was noted in the interaction with IPO-0. This is in line with previous findings that *R1* functions as a "strong" *R* gene and *R10* as a "weak" *R* gene (Turkensteen, 1987; Vleeshouwers *et al.*, 2000). Statistical analyses of LGR and IE between *Solanum* clones and *P. infestans* isolates also revealed a highly significant interaction ($P < 0.001$) when cultivars bearing *R1* and *R10* were excluded, suggesting that novel undefined *R* genes may occur in the examined set of *Solanum* plants. However, strong isolate-specific resistance (Vleeshouwers *et al.*, 2000) comparable to *R1* was not evident. In contrast, weaker isolate-specific responses were common. For example, *S. microdontum*-167 was more susceptible to isolate 90128 than to the other isolates. *S. sucrense*-23 was more often infected by IPO-0 and Mex580 (IE = 88% and 85% respectively) than by other isolates (IE = 15% to 45%), although high LGRs were noted in all interactions. Potato cv. Estima and Première exhibited similar IEs and LGRs to isolates IPO-0, 90128, Mex580 and BIN-16, but Estima was remarkably resistant to isolate ME93-2A.

Low LGR values are considered as indicators of nonspecific, partial resistance (Colon *et al.*, 1995). In addition to isolate-specific responses, *Solanum* clones differed in LGRs independently of the isolate tested. For example, potato cv. Robijn showed lower LGR values than Bintje with all tested isolates. Cultivar Estima and Première, and to a lesser extent cv. Ehud, showed intermediate LGRs, indicating a certain level of partial resistance for these cultivars. In *S. microdontum*, clone 167 and 178 displayed lower LGRs than clone 265, and in *S. arnezii x hondelmannii*, clone 63 generally displayed slightly lower LGRs than clone 72, independently of the isolate. This suggests that most of the examined *Solanum* plants display significant and variable levels of race/isolate-nonspecific, partial resistance to five *P. infestans* isolates of diverse origin.

Occurrence of PR genes in Solanum

To check whether sufficient cross-hybridization occurs between the *PR* gene probes and the selected plants, a Southern blot containing genomic DNA from the different *Solanum* species was hybridized with probes from a potato *PR-1* gene, a tomato *PR-2* gene, and a tobacco *PR-5* gene. As shown in (Figure 4-1), the three probes cross-hybridized with DNA from all *Solanum* clones. In all cases multiple hybridizing bands were detected revealing the presence of multi-gene families for *PR-1*, *PR-2*, and *PR-5* in *Solanum* species. In addition, there was variation in signal intensity among the hybridizing bands, suggesting sequence diversity or differences in copy number among the family members. Multi-gene families have been reported for *PR-2* in *S. tuberosum* (Beerhues and Kombrink, 1994), for *PR-5* in *S. commersonii* (Zhu *et al.*, 1995), and for *PR-1*, *PR-2*, and *PR-5* in tobacco (van Loon and van Strien, 1999).

For each of the three *PR* genes, the hybridization pattern within the species was reasonably conserved. Between species however, hybridization patterns were quite diverse. For *PR-2*, a certain specificity can be noted, as the hybridization patterns of the closely related species *S. berthaultii* and *S. arnezii x hondelmannii* (Spooner and Castillo, 1997) were quite similar. In addition, the hybridization of the tomato *PR-2* probe to *S. nigrum* DNA was exceptionally weak, whereas hybridization of the same blot with the *PR-1* and *PR-5* probes resulted in stronger signal intensities. This suggests that the *PR-2* genes from *S. nigrum* are quite divergent from those of the other tested *Solanum* species. This is not surprising since *S. nigrum* is the most distantly related species in the examined set. *S. nigrum* is classified in the subgenus *Solanum*, whereas the other tested *Solanum* species and tomato belong to the subgenus *Potatoe* (Spooner *et al.*, 1993). In summary, the Southern blot hybridizations showed that the heterologous *PR* probes are suitable for analyzing expression of *PR-1*, *PR-2*, and *PR-5* genes in the *Solanum* plants.

Expression analysis of Solanum PR genes

To monitor constitutive SAR in the *Solanum* plants, the basal expression levels of the *PR-1*, *PR-2*, and *PR-5* genes were determined in two independent experiments using uninoculated plants cultivated under defined conditions in growth chambers. The results of the first experiment are shown in Figure 4-2. The autoradiographs showed that *PR-1*, *PR-2*, and *PR-5* mRNAs were present at detectable levels in the majority of the tested plants. Interestingly, there is variation in *PR* mRNA levels between the different clones. In addition, within the entire *Solanum* set, the patterns of mRNA levels of the three *PR* genes were not identical. This is in line with the separation of *PR-1*, *PR-2*, and *PR-5* gene activation pathways previously shown for *Arabidopsis* enhanced disease susceptibility mutants *eds5* (Reuber *et al.*, 1998). The absence of coordinated regulation between the different *PR* genes was also observed in salicylic acid induction deficient mutants *sid1* and *sid2*, in which the pathway leading to *PR-1* expression was blocked, whereas *PR-2* and *PR-5* were expressed at wildtype levels (Nawrath and Metraux, 1999). Thus unequal mRNA accumulation of the different *PR* genes could reflect different pathways of regulations for individual members of the three *PR* gene families, perhaps

reflecting the wide range of physiological responses in which these proteins are involved (Ori *et al.*, 1990; Zhu *et al.*, 1995; Tornero *et al.*, 1997).
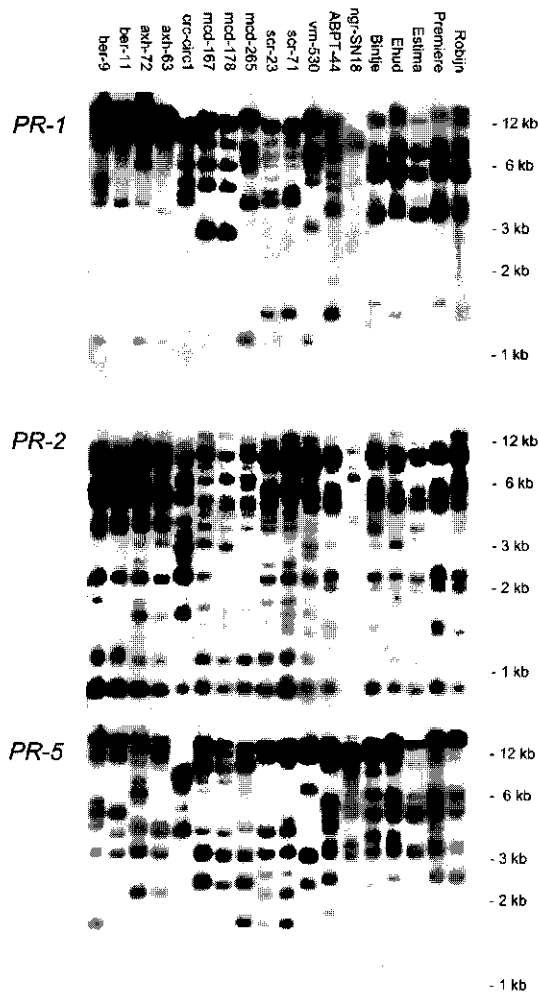

Figure 4-1

Occurrence of *PR* genes in *Solanum* clones (for abbreviation of clones see Table 4-2). A Southern blot containing *Eco*RV digested genomic DNA isolated from *Solanum* clones was sequentially hybridized with *PR-1* probe from potato, a *PR-2* probe from tomato, and a *PR-5* probe from tobacco.

PR mRNA levels were quite similar in the two experiments. However, in potato cv. Ehud and *S. vernei*-530, the mRNA levels observed for all three *PR* genes were higher in the second experiment compared to the first experiment. We assume that despite the precautions we took the Ehud and *S. vernei* plants in the second experiment may have been in a stressed state. In other experiments we noted high *PR-1* mRNA levels in plants grown in the greenhouse (data not shown), where they were exposed to heat and drought stress.
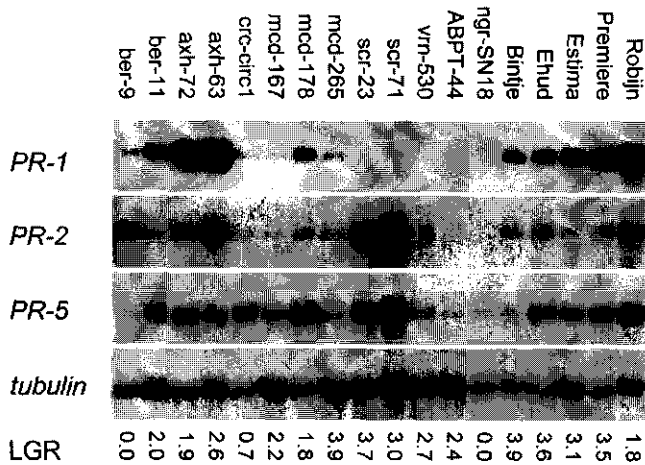
Figure 4-2

Expression analyses of *PR* genes in uninoculated *Solanum* plants (for abbreviation of clones see Table 4-2). A northern blot containing total RNA from *Solanum* clones was sequentially hybridized with *PR-1*, *PR-2*, *PR-5* and tubulin probes. The average LGRs (mm.day^{-1}) based on *P. infestans* isolates IPO-0, 90128, Mex580, BIN-16, ME93-2A) are indicated for each clone (LGR = 0 excluded).

Does PR gene expression correlate with resistance?

To test whether isolate-nonspecific, partial late blight resistance in *Solanum* is associated with a constitutive SAR, the obtained LGRs were compared to basal mRNA levels of *PR* genes. *PR-1*, *PR-2*, and *PR-5* mRNA levels were quantified and normalized using tubulin mRNA levels as a reference. At the genus level, there was no indication for a correlation between nonspecific resistance and *PR* expression levels. This may be explained by the different genetic background of the different plant species and the noted complexity of the three *PR* gene families. In contrast, significant correlation was observed at the species level. In *S. arnezii x hondelmannii*, *S. microdontum*, *S. sucrense* and *S. tuberosum*, partially resistant clones

exhibited higher levels of *PR* mRNA than more susceptible ones. In *S. sucrense* and *S. amezii x hondelmannii,* this correlation was evident for all three *PR* genes in the two independent experiments. In *S. circaeifolium, S. vernei,* ABPT, and *S. nigrum,* only one clone was used, and thus correlations at the species level could not be tested. No correlation was observed between *PR* mRNA levels and resistance in *S. berthaultii.* However, the full resistance observed in clone 9 is HR-mediated (Vleeshouwers *et al.,* 2000) and suspected to operate through a novel *R* gene as recently observed for another accession of *S. berthaultii* (Ewing *et al.,* 2000).
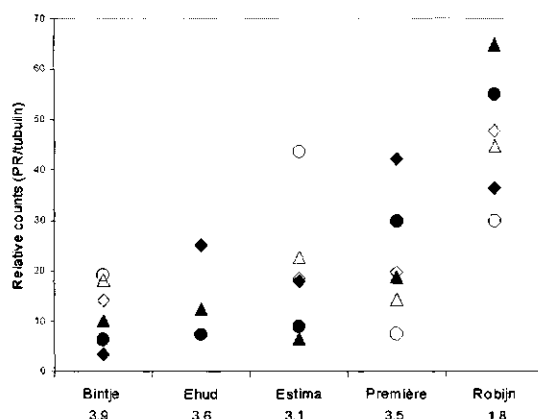
Figure 4-3

Messenger RNA levels of *PR* genes and isolate-nonspecific resistance in *S. tuberosum. PR-1* (●), *PR-2* (▲), and *PR-5* (♦) mRNA levels from uninoculated leaves from potato cultivars Bintje, Ehud, Estima, Première and Robijn were quantified on a phosphor-imager, and the PR signals were normalized using the tubulin mRNA level as reference. Messenger RNA levels were determined in two independent experiments (first experiment closed, second open symbols); the data from Ehud in the second experiment were excluded. On the X-axis, average LGRs (mm.day^{-1}, see Table 4-2) determined after inoculation with *P. infestans* isolates IPO-0, 90128, Mex580, BIN-16, ME93-2A are indicated for each cultivar (LGR = 0 excluded).

Within *S. tuberosum,* the relationship between nonspecific resistance and *PR* mRNA levels was examined for the five tested cultivars (Figure 4-3). Highly resistant cv. Robijn accumulated the highest levels of *PR-1, PR-2,* and *PR-5* mRNA, whereas partially resistant Ehud, Estima and Première displayed intermediate levels, and susceptible Bintje very low levels. These results suggest a correlation between *PR* gene expression levels and resistance levels in potato. These results are consistent with those obtained with *S. amezii x hondelmannii, S. microdontum,* and *S. sucrense* suggesting that an enhanced constitutive

expression of SAR may be a component of the partial-nonspecific resistance noted in *Solanum* species.

Concluding remarks

Basal SAR may function as an independent resistance mechanism, but more likely contributes to the complex network of defense reactions that take place following pathogen attack (Graham and Graham, 1999; Pieterse and van Loon, 1999). Resistance in the *Solanum* - *P. infestans* interaction often exhibits a quantitative nature, which can be explained by the extent of an ambiguous HR. Per infection event, a fine balance between invading hyphae and plant cells exhibiting an HR, determines whether infection will be aborted (Vleeshouwers *et al.*, 2000). Basal level of SAR may increase the sensitivity of plant cells to HR elicitation (Shirasu *et al.*, 1997), or may slow pathogen invasion by creating physiological conditions that limit pathogen growth.

The identification of molecular markers linked to partial resistance to *P. infestans* in *Solanum* species, is of great value for potato late blight resistance breeding. High levels of PR mRNAs could serve as useful molecular markers for screening breeding populations and germplasm. Assays based on quantitative reverse transcriptase (RT-PCR) of *PR* genes could be developed to assist potato breeders and geneticists in identifying promising genotypes and could supplement other assays based on quantitative trait loci (QTL) (Leonards-Schippers *et al.*, 1994). Using molecular marker genes for known resistance mechanisms such as SAR may provide a novel prospective for marker-assisted breeding.

Acknowledgements

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Ancient diversification of the Pto *kinase family preceded speciation in* Solanum

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Summary

Recent phylogenetic analyses of the NBS-LRR class of plant disease resistance (*R*) genes suggested that these genes are ancient and coexist next to susceptibility alleles at resistance loci. Another class of *R* genes encodes serine/threonine protein kinases related to *Pto*, which were originally identified from wild relatives of tomato. In this study, we exploit the highly diverse genus *Solanum* to identify *Pto*-like sequences and test various evolutionary scenarios for *Pto*-like genes. Polymerase chain reaction amplifications using primers based on conserved and variable regions of *Pto* revealed an extensive *Pto* gene family and yielded 32 intact *Pto*-like sequences from six *Solanum* species. *Pto*-like transcripts were also detected in leaf tissue of all tested plants. The kinase consensus and autophosphorylation sites were highly conserved, in contrast to the kinase activation domain which is involved in ligand recognition in Pto. Phylogenetic analyses distinguished nine classes of *Pto*-like genes, and revealed that orthologues were more similar than paralogues, suggesting that the *Pto* gene family evolved through a series of ancient gene duplication events prior to speciation in *Solanum*. Thus, in line with the NBS-LRR class, the kinase class of *R* genes is highly diverse and ancient.

Introduction

The occurrence of conserved structural features in plant disease resistance (*R*) genes provides a great potential for the isolation of novel analogues of these *R* genes using the polymerase chain reaction (PCR). In most *R* genes cloned so far, one or more characteristic functional domain is conserved across plant families. Such domains include the leucine-rich repeats (LRR), nucleotide binding sites (NBS), leucine-zippers (LZ), transmembrane (TM) and kinase domains, and suggest that *R* genes encode components of conserved and ubiquitous signal transduction pathways in plants (Hammond-Kosack and Jones, 1997). A PCR-based strategy using degenerate primers corresponding to NBS motifs was applied to *Arabidopsis*, soybean, potato, lettuce, and several other plants, and resulted in cloning of a large collection of resistance gene candidates (RGAs or *R* gene analogs) from the NBS-LRR class (Kanazin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996; Aarts *et al.*, 1998; Shen *et al.*, 1998). *R* genes from other classes, i.e. the LRR, LRR-TM, LRR-TM-kinase, and kinase class (Bent, 1996; Richter and Ronald, 2000), do not possess an NBS domain. Yet a similar gene discovery approach can be applied using primers based on other conserved domains, such as the kinase domain.

       The *Pto* gene from tomato confers hypersensitive response (HR)-mediated resistance to *Pseudomonas syringae* pv. *tomato* (Martin *et al.*, 1993a). *Pto* is a member of a multi-gene family that is clustered within a 400 kb region on chromosome 5 of tomato (*Lycopersicon*) (Martin *et al.*, 1993b). Another member of this family is *Fen*, which confers sensitivity and HR-like lesions to the insecticide fenthion (Martin *et al.*, 1994). Characterization of the Pto and Fen

proteins revealed that they are 80% identical and 87% similar; that both encode active serine/threonine kinases, and that they participate in the same signal transduction pathway leading to the HR (Martin et al., 1993a; Martin et al., 1994; Loh and Martin, 1995). Pto and Fen originate from the wild species Lycopersicon pimpinellifolium, and were introgressed into tomato cultivars by classical breeding approaches (Pitblado and Kerr, 1980). Alleles of Pto and Fen genes, pto and fen respectively, were cloned from the cultivated tomato Lycopersicon esculentum. They encode active kinases, but do not confer bacterial speck resistance or fenthion sensitivity (Jia et al., 1997). Pto and pto, and Fen and fen alleles differ in only a few amino acids, which presumably cause conformational changes that affect the ability to physically interact with other proteins (Jia et al., 1997). Pto specifically phosphorylates other kinases, such as Pto interacting kinase 1 (Pti1) (Zhou et al., 1995) and physically interacts with the avirulence gene product AvrPto (Scofield et al., 1996; Tang et al., 1996). The activation domain (the region between amino acid 182 and 211 of Pto) plays a critical role for recognition. In the Pto-AvrPto interaction, S198 is required for elicitation of the HR (Sessa et al., 2000b), T204 is required for recognition specificity, Y205 plays a subsidiary role in recognition (Frederick et al., 1998), and Y207 influences binding properties (Rathjen et al., 1999). In addition, autophosphorylation sites appear to be required for kinase activity or physical interaction with amongst others AvrPto and several Pto interacting (Pti) proteins (Sessa et al., 2000b).

Despite tremendous advances in structural molecular genetics of R genes (Baker et al., 1997), the evolution of recognitional specificities remains poorly understood. Genomic sequencing and genetic mapping studies have revealed the occurrence of clusters of R genes in the genome (Michelmore and Meyers, 1998; Richter and Ronald, 2000). In tomato, the Pto locus has evolved through a series of gene duplications and deletions, resulting in five Pto homologues (Michelmore and Meyers, 1998). Also other R gene families, such as those at the tomato Hcr9 loci, which harbor homologues of Cladosporium fulvum resistance gene Cf9, and the lettuce Dm3 locus, which contains Bremia lactucae resistance genes, occur in duplicated gene clusters (Meyers et al., 1998; Parniske and Jones, 1999). A wide diversity of R genes of the NBS-class occurs in the plant kingdom and elegant phylogenetic analyses of an extensive set of NBS-containing R gene-like sequences pointed to an ancient evolutionary history for these genes (Meyers et al., 1999). In addition, recent population genetic data suggested that R genes of the NBS-LRR class co-exist next to susceptibility alleles (Stahl et al., 1999). The results of both of these studies conflict with the widely accepted 'arms-race' model for rapid evolution of resistance genes as a response to an adapting pathogen. A novel 'trench warfare' theory is emerging: a dynamic process of advances and retreats of ancient resistance alleles maintains diversity at a disease resistance locus (Stahl et al., 1999).

The genus Solanum is highly diverse, consisting of about 1100 species (D'Arcy, 1991). Based on chloroplast DNA (cpDNA) restriction fragment analysis, Solanum is thought to form a monophyletic lineage that includes Lycopersicon (Spooner et al., 1993), and here we will refer to Solanum in the broad sense. The center of origin of Solanum is thought to be in Mexico, from where species migrated southwards and evolved into a separate gene pool in South America (Hawkes, 1990). The cultivated potato S. tuberosum arose in this area, probably from a

complex of diploid domesticated *Solanum* species. A selection of wild *Solanum* species has been incorporated in modern breeding programs to introgress resistance against a broad spectrum of potato pathogens. For example, *S. demissum* has supplied the 11 known *R* genes that confer HR-mediated resistance to the potato late blight pathogen *Phytophthora infestans.* Several other tuber-bearing *Solanum* species also show HR-mediated resistance, which suggests the involvement of *R* genes in late blight resistance (Vleeshouwers *et al.*, 2000).

In this study, we take advantage of the genetic diversity of the genus *Solanum* to test the co-evolutionary arms-race hypothesis for the *Pto* gene family. We amplified *Pto*-like sequences from a diverse set of *Solanum* species with primers based on conserved and variable regions of known *Pto*-like genes. Sequencing and Southern blot analyses revealed an extensive *Pto*-like gene family in *Solanum*, and northern blot analyses showed expression of *Pto*-like genes in leaves. Phylogenetic analysis revealed that orthologues (homologues separated by a speciation process) are more similar than paralogues (homologues generated by a gene duplication event), suggesting that ancient duplications of the common ancestor of *Pto*-like genes probably occurred prior to *Solanum* speciation.

Materials and methods

Plant material

The *Solanum* clones used in this study are presented in Table 5-1. Wild *Solanum* accessions contain resistance to various bacterial, viral, nematode, fungal and oomycete pathogens (Hoekstra and Seidewitz, 1987; Vleeshouwers *et al.*, 2000; evaluation data from the Center of Genetic Resources, the Netherlands, http://www.plant.wageningen-ur.nl/cgn/potato).

Southern and northern blot analysis

DNA was isolated from leaves of *Solanum* plants (Shure *et al.*, 1983). The DNA was digested with *Eco*RV, electrophorized and transferred to Hybond-N$^+$. For expression analyses, leaf material was harvested and RNA was isolated (Verwoerd *et al.*, 1989). From 500 µg total RNA, poly A$^+$ RNA was extracted using the Oligotex mRNA kit (Qiagen). For northern blot analyses, the poly A$^+$ RNA was denatured at 50°C in 1 M glyoxal, 54% (v/v) DMSO, and 10 mM sodiumphosphate buffer (pH 7.0), electrophorized and transferred to Hybond-N$^+$. As DNA templates for probe synthesis the following fragments were used: a 600 bp *Sac*II/*Pst*I fragment of berDF1, a 600 bp *Sac*II/*Pst*I fragment of berDF4, and a 1800 bp *Eco*RI/*Xho*I fragment from cDNA clone pFB19 encoding tubulin from potato. The Southern and poly A$^+$ northern blot were hybridized with ^{32}P-labelled probes at 65°C, and washed at 0.1x or 1x SSC stringency for the berDF1 and the tubulin hybridization respectively. Messenger RNA levels were determined using a Fujix Bio-Imaging analyzer (BAS 2000), and the signals were quantified in photo-stimulated luminescence (PSL) per mm^2.

Table 5-1

Solanum species and hybrids used in this study

Solanum species / hybrids	Code	Accession / origin	Country of collection	plant clones / cultivars
ABPT-hybrid[1]	ABPT	Plant Breeding, WUR	-	44
S. arnezii x hondelmannii	axh	BGRC[2] 27308	Bolivia	63, 72
S. berthaultii	ber	BGRC10063	Bolivia	9, 11
S. circaeifolium ssp. circaeifolium	crc	BGRC 27058	Bolivia	circ1
S. demissum	dms	CPC[3] 2127	Mexico	PBL[4]
S. microdontum	mcd	BGRC 24981	Argentina	167, 178
S. microdontum var. gigantophyllum	mcd	BGRC 18570	unknown	265
S. nigrum	ngr	Plant Res. Int., WUR	Netherlands	SN18
S. nigrum x tuberosum[5]	nxt	Plant Res. Int., WUR	-	SN18xDes
S. sucrense	scr	BGRC 27370	Bolivia	23, 71
S. tuberosum	tbr	potato cultivars	-	Bintje, Ehud, Estima, Première, Robijn
S. vernei	vrn	BGRC 24733	Argentina	530

[1] Double-bridge hybrid (30x33) of S. acaule, S. bulbocastanum, S. phureja, S. tuberosum (Hermsen and Ramanna, 1973).
[2] Braunschweig Genetic Resource Center (Germany), which moved to Center of Genetic Resources, the Netherlands.
[3] Commonwealth Potato Collection, Scottish Crop Research Institute, UK.
[4] Plant Breeding Line, obtained from DJ Huigen, Laboratory of Plant Breeding, Wageningen University.
[5] Hybrid of S. nigrum-SN18 x S. tuberosum cv. Désirée (Eijlander and Stiekema, 1994).

Primer design, PCR amplification and DNA sequencing

The primers used in this study are presented in Figure 5-1. For primer combinations F1-R1, F4-R1, F2-R2, and F5-R2, PCR amplification was performed on 60 ng genomic DNA in 50 or 100 µl reaction volumes. The templates were denatured by heating to 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, with a final extension of 10 min at 72°C. For primer combinations F1-R2 and F2-S1, the PCR was conducted in 25 µl reaction volumes, denaturation was performed at 94°C for 7 min, followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 50°C, 1 min at 72°C, with a final extension of 5 min at 72°C.

PCR products were cloned in pGEM-T or pGEM-T Easy (Promega). Recombinants were selected using X-Gal in the selection medium or by colony hybridization at 65°C using a radio-labeled PCR product as a probe. After selection of positive clones, plasmid DNA was isolated and digested with SacII/PstI to estimate the size of the cloned insert. Alternatively, the insert sizes were checked by PCR. The DNA sequences were determined by automated DNA sequencing.

Primer	Sequence (5'-3')	Location on Pto (aa)	Specificity
F1	CAAATTCGATAAATGATGC	9-15	conserved
F2	AGATCTACGTCTTCCCACT	130-136	specific to dmsFD2
F4	TTTAAACTCGAGTTATCGC	16-21	conserved
F5	TTGTATGGATCAGATCTAC	126-132	semi-conserved
R1	CCGAAAGAATAAACATCAG	222-228	conserved
R2	GTGCATACTCCAGTTTCCA	308-313	conserved
S1	CCCTTCTTCATCCAATTCATT	257-262	specific to berS1
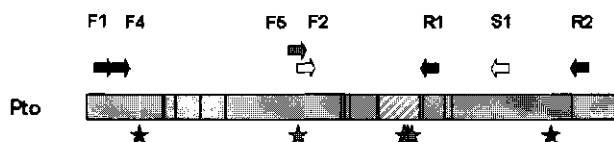
Figure 5-1

Primers designed to amplify *Pto*-like sequences from *Solanum*. Nucleotide sequences, position on *Pto* (Martin *et al.*, 1993a) and degrees of specificity of the primers. In the schematic representation of *Pto*, the orientation of the primers is indicated by arrows, the 15 invariant kinase amino acid residues by vertical bars, the activation domain is dashed, and conserved autophosphorylation sites are indicated by asterisks.

Phylogenetic analyses

Sequence data were evaluated with the DNA-Star software package (Lasergene, Madison, Wisconsin). The sequences were aligned in ClustalX 1.64b (Thompson *et al.*, 1997). Ambiguous regions in the alignment were removed from the data set. Phylogenetic trees were constructed in PAUP 4.0b4a (Swofford, 2000) by the neighbor-joining method (Saitou and Nei, 1987) using default settings. Heuristic bootstrapping was performed to evaluate the degree of support for grouping in the neighbor-joining analyses.

Results

Pto-like sequences are diverse in Solanum

A set of diverse potato cultivars and wild *Solanum* species was selected (Table 5-1). To explore the presence of *Pto*-like sequences, a Southern blot containing *Eco*RV digested genomic DNA from the selected *Solanum* plants was hybridized with a probe from *Pto*-like clone berDF1 from *S. berthaultii*, (Figure 5-2, berDF1 was obtained by PCR with primers based on *Pto*, see below). In each lane, four to nine bands were detected, revealing the presence of an extensive family of *Pto*-like sequences in *Solanum*. Variation in the signal intensity among the hybridizing bands suggests sequence diversity or presence of multiple copies (per *Eco*RV fragment).
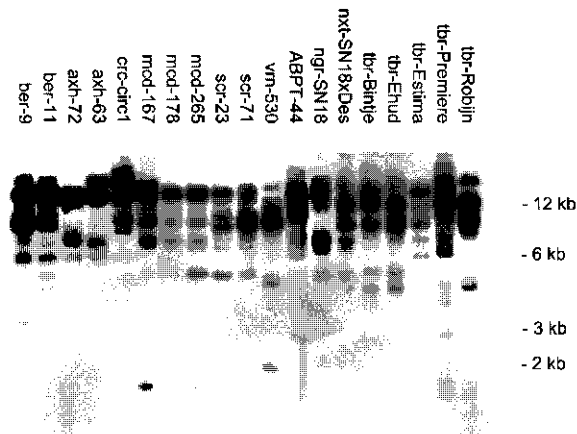
Figure 5-2

Multiple *Pto*-like sequences in *Solanum*. Autoradiograph of a Southern blot containing *Eco*RV-digested genomic DNA from *Solanum* plants hybridized with a berDF1 probe. For abbreviations of the plant codes see Table 5-1.

Within species, the hybridization patterns were partly conserved suggesting conservation of the *Pto*-like sequences. Intraspecific polymorphisms were also revealed for the different *Solanum* species. Hybridization of the same Southern blot with a probe from a distinct member berDF4 of the *Pto* family (see below) yielded a similar banding pattern with only slight differences in intensity of some bands (data not shown), indicating that most family members share a high level of identity at the DNA level.
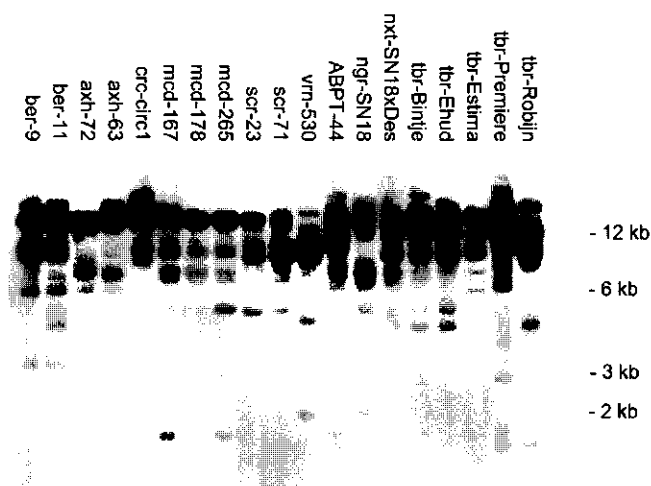
Pto-like genes are expressed in Solanum leaves

To examine expression of *Pto* homologues in *Solanum*, a northern blot was prepared containing poly A⁺-RNA isolated from leaves from a subset of the selected set of plants (Figure 5-3). Hybridization with the berDF1 probe revealed the presence of *Pto*-like transcripts in all tested *Solanum* plants. Several bands with slight size differences could be discriminated for individual plant genotypes, suggesting the presence of a mixture of *Pto*-like mRNAs. The same blot was hybridized with a potato tubulin probe, and signals from *Pto*- and tubulin-hybridizing bands were quantified with a phosphor-imager. Calculation of the relative amounts of *Pto*-like mRNA revealed similar levels of *Pto*-like mRNA in the wild *Solanum* species (data not shown). Within the potato cultivars, the relative mRNA levels were also similar, but overall they were higher than in the wild relatives. In summary, these results suggest that *Pto*-like genes are expressed in all tested *Solanum* plants.
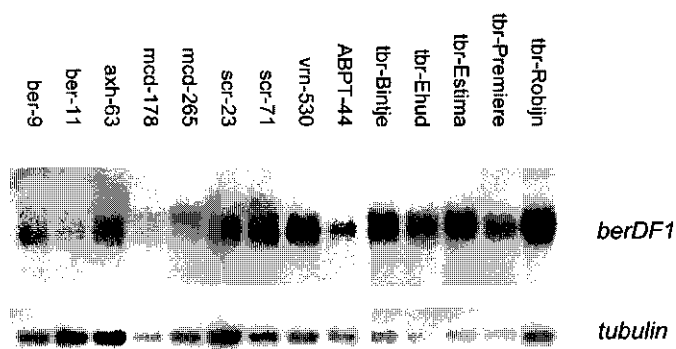
Figure 5-3

Pto-like transcripts in Solanum leaves. Autoradiographs of a poly A$^+$ northern blot of Solanum leaves hybridized with a berDF1 and a tubulin probe. For abbreviations of the plant codes see Table 5-1.

High levels of diversity of Pto-like sequences from Solanum

An iterative PCR-based approach was adopted to identify Pto-like sequences from the diverse set of Solanum species. Forward primer F1 and reverse primers R1 and R2 were designed based on sequences conserved in Pto and Fen from L. pimpinellifolium but divergent in other kinases (Figure 5-1). An initial set of Solanum DNA fragments was amplified and sequenced. Using this sequence information, additional forward primers F4 and F5 were designed based on a semi-conserved region. In addition two specific primers were designed, i.e. forward primer F2 based on dmsFD2 and a common 2 amino acid indel (insertion/deletion), and reverse primer S1 based on berS1 and a highly variable region in the 3' part.

PCR with the conserved, semi-conserved and specific primers yielded a large and diverse pool of Pto-like sequences in Solanum. A total of 66 clones were sequenced. Of these, 20 were found to contain frameshifts and stopcodons, and were excluded from further analyses. Among the remaining 46 sequences, 14 were redundant. The 32 remaining Pto-like sequences were included for further study (Table 5-2). Sequence alignments were performed and revealed higher conservation in the 5' part of the gene than in the 3' part (not shown).

Table 5-2

Overview of *Pto*-like sequences obtained by PCR. *Solanum* from which the template DNA was derived, the primers used for amplification, the size of the PCR fragment, and the GenBank accession numbers are indicated.

Pto-like sequence	*Solanum* clone	Primers[1]	size (bp)	GenBank accession number
axhBF1	*S. arnezii x hondelmannii*-63	F1 – R1	614	AF288538
ber2H	*S. berthaultii*-9	F1 – R2	871	AF288539
ber7A	*S. berthaultii*-9	F2 – S1	362	AF288540
berBD3	*S. berthaultii*-9	F1 – R1	614	AF288541
berDF1	*S. berthaultii*-9	F5 – R2	512	AF288542
berDF4	*S. berthaultii*-9	F5 – R2	530	AF288543
berDJ2	*S. berthaultii*-9	F4 – R1	595	AF288544
berS1	*S. berthaultii*-9	F2 – R2	516	AF288545
dmsFD2	*S. demissum*-PBL	F1 – R2	877	AF288546
scr1A	*S. sucrense*-71	F1 – R2	871	AF288547
scr1C	*S. sucrense*-71	F1 – R2	871	AF288548
scr1G	*S. sucrense*-71	F1 – R2	871	AF288549
scr8A	*S. sucrense*-71	F2 – S1	362	AF288550
scr8B	*S. sucrense*-71	F2 – S1	377	AF288551
scr8C	*S. sucrense*-71	F2 – S1	362	AF288552
scr8D	*S. sucrense*-71	F2 – S1	377	AF288553
scr8E	*S. sucrense*-71	F2 – S1	377	AF288554
scr8I	*S. sucrense*-71	F2 – S1	377	AF288555
scrDG1	*S. sucrense*-71	F5 – R2	521	AF288556
scrDG4	*S. sucrense*-71	F5 – R2	530	AF288557
scrDK1	*S. sucrense*-71	F4 – R1	595	AF288558
scrY10	*S. sucrense*-71	F1 – R1	614	AF288559
tbrBA3	*S. tuberosum*-Ehud	F1 – R1	614	AF288560
tbr4A	*S. tuberosum*-Robijn	F1 – R2	871	AF288561
tbr4D	*S. tuberosum*-Robijn	F1 – R2	871	AF288562
tbr5A	*S. tuberosum*-Robijn	F2 – S1	377	AF288563
tbrDI2	*S. tuberosum*-Robijn	F5 – R2	521	AF288564
tbrDI4	*S. tuberosum*-Robijn	F5 – R2	530	AF288565
tbrW1	*S. tuberosum*-Robijn	F1 – R1	614	AF288566
vrn6A	*S. vernei*-530	F2 – S1	386	AF288567
vrnBB1	*S. vernei*-530	F1 – R1	614	AF288568
vrnDL2	*S. vernei*-530	F4 – R1	595	AF288569

[1] See Figure 3
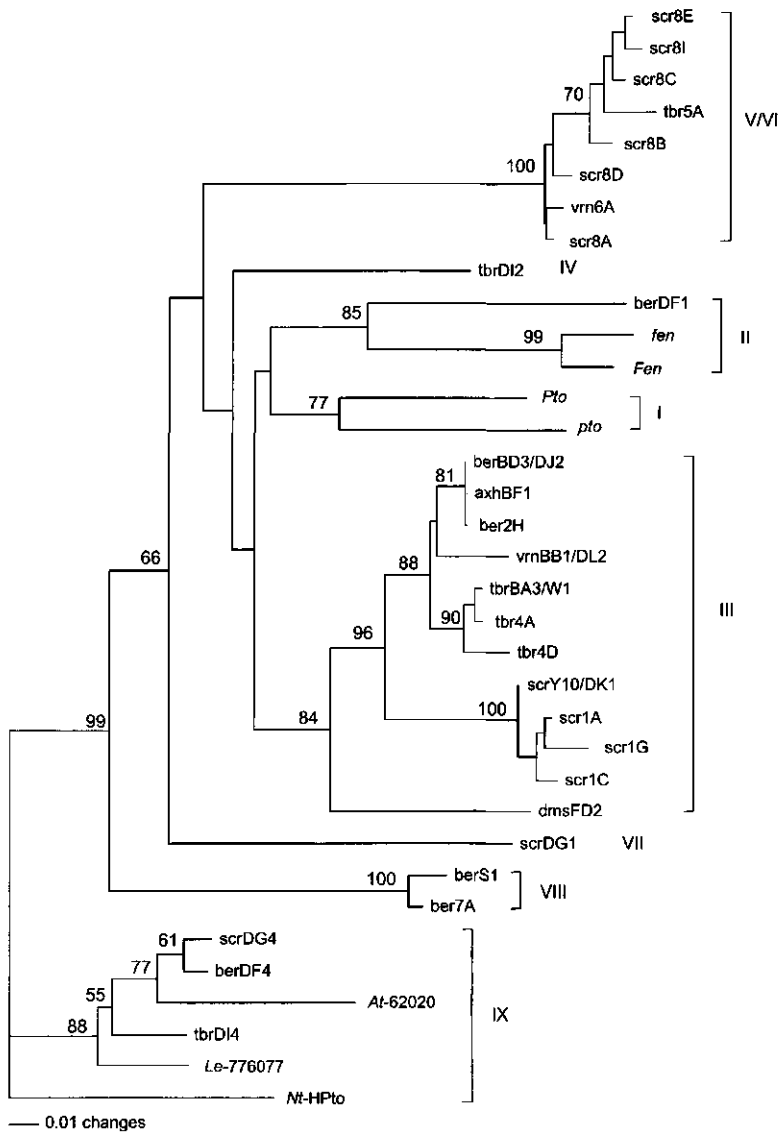
Figure 5-4

Phylogenetic tree of Pto-like sequences. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the aligned amino acid sequences. The tree was rooted with class IX. For the sequence codes, see Table 5-2 and results section. The branch lengths are proportional to average substitutions per site as indicated by the scale. Gaps were treated as missing characters. Bootstrap values are indicated at nodes supported with >50% of 100 replicates. Branches were supported by the phylogenetic tree generated using parsimony analysis (not shown).

Phylogenetic analyses of the Pto gene family

To develop a data set for phylogenetic analyses, we mined the NCBI and TIGR databases using BLAST searches with the *Pto* sequence (Altschul *et al.*, 1997). This resulted in a large set of (putative) kinases, predominantly receptor-like kinases and serine-threonine kinases, from various plants, including *Arabidopsis thaliana, Catharanthus roseus, Zea mays* and *Oryza sativa.* Other homologous sequences, i.e., a receptor-like kinase homologue from *Arabidopsis* (*At*-62020, accession CAB62020) and a *L. esculentum* EST clone (*Le*-776077, accession AI776077), in addition to the *Pto/Fen* homologues from *Lycopersicon* and *Nicotiana* (*Nt*-HPto), and the *Solanum* PCR fragments were included in a data set (Martin *et al.*, 1993a; Martin *et al.*, 1994; Martin *et al.*, 1996; Jia *et al.*, 1997). *Pto*-like sequences were also reported from *Capsicum annuum* (Pflieger *et al.*, 1999), however since they represent pseudogenes, they were not included. Phylogenetic analyses were performed using both the Neighbor-Joining and maximum parsimony algorithms and statistical significance of the identified groups was evaluated using bootstrapping. Similar clustering of sequences was obtained with both methods (Figure 5-4 and not shown) and nine different classes (class I-IX) could be distinguished based on phylogeny and indel distribution (Table 5-3).

Class IX includes disparate Solanaceae and Crucifereae sequences, which suggests that this class diverged prior to the radiation of the Solanaceae. In addition, all class IX sequences contain a 3 amino acid indel (Table 5-3), that is conserved in a broader range of serine/threonine receptor-like kinases from *Arabidopsis, Catharanthus, Oryza,* and *Zea,* (obtained using BLAST searches with *Pto,* not shown). Therefore, class IX was used to root the tree.

Sequence features of classes of Pto-like sequences

The sequence characteristics of the nine classes of *Pto*-like sequences were carefully analyzed and are summarized in Table 5-3. Class I contains the two *Pto* alleles from *Lycopersicon.* Class II includes the *Fen* alleles from *Lycopersicon* as well as berDF1 from *S. berthaultii.* In class IX, two non-*Solanum* sequences from *Nicotiana* and *Arabidopsis* cluster together with four *Solanum/Lycopersicon* sequences. All members of this class share a 3 amino acid indel at position 159 (numbered according to the *Pto* sequence), in contrast to all other *Solanum Pto*-like sequences. Another multiple amino acids indel AVGRY was evident at position 245 in scr8E, scr8I, scr8B, scr8D and tbr5A, and all these sequences are classified as classes V/VI in the phylogenetic tree. Although these indels were excluded from the phylogenetic analysis, their occurrence is consistent with the classification.

In the *Pto*-like sequences from *Solanum,* the 15 amino acid residues of the protein kinase consensus (Hanks and Quinn, 1990) were highly conserved (Table 5-3). Autophosphorylation sites previously identified in *Pto* show a high degree of conservation, for example T38 and T288 are conserved in all sequences. At position 133, the threonine was often replaced by a serine, thereby providing an alternative phosphorylation site. Also T195,

S198 and T199 are highly conserved, except for class V/VI sequences. Furthermore, the kinase activation consensus residues domain appeared well conserved compared to other plant serine/threonine kinases (Sessa *et al.*, 2000a), and showed only one amino acid change in class V/VI, VII and VIII sequences. In contrast, there was more variation in the entire activation domain than in the 5' part of the *Pto*-like sequences (Table 5-3).

Discussion

In this study, a diverse pool of 32 *Pto*-like sequences was identified and characterized from two potato cultivars and five wild *Solanum* species, revealing a complex family of *Pto*-like genes in *Solanum*. Several *Pto*-like transcripts of varying sizes were detected in all tested *Solanum* plants suggesting that various members of the gene family are expressed in *Solanum* leaves. All amplified *Solanum* sequences displayed fully conserved serine/threonine kinase motifs suggesting that the uncovered genes are likely to encode active kinases and are members of a large family of *Pto*-like kinases.

Most cloned *R* genes are members of multi-gene families, indicating that gene duplication and subsequent diversification are common processes in *R* gene evolution (Ronald, 1998). For example, the *Xa21* gene family evolved through gene duplication, recombination and diversification into two distinct classes of genes in rice (Song *et al.*, 1997). In the present study, phylogenetic analyses of the *Pto*-like sequences revealed nine different classes in *Solanum*, several of which occur in a single *Solanum* species and represent paralogous sequences. The observation that orthologues are more similar than paralogues, indicates that gene duplications, the most likely source of diversification of *Pto*-like genes, probably occurred prior to *Solanum* speciation.

The phylogenetic analyses provided an insight into the evolution of *Pto*-like genes in the Solanaceae. Using a combination of sequence based phylogenies and the distribution of indels in the various classes and across various plant families, we attempted to reconstruct the evolutionary history of *Pto*-like sequences (Figure 5-5). We hypothesize that prior to the divergence of Solanaceae from other plants, at least one gene duplication event of a common ancestral *Pto* gene occurred. A three amino acid deletion diagnostic of *Pto*-like sequences from the Solanaceae but not from other dicots and monocots may have occurred early in Solanaceae evolution and distinguishes classes I-VIII. Subsequently, several gene duplication and diversification events gave rise to the nine identified *Pto*-like classes in *Solanum*.

Class	Pto-like sequence	indel 159	indel 245	Kinase consensus[1]	Phosph[2] p p p	Activation domain[3]
I	Pto	-	-	G G V K E D K N D G P E D G R	T S T	D F G S K K R T E L D Q T H L S T · V K G T G Y D P E
	pto	-	-	G G V K E D K N D G P E D G R	T S T	D F G S K K R T E L Y Q T · · T D V K G T G Y D P E
II	Fen	-	-	G G V K E D K N D G P E D G R	T S T	D F G S K M T E L D Q T H L S T V K G T G Y A P E
	fen	-	-	G G V K E D K N D G P E D G R	T S T	D F G S K M T E L D L Q T H L S T V K G T G Y A P E
	berDF1	-	-	? ? ? ? ? ? ? ? D K N D G P E D G R	? S T	D F G S K L T E L D Q T H L S T V K G T G Y D P E
III	berBD3/DJ2	-	-	G G V K E D K N D G P E E ? ? ?	T S T ?	D F G S K K G T E L D Q T H L S T L V G T I G Y L D P E
	axhBF1	-	-	G G V K E D K N D G P E ? ? ?	T S T ?	D F G L S K K G T E L D Q T H L S T L V G T I G Y L D P E
	vrnBB1/DL2	-	-	G G V K E D K N D G P E E D G R	? ? ?	D F G L S K K G T E L D Q T H L S T L V G T I G Y L D P E
	tbrBA3W1	-	-	G G V K E D K N D G P E E D G R	T S T ?	D F G S K K G T E L D Q T H L S T L V G T I G Y L D P E
	scrY10/DK1	-	-	G G V K E D K N D G P E E D G R	? ? ?	D F G L S K K R T E L D Q T H L S T L V G T I G Y L D P E
	scr1A	-	-	G G V K E D K N D G P E E D G R	T S T	D F G L S K K R T E L D Q T H L S T L V K G T G Y L D P E
	scr1G	-	-	G G V K E D K N D G P E E D G R	T S T	D F G L S K K R T E L D Q T H L S T L V G T G Y L D P E
	scr1C	-	-	G G V K E D K N D G P E E D G R	T S T	D F G L S K K G T E L D Q T H L S T V G T G Y L D P E
	tbr4A	-	-	G G V K E D K N D G P E E D G R	T T	D F G L S K F K K G E L D Q T H L S T L V K G T G Y L D P E
	tbr4D	-	-	G G V K E D K N D G P E E D G R	T T	D F G L S K F K K G E L D Q T H L S T L V K G T G Y L D P E
	ber2H	-	-	G G V K E D K N D G P E D G R	? S T ?	D F G L S K F K K G E L D Q T H L S T L V G T G Y L D P E
	dmsFD2	-	-	G G V K E D K N D G P E E D G R	T	D F G I S K K E L D Q T H L S T V K G T I G Y D P E
IV	tbrDI2	-	-	? ? ? ? ? ? ? ? D K N D G P E E D G R	T	D F G L S K R T E L D Q T H S T V K G T G Y L D P E
V	scr8E	AVGRY	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L S K F K K G E L D K G T K G T G Y L D P E
	scr8I	AVGRY	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L S K F K K G E L D K G T K G T G Y L D P E
	scr8B	AVGRY	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L S L K G E L D K G T K G T G Y L D P E
	tbr5A	AVGRY	-	? ? ? ? ? ? ? ? D K N D G P E E D G ?	? ?	D F G L S K F K K G E L D K G T K G T G Y L D P E
	scr8D	AVGRY	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L S K K G E L D K G T K G T G Y L D P E
VI	scr8C	-	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L G I S K K G E L D H K G T G Y L D P E
	vrn6A	-	-	? ? ? ? ? ? ? ? D K N D G P E D G ?	? ?	D F G L S K K G E L D H K G T G Y L D P E
	scr8A	-	-	? ? ? ? ? ? ? ? D K N D G P E E D G ?	? ?	D F G L S K K G E L D H K G T G Y L D P E
VII	scrDG1	-	-	? ? ? ? ? ? ? ? D K N D G P E E D G R	? S T	D F G L S K G T E P D Q T T V N V K G T G Y L D P E
VIII	berS1	-	-	G G V K E D K N D G P E E D G	? T ?	D F G L S K R T E L D Q T H S T D V K G Y L D
	ber7A	-	-	G G V K E D K N D G P E E D G R	? ? ?	D F G L S K G E L D Q T H S T · D V K G Y L D P E
IX	scrDG4	YAK	-	? ? ? ? ? ? ? ? D K N D G P E E D G R	? ?	D F G L S K G E L D Q T H S T · V K G Y L D P E
	berDF4	YAK	·	? ? ? ? ? ? ? ? D K N D G P E E D G R	? S T ?	D F G L S K G E L D Q T H S T · V K G G Y L D P E
	tbrDI4	DAK	-	? ? ? ? ? ? ? ? D K N D G P E E D G R	? S T	D F G L S K A G E L D Q T H S T V K G G Y L D P E
	Le-776077	YAK	-	? ? ? ? ? ? ? ? D K N D G P E E D G R	? S T ?	D F G L S K G E L D Q T H S T · V K G G Y L D P E
	Nt-HPto	YAN	·	G G V K E D K N D G P E E D G R	? S T	D F G L S K R E L D Q T H S T V K G G Y L D P E
	At-62020	DSK	·	G G V K E D K N D G P E E D G R	T S T	D F G L S K G E L D Q T H · S T V K G G Y L D P E
	Plant serine/threonine consensus[4]			G	T	D F G S T G Y P E

[1] Kinase consensus sequence, at positions 48, 50, 55, 69, 84, 164, 166, 169, 182, 184, 210, 211, 223, 228, 300 (Hanks and Quinn, 1990); [2] Pto autophosphorylation sites, outside (position 38, 133, 288) and inside (position 195, 198, 199) the activation domain (Sessa et al., 2000b); [3] Kinase activation domain, between position 182-211 of Pto (Hanks and Quinn, 1990); [4] Consensus of the activation domain for plant serine/threonine kinases (Sessa et al., 2000a); * Dashes indicate gaps introduced to maximize alignment, ? Question marks indicate unknown amino acid residues

Table 5-3

Characteristics of the *Pto*-like sequences from *Solanum* (see Table 5-2) of class I-IX. Two multiple amino-acid indel sites (positions 159 and 245), the kinase consensus sequence, autophosphorylation sites (marked with 'p'), and the activation domain are shown. Level of conservation is indicated by box shading: conserved consensus residues are in black, residues with similar or different physicochemical properties (Grantham, 1974) to the consensus residue are in gray or white respectively. Numbering of amino acid positions corresponds to the *Pto* sequence (Martin *et al.*, 1993a).

In the phylogenetic tree, a diverse set of *Solanum* species is represented in class III (Figure 5-4). The clustering of these sequences is in agreement with the geographical distribution, current evolutionary hypotheses (Hawkes, 1990) and cpDNA-based phylogeny (Spooner and Castillo, 1997) of *Solanum*. The two, three and four sequences from the diploid *S. berthaultii*, tetraploid *S. tuberosum* and tetraploid *S. sucrense* respectively, could be different alleles, although genetic analyses are required to prove this. The *S. berthaultii*, *S. hondelmannii, S. tuberosum, S. vernei*, and *S. sucrense* orthologues cluster together, in line with their taxonomic classification in the Tuberosa Series (Hawkes, 1990; Spooner and Castillo, 1997). These species all evolved as diploid populations in the central Andes, except *S. sucrense*, which evolved in a population of polyploid species at higher altitudes. *S. demissum,* a member of the polyploid Demissa Series may have arose following a return migration to Mexico and subsequent amphipolyploidisations with primitive Mexican ancestors (Hawkes, 1990). In line with this migration hypothesis, the *S. demissum* sequence clustered more distantly.

Recently, Lavelle *et al.* submitted the full sequence of *Pto* loci of *L. pimpinellifolium* and *L. esculentum* to GenBank (accession AF220602 and AF220603), thereby revealing three novel paralogues besides *Pto* and *Fen*. Adding these sequences to our data set did not significantly alter the phylogenetic analyses. Clustering in sets of two paralogues (orthologues from the two *Lycopersicon* species) in class I, II and IV was obtained and confirmed the robustness of the tree and the observation that orthologues are more similar than paralogues (data not shown). In addition, the fact that these paralogues cluster with a subset of classes suggests that genes from these classes may also be localized at a syntenous position on chromosome V in *Solanum*. Other classes of *Pto*-like kinases may be located at different chromosomal positions, as described in *Capsicum* (Pflieger *et al.*, 1999), and additional studies are needed to test whether more than one cluster of *Pto*-like genes occurs in *Solanum*.
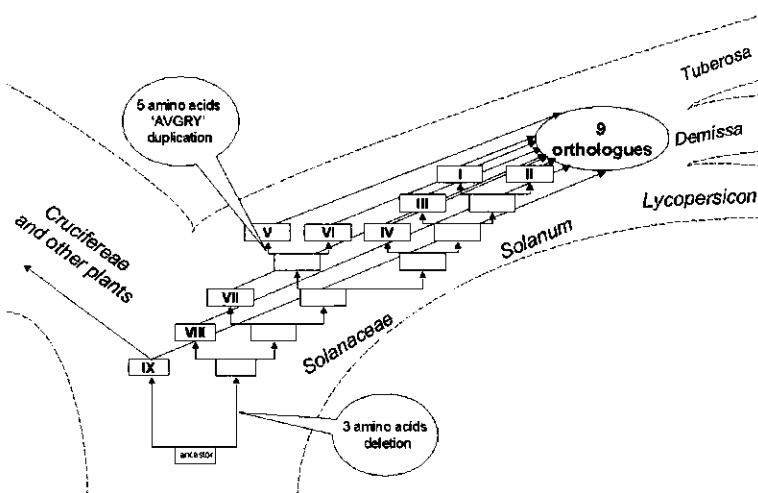
Figure 5-5

Hypothetical model for the evolution of the *Pto* family. The model illustrates a series of gene duplications that could explain the radiation of *Pto*-like genes in Solanum. The occurrence of indels is indicated for the three amino acid deletion (position 159), and the five amino acid duplication (AVGRY at position 245). The numbers in the boxes refer to the nine classes as depicted in Figure 5-4 and Table 5-3.

The Pto-like kinases appear to be encoded by members of an ancient gene family, since in *Solanum* (this study) and in *Lycopersicon* (Michelmore and Meyers, 1998), *Pto* orthologues are more similar than paralogues. Similar conclusions were also reported for other classes of *R* genes. These observations raise questions about the "arms-race" hypothesis for the evolution of *R* gene specificity (Michelmore and Meyers, 1998). For example, although *Cf* paralogues of the LRR-TM-class have been subjected to unequal crossing-over and gene conversion (Parniske *et al.*, 1997), these events did not occur frequently enough to homogenize them within a haplotype (Michelmore and Meyers, 1998). Individual NBS-LRR *R* genes are thought to have evolved mainly by divergent evolution, proposing a birth-to-death process similar to that envisaged for the vertebrate major histocompatibility complex (MHC) (Nei *et al.*, 1997; Michelmore and Meyers, 1998). In addition, minimal sequence changes in LRRs drastically altered recognition specificity in the flax *L* gene (Ellis *et al.*, 1999), and diversifying selection appears to have created extreme variation in rice *Xa21* (Wang *et al.*, 1998) and tomato *Cf* genes (Parniske *et al.*, 1997). This suggests that *R* gene evolution towards recognitional specificity occurs relatively rapidly within regions involved in recognition, thus in LRR or possibly the activation domain for kinase *R* genes (Grube *et al.*, 2000; Pan *et al.*, 2000a), and a balance in creating new specificities and conserving old ones is established (Stahl and Bishop, 2000; Young, 2000). Likewise, evolution for creating novel specificities in the

activation domain may have occurred relatively rapid for the Pto-like genes, but their ancient origin and strong conservation of kinase characteristics implies that they may not have evolved following the arms-race model. This assessment is consistent with the view that emerged for the NBS-LRR genes, that suggests that these genes occur in reservoirs of ancient and highly diverse families, with fine-tuning of recognition specificity occurring at various layers of antagonistic co-evolution (Meyers et al., 1999; Pan et al., 2000b; Stahl and Bishop, 2000). Further characterization of the genetic structure of Pto-like gene clusters in representative Solanum species should help test the various evolutionary scenarios.

More than 3,000 plant kinases have now been deposited in GenBank. Sequence comparison of Pto-like sequences with those of other kinases suggest that Pto forms a unique family of kinases in plants. In almost all Pto-like sequences, the invariant residues characteristic of functional protein kinases are fully conserved (Hanks and Quinn, 1990), suggesting that these Pto-like genes are likely to encode active kinases. The activation domain, which in Pto is involved in ligand binding, shows significant conservation of the plant serine/threonine kinase consensus (Sessa et al., 2000a), but considerable variation in other residues. For example, in this region, the AvrPto-non-responsive pto allele from L. esculentum shows aberrant signature sequences compared to Pto and all other Solanum Pto-like sequences, such as a gap at positions 196-198 and QLY in stead of ELD at 191-193. A particularly attractive hypothesis is that each member of the family might be interacting with different ligands, whether pathogen elicitors and/or signal transduction pathway components. The Lycopersicon genes Pto and Fen both act in the same signal transduction pathway leading to HR and defense responses, but are activated by different signals, i.e. AvrPto and fenthion, respectively (Ronald et al., 1992; Martin et al., 1994). Other characteristics of protein kinases are autophosphorylation sites, which fulfill an important regulatory function. The autophosphorylation sites which are essential for kinase activity or AvrPto-Pto-mediated HR induction (Sessa et al., 2000b) are highly conserved in the Solanum Pto-like sequences, except for class V/VI sequences, which show weaker conservation in the rest of the activation domain. In summary, the conservation of kinase consensus residues and autophosphorylation sites suggest that the uncovered genes are likely to encode active kinases. It remains to be determined whether all members of the Pto family are involved in similar signal transduction pathways leading to disease resistance. Functional assays to test whether the Pto-like sequences are involved in resistance can be performed by complementation, particularly using virus or Agrobacterium-mediated transient assays (Bendahmane et al., 2000; Sessa et al., 2000b), or by performing loss of function experiments, e.g. through virus-induced gene silencing (VIGS) (Baulcombe, 1999).

Using amplification with conserved primers, we obtained a great diversity of sequences and identified novel classes of Pto-like sequences. Increasing the specificity of the PCR primers, by designing them based on variable domains, biased the amplification of certain classes, for example, all class V/VI sequences were amplified with the F2-S1 primer set. Such specific primers may be applied in resistance breeding, for example for cloning specific candidate resistance genes, or following such genes in a breeding progeny. In pepper, quantitative trait loci (QTL) mapped close to Pto homologues obtained by PCR (Pflieger et al., 1999). However, these sequences were pseudogenes, but R gene -like sequences with

uninterrupted reading frames, as obtained in our study, are putative candidates for *R* genes. PCR-based cloning of *Pto*-like sequences proved successful in *Solanum,* suggesting that cloning novel *R* genes by this approach has great potential.

Acknowledgements

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Chapter 6

General discussion

Introduction

The research described in this thesis contributes to the ultimate goal to achieve durable resistance to late blight in potato, a key component for sustainable agriculture.

Durable resistance

The traditional view on durable resistance arose through lack of knowledge. In the past, non-durable resistance in potato cultivars bearing the race-specific resistance genes from *S. demissum* was found mediated by the HR. Without investigating the mechanisms of other types of resistance (durable resistance, nonhosts, etc) a premature conclusion was drawn suggesting that 'HR-based resistance is not durable'. This resulted in the irrational thought that thus mechanisms other than HR would confer durable resistance. In this thesis, we try to fill the gap of knowledge: we studied and compared a diversity of *P. infestans* resistances in various *Solanum* species, including durably resistant plants.

The basis of durability of genetic resistance, or the ability of pathogens to evolve new races is linked to epidemiological events. In soybean, single resistance (*R*) genes targeted against the soil pathogen *Phytophthora sojae* tend to last for several years (Athow, 1987), whereas *R* genes to the aerial *P. infestans* are quickly overcome by new races. The disease epidemiology and pathogen dispersal of these two *Phytophthora* species is different. In addition, plants that exhibit partial resistance may not impose sufficient selection pressure to allow novel virulent races to quickly dominate populations of the pathogen (James and Fry, 1983). In this case, partial resistance could prove durable regardless of whether it is mediated by *R* genes or by other mechanisms.

Resistance to oomycetes

Plants are attacked by a diversity of pathogens, including bacteria, viruses, nematodes, fungi and oomycetes. In all these interactions, common themes of both attack and defense can be identified. However, generalization should not overrule, and when learning from other systems, diversity in biological systems should be recognized.

Oomycetes include saprophytic and parasitic species that infect algae, fungi, animals or plants (Fuller and Jaworski, 1987). The plant pathogenic oomycetes comprise species with a narrow host range (downy mildews, several *Phytophthora* species) and a broad host range (e.g. *Pythium* species). Here, we only discuss plant interactions involving biotrophic oomycetes with a narrow host-range.

It is now generally accepted that oomycetes are a distinct group of pathogens, which evolved their strategies to attack plants independently from fungi and other pathogens. Also plants may have evolved specific defense mechanisms to diverse pathogens. In this chapter, we relate our findings in the *Solanum* - *P. infestans* interaction to several other plant-oomycete interactions, rather than to plant - fungus interactions. First, we describe the infection events,

where the 'go - no go' interaction is determined. Then we comment on the different types of resistance, and the *R* genes. We conclude by discussing approaches to obtain late blight resistance and the future prospects. For these different topics, we first discuss oomycete-plant pathosystems in a broader perspective, and subsequently focus specifically on the *Solanum - P. infestans* interaction.

Infection events

The disease cycle of various plant pathogenic biotrophic oomycetes, such as *P. infestans*, *P. medicaginis*, *P. sojae*, *P. palmivora*, *Peronospora parasitica*, *Bremia lactucae*, and *Albugo candida* is well characterized (Hohl and Suter, 1976; Maclean and Tommerup, 1979; Stössel *et al.*, 1980; Coffey and Wilson, 1983; Miller and Maxwell, 1984; Feuerstein and Hohl, 1986; Koch and Slusarenko, 1990; Holub *et al.*, 1995). Early infection events are similar in both susceptible and resistance interactions. Typically, infection starts when zoospores (motile spores), encyst and germinate on root or leaf surfaces (Figure 6-1, Figure 6-2). Alternatively, in some species, asexual spores, sporangia, may germinate directly. Germ tubes penetrate an epidermal cell to form an infection vesicle. In susceptible plants, branching hyphae with feeding structures known as haustoria, expand from the site of penetration to neighboring cells through the intercellular space (Figure 6-2A). In resistant plants, the major defense reaction is the hypersensitive response (HR) (Figure 6-2B), and the timing and extent of the HR varies depending on the interacting pathogen and plant genotypes. The quantitative nature of resistance is illustrated for the *P. infestans - Solanum* interaction (Chapter 3). In some cases, such as in nonhost interactions, the HR remains limited to one or a few Figure 6-1; Figure 6-3). In other cases, for example in potato cultivars carrying the race-specific *S. demissum* resistance (*R*) genes, or in potato cultivars with high partial resistance levels, a group of cells display the HR and the infection is blocked at a later stage (Figure 6-2C). Interestingly, interactions displaying lower partial resistance are also associated with HR and slowly growing lesions (Figure 6-2D). In these cases, the HR appears ineffective in blocking the pathogen resulting in numerous escaping hyphae and a typical phenotype of trailing HR, in which the growing hyphae remain ahead of the plant response.
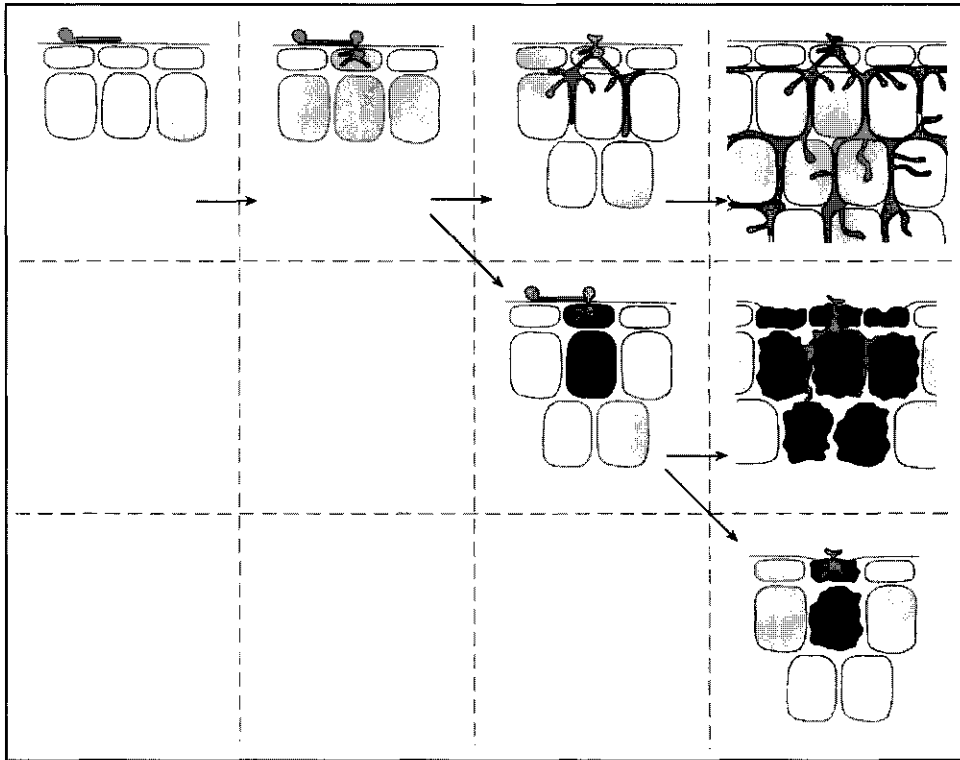
Figure 6-1

Schematic view of the early infection events in susceptible and resistance interactions between *Phytophthora infestans* and plants. Early stages are similar in all types of interactions. In susceptible plants, no visible defense responses occur (upper row), secondary hyphae grow into the intercellular space and form haustoria inside mesophyll cells. The hyphae branch and rapidly colonize the mesophyll tissue, which will finally result in disease. In resistant plants, cells are activated after penetration by the pathogen (middle row). The HR is induced and the pathogen is contained within a group of dead plant cells (middle row) or within the penetrated epidermal cell (lower row) depending on the genotypes of the interacting plant and pathogen. The HR lesions in the middle row are visible macroscopically as brownish-black spots. In many nonhost plants (lower row), the HR is induced extremely fast, and only one or two plant cells are sacrificed. Macroscopically no symptoms are visible.
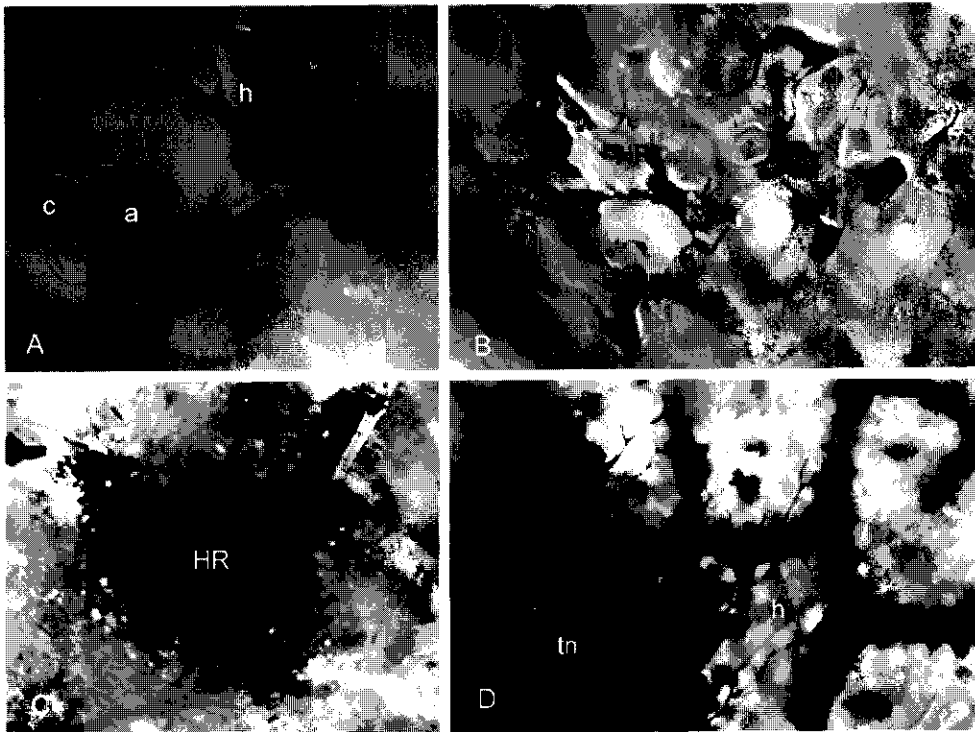
Figure 6-2

Cytology of *Phytophthora infestans* and *Solanum* host plant interactions. (**A**) Penetrated plant cells of a susceptible potato cultivar 'Bintje' do not show a visible plant response and pathogen hyphae proliferate through the plant tissue (1000x). (**B**) In potato cultivar 'Ehud' carrying the *R1* resistance gene, a few mesophyll cells beneath the penetration site have collapsed following the induction of the HR (640x). (**C**) In the partially resistant cultivar 'Robijn', several mesophyll cells are involved in the HR before the pathogen is restricted (200x). (**D**) Containment of hyphae in HR lesions is not always achieved in partially resistant plants, such as *S. arnezii x hondelmannii*. In some infection sites, the expanding hyphae are followed by a trailing necrosis (200x). Hyphae and reacting plant cells were visualized by trypan blue staining (**A, B, D**), and phenolic compounds were visualized as brown spots in aniline blue stained tissue (**C**) with DIC optics. Abbreviations: a, appressorium; c, cyst; h, hypha; HR, hypersensitive response; tn, trailing necrosis

Resistance

Diverse resistance processes occur in plants. It has been argued that the various types of resistance may have distinct values in disease management strategies because of differences in durability in the field. For example, race-specific resistance mediated by single resistance (*R*) genes introgressed from *S. demissum* appeared of limited value in the field, due to rapid

adaptation of *P. infestans*. On the other hand, nonhost and partial resistance appear more durable Figure 6-3). However, the extent to which durable nonhost or partial resistance involves genetic components that are distinct from *R* genes remains unclear.

Race-specific resistance

In many pathosystems, race-specific resistance is explained by the gene-for-gene model. In this concept, presence of both the *R* gene and the corresponding avirulence (*Avr*) gene results in HR-mediated resistance, whereas absence of at least one of the two results in disease. For potato - *P. infestans*, the gene-for-gene model has been proposed based on genetic analyses, as the eleven *S. demissum R* genes provide strong resistance to specific races only. (Malcolmson and Black, 1966). Various other oomycete pathosystems confirm the gene-for-gene model, for example at least 15 *Dm* resistance genes in lettuce match avirulence genes in *Bremia lactucae* (Farrara *et al.*, 1987). The extensive family of *RPP* genes in *Arabidopsis* were shown to recognize distinct avirulence determinants of *Peronospora parasitica* (Botella *et al.*, 1998; Noel *et al.*, 1999; Bittner-Eddy *et al.*, 2000).

Several race-specific *R* genes targeted against oomycetes have been isolated from lettuce and *Arabidopsis* (see below), but the pathogen *Avr* factors that these are thought to interact with have yet to be identified. Resistance mediated by *R* genes in *Arabidopsis* and lettuce is always associated with the HR, which is generally visible as a distinct necrosis and correlates with the accumulation of autofluorescent compounds and irreversible membrane damage (Bennett *et al.*, 1996; Reignault *et al.*, 1996). The extent, timing and severity of the HR vary depending on the *R* gene examined and the pathogen strain.

Nonhost resistance

Nonhost resistance to specialized biotrophic pathogens occurs in most plant species. Weeds, like *Arabidopsis* plants, that are able to grow in infected fields are excellent examples of nonhost resistance. A particularly interesting example is a relative of potato, the black nightshade (*S. nigrum*) which survives in *P. infestans*-infected potato fields (Figure 6-3).

Parsley is a nonhost of *P. infestans* and *P. sojae*. Following inoculation with *Phytophthora*, parsley cells exhibit a complex and coordinated series of morphological and biochemical defense responses that culminate into HR cell death (Hahlbrock *et al.*, 1995; Naton *et al.*, 1996). An extracellular 42 kD glycoprotein elicitor from *P. sojae* or, more specifically a 13 amino-acid oligopeptide (Pep-13) derived from this protein, is sufficient to induce changes in plasma membrane permeability, an oxidative burst, activation of defense genes, and accumulation of defense compounds (Nürnberger *et al.*, 1994). In addition to signaling molecules, local mechanical stimulation, perhaps similar to that caused by the invading pathogen, induces some of the early morphological reactions and potentiates the response to the elicitor (Gus-Mayer *et al.*, 1998).
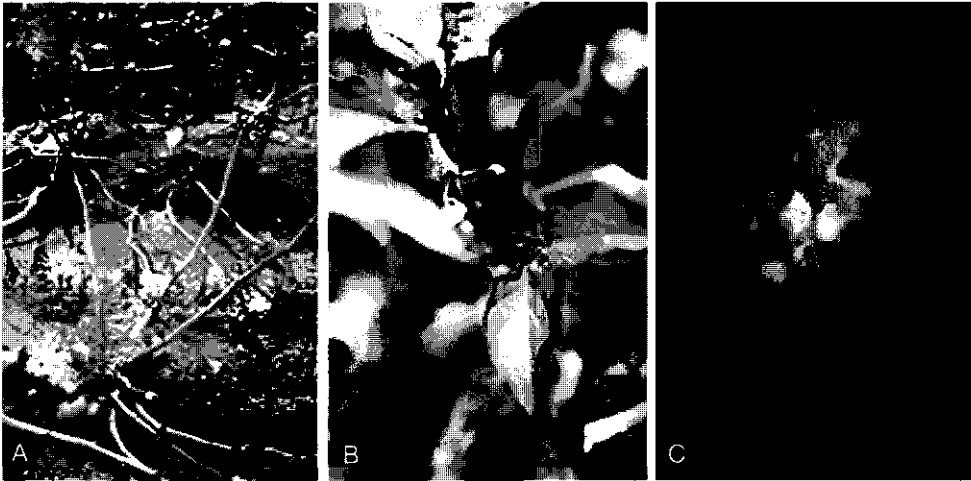
Figure 6-3

Nonhost resistance in weeds. (**A**) *Phytophthora infestans*-infected potato plants that were not treated with chemicals and were under highly favorable environmental conditions show systemic blight symptoms. The leaves turn black, drop to earth, and ultimately the plants will die. When the field is examined in more detail, healthy green weeds can be seen among the blighted potatoes. These weeds exhibit nonhost resistance to the late blight pathogen. One indigenous weed commonly seen in infected European fields is black nightshade (*Solanum nigrum*) (**B**). Even though this plant has been continuously exposed to late blight since the introduction of *P. infestans* to Europe 150 years ago, it has remained resistant. Laboratory inoculation of *S. nigrum* with *P. infestans* shows penetration of the leaf epidermis accompanied by rapid cell death (HR) of the penetrated plant cells (**C**). Phenolic compounds that accumulate in HR cells show a bright yellowish autofluorescence when illuminated with UV (1000x magnification). A future challenge is to engineer potatoes into nonhosts of *P. infestans*, just like all the weeds that grow in infected fields.

In tobacco and other species of the genus *Nicotiana*, resistance to *P. infestans* is diverse and the HR varies in intensity depending on the plant species examined (Kamoun *et al.*, 1998). *P. infestans* as well as other *Phytophthora* species produce 10 kD extracellular proteins, termed elicitins, which induce the HR and other biochemical changes associated with defense responses in *Nicotiana* (Ricci *et al.*, 1989; Kamoun *et al.*, 1993; 1997b). *P. infestans* strains deficient in the elicitin protein INF1, induce disease lesions on *Nicotiana benthamiana*, suggesting that INF1 functions as an *Avr* factor that conditions resistance in this plant species (Kamoun *et al.*, 1998). In contrast, INF1 deficient strains remained unable to infect other *Nicotiana* species, such as tobacco. In this case, tobacco may react to additional elicitors, perhaps other members of the complex INF elicitin family of *P. infestans* (Kamoun *et al.*, 1997a; 1999b). Similar to the phenotypic expression of resistance, the genetic basis of *Nicotiana* resistance to *P. infestans* could be diverse. In the case of *N. benthamiana*, a single component,

the recognition of INF1, is the main determinant of resistance, but in other *Nicotiana* species a more complex genetic control, perhaps an array of *R* genes with different specificities, might be responsible. In the light of this diversity, the *P. infestans-Nicotiana* system appears an ideal model to dissect and compare the molecular basis of nonhost recognition in closely related species (Kamoun *et al.*, 2000).

Phytophthora mirabilis, a host-specific species closely related to *P. infestans*, infects *Mirabilis jalapa* (four-o'clock) but is unable to infect potato and tomato. Interspecific hybrids between these two *Phytophthora* species were essentially unable to infect the original host plants suggesting that avirulence on the nonhosts is dominant (Goodwin and Fry, 1994). In contrast to the parental strains, large HR lesions were induced by several of the hybrids on tomato indicating an alteration of the extent of the HR. Future genetic work could help identify the components of host-specificity in these interactions.

Partial resistance

Partial resistance to *P. infestans* commonly occurs in wild *Solanum* species (Colon *et al.*, 1995). Cytological examination of these plants infected by *P. infestans* revealed HR-like reactions and in numerous occasions, late or trailing HR was observed (Chapter 3, and Figure 6-2). Interestingly, a similar phenotype was observed in transgenic *Arabidopsis* plants homozygous for the *R* gene *RPP1-WsB* following inoculation with *Peronospora parasitica* (Botella *et al.*, 1998). These plants show partial resistance to *P. parasitica*, as illustrated by a trail of HR cells adjoining the invading hyphae. This suggests that in some cases, weak *R* gene-*Avr* gene interactions or gene dosage effects could result in ineffective HR reactions resulting in partial resistance phenotypes.

Partial resistance to two races of *P. infestans* was found to segregate in a cross between diploid potato lines, and quantitative trait loci (QTLs) contributing to resistance to late blight were identified (Leonards-Schippers *et al.*, 1994). Also in *S. berthaultii* and *S. microdontum,* QTLs for late blight resistance were found (Ewing *et al.*, 2000; Sandbrink *et al.*, 2000). Genetic mapping revealed that QTLs correspond to regions of the genome that contain clusters of *R* genes and *R* gene analogs (RGAs) (Leister *et al.*, 1996), which raises the possibility that these QTLs may represent genes homologous to typical *R* genes. Similarly, in pepper, RGAs mapped closely to a QTL involved in partial resistance to cucumber mosaic virus (Pflieger *et al.*, 1999). Colocalizations of RGAs and QTLs suggest that qualitative and quantitative resistance may share a similar genetic basis. Molecular cloning of the sequences determining the QTLs for late blight resistance should help understand the molecular basis of partial resistance to *P. infestans*.

Resistance genes

Resistance genes targeted to oomycetes

A number of *R* genes targeted against the downy mildews *Peronospora parasitica* (*RPP* genes, Parker *et al.*, 1997; Botella *et al.*, 1998; McDowell *et al.*, 1998) and *Bremia lactucae* (*Dm* genes, Meyers *et al.*, 1998) have been isolated from *Arabidopsis* and lettuce, respectively. All identified genes encode receptor-like proteins that contain a nucleotide binding site (NBS) and several leucine-rich-repeats (LRR). This NBS-LRR structure is typical of *R* genes active against other pathogens; moreover, most identified *R* genes belong to this large class (Young, 2000). Until now, at least two subclasses of NBS-LRR *R* proteins targeted against oomycetes are known. They are distinguished by their N-terminal regions, which show homology to the Toll-Interleukin 1-receptor (TIR) domain (*RPP1* and *RPP5* clusters), or contain a coiled-coil motif of which the leucine-zipper is a specific example (*RPP8* cluster) (Meyers *et al.*, 1999; Pan *et al.*, 2000b). However, specificity of recognition may lie in the hypervariable LRR regions (Botella *et al.*, 1998; McDowell *et al.*, 1998; Meyers *et al.*, 1998).

The cloning of *R* genes to *P. infestans* is in progress. In addition to PCR-based approaches, the isolation of *R* genes through transposon tagging is an appealing strategy. Genetic maps of potato are available, and both QTLs and *R* genes conferring *P. infestans* resistance have been positioned on the map (El Kharbotly *et al.*, 1994; 1996b; Gebhardt *et al.*, 1994; Jacobs *et al.*, 1995; Leonards-Schippers *et al.*, 1992; 1994; Li *et al.*, 1998). A tagging population was obtained and plants with an altered *R1* resistance response were identified (El Kharbotly *et al.*, 1996a; van Enckevort, 2000). Further analyses will reveal more insight in the *R* gene biology.

Resistance genes in Solanum

Most known *R* genes belong to the NBS-LRR class, but also other classes of *R* genes are known. Most of these classes have been identified in the genus *Solanum*. The NBS-LRR class is abundantly present, and active against bacterial (*Prf, Pseudomonas syringae*, Salmeron *et al.*, 1996), viral (*Rx*, Potato Virus X, Bendahmane *et al.*, 1999), fungal (*I2, Fusarium oxysporum*, Simons *et al.*, 1998) and nematode (*Gpa2, Globodera rostochiensis*, Stiekema *et al.*, 2000) pathogens. Also the LRR-transmembrane (LRR-TM) class is represented in *Solanum*, for example the *Cf* genes, conferring resistance to the fungus *Cladosporium fulvum* (Jones *et al.*, 1994). *Pto*, conferring resistance to *P. syringae*, represents the kinase class of *R* genes (Martin *et al.*, 1993).

Evolution of resistance genes

The *Pto* gene family in *Solanum* arose early in evolution through a series of gene duplications, followed by gene diversification into at least nine classes. Phylogenetic analyses suggested

that the *Pto* homologues probably arose from a common ancestor that existed before speciation in *Solanum*, perhaps Solanaceae (Chapter 5). Similar conclusions were drawn for solanaceous NBS-LRR *R* genes: potato and tomato NBS-LRR *R* genes and RGAs form tight clusters and are well distributed among the different branches in a phylogenetic tree, indicating that they arose from common ancestral genes before speciation in Solanaceae (Pan *et al.*, 2000a). The NBS-LRR class of *R* genes can be divided into two subclasses: the TIR and the non-TIR subclass (Meyers *et al.*, 1999; Pan *et al.*, 2000a). The non-TIR subclass is present in all of the angiosperm species tested, but the TIR subclass appears to be absent in Poaceae. The current model predicts that the common ancestor of angiosperms and gymnosperms contained both types of NBS-LRR sequences, with the branch leading to modern grasses losing the TIR class after divergence (Pan *et al.*, 2000b). In conclusion, both the *Pto* kinase class and the NBS-LRR class of *R* genes are of ancient origin, and diversification into distinct lineages occurred prior to or since speciation (Young, 2000).

It is tempting to draw parallels between plant *R* gene biology and defense mechanisms in other Kingdoms. In the NBS-LRR *R* genes, diversifying selection occurs in the solvent-exposed LRR domains that form the binding site for the ligand. Here, an intriguing resemblance is found between the plant disease *R* gene system and the major histocompatibility complex (MHC) of mammals (Nei *et al.*, 1997): both comprehend large gene clusters, and both show hypervariability in a solvent exposed region through diversifying selection (Michelmore and Meyers, 1998; Laugé, 1999). Other parallels are revealed by sequence characteristics of *R* gene domains. The NBS domain shares similarities with human APAF-1 and nematode CED-4 (Kumar and Colussi, 1999). These genes play a role in apoptosis, a form of programmed cell death, which shares similarities with the HR (Mittler and Lam, 1996; Ryerson and Heath, 1996; Heath, 1998). The TIR domain also occurs in mammalian and invertebrate proteins involved in innate immunity, thereby suggesting an ancient origin for mechanisms of cellular defense (Baker *et al.*, 1997).

Pathogenesis-related genes and systemic acquired resistance

Upon execution of the HR, a set of proteins collectively termed pathogenesis-related (PR) proteins, accumulate locally and systemically (Kombrink *et al.*, 1988). PR proteins occur in complex gene families, including glucanases (PR-2), chitinases (PR-3), thaumatin/osmotin-like (PR-5) proteins and PR-1 type proteins. For PR-1 and PR-5, an anti-*Phytophthora* function has been proposed (Woloshuk *et al.*, 1991; Alexander *et al.*, 1993; Liu *et al.*, 1994).

Systemic acquired resistance (SAR) is associated with *PR* gene expression, and therefore, PR genes can be used as marker genes for SAR (Ryals *et al.*, 1996). SAR can either be induced upon HR, but can also be active at basal levels. In *Arabidopsis*, the *cpr* (constitutive expresser of *PR* genes) and *dnd* (defense with no death) mutants displayed a SAR phenotype: they showed elevated mRNA levels for *PR* genes, and were more resistant to a broad spectrum of pathogens (Cao *et al.*, 1994; Yu *et al.*, 1998). Similar to this type of *Arabidopsis* mutants, basal isolate-nonspecific resistance levels in *Solanum* were associated with mRNA levels of *PR* genes, suggesting that the constitutive SAR-phenotype of some

Solanum plants, such as cultivar Robijn, may explain the high levels of basal resistance (Chapter 4). Additional experiments, for example induction with exogenous application of salicylic acid, may answer whether basal *PR* gene expression in *Solanum* really contributes to nonspecific resistance to *P. infestans*.

Although SAR is considered a defense mechanism different from the *R* gene-mediated HR, the two systems can be connected by the action of transcription factors. The first evidence for this was demonstrated for the Pto interacting proteins Pti4/5/6, which physically interact with the Pto resistance protein and specifically recognize a promoter region of a large number of *PR* genes. A direct link between an *R* gene signal transduction pathways and *PR* gene expression is thereby established (Zhou *et al.*, 1997).

How to obtain *P. infestans* resistance?

A general role for the hypersensitive response

The HR is associated with all known forms of genetic resistance to *Phytophthora* and downy mildew oomycetes. There is an emerging body of evidence that suggests that *R* gene receptors triggered by pathogen elicitors might also mediate nonhost resistance and partial resistance phenotypes. This should lead to a reassessment of the potential usefulness in the field of resistance reactions mediated by *R* genes and involving the HR. Even though *R* genes are thought to be ineffective in the field over long periods of time, there are plausible hypotheses that suggest that these genes could mediate durable resistance. For example, an arsenal of *R* genes recognizing a number of unrelated *Avr* targets could be difficult to overcome as the pathogen would require multiple independent mutations to become virulent (Staskawicz *et al.*, 1995; Crute and Pink, 1996). This model of genes-for-genes interactions is sufficient to explain nonhost resistance of *Nicotiana* to *Phytophthora*, a system in which multiple elicitor signals have been identified in the pathogen (Baillieul *et al.*, 1995; Kamoun *et al.*, 1997a; 1998; Mateos *et al.*, 1997). Alternatively, a durable *R* gene could recognize an *Avr* gene that is essential to the pathogen (Staskawicz *et al.*, 1995; Swords *et al.*, 1996; Laugé *et al.*, 1998). Targeting such pathogen "Achilles heel" is expected to lead to durable resistance because mutations in the dual *Avr*-virulence gene would result in a severe fitness penalty for the pathogen. In the tomato fungal pathogen *Cladosporium fulvum*, the application of this concept led to the identification of a tomato *R* gene targeted against ECP2, an important virulence factor (Laugé *et al.*, 1997; 1998) . Widespread cultivation of tomato lines harboring *R-Ecp2* should help determine the long-term resistance of this genotype, and the future potential of the strategy.

Classical breeding and genetic engineering

Nowadays, several procedures have been developed to produce potato cultivars with late blight resistance, each method with its own advantages and disadvantages. The power of classical breeding programs should not be underestimated, as the genetic diversity maintained in

breeding material is of major importance for the future. This diversity however, also implicates a main drawback, as extremely high demands for early maturity, potato size, taste, flesh color after baking and frying, etc often interfere with resistance properties. Therefore, classical breeding for resistance is often a long-term procedure. The use of molecular markers, such as QTLs (Leonards-Schippers *et al.*, 1994), or *PR* gene expression levels (Chapter 4) may speed up the process. Although the essentially empirical approach of classical potato breeding has yet yielded moderate success, now new gene technologies may increasingly be integrated.

Presence of shared motifs among *R* proteins suggests that downstream signaling pathways contain similar or compatible components (Martin, 1999). If so, then *R* genes from one plant species might be used to engineer resistance specificity in other economically important species. In addition, overexpression of signaling components downstream of *R* genes can be used to increase partial, but broad-spectrum resistance, such as the *npr1* from *Arabidopsis* (Cao *et al.*, 1998).

Since resistance genes occur in clusters at certain chromosomal locations (Grube *et al.*, 2000), it could be envisaged that by classical crossings, an introgressed resistance gene (cluster) could replace yet another set of *R* genes in the new cultivar. It is not unthinkable, that the 'lost cluster' may even contain *R* genes against other pathogens present in the field. In this scenario, direct transfer of *R* genes to cultivars appears much more appealing.

Nonhost resistance genes from Arabidopsis?

An untapped source of nonhost resistance genes may be the model plant *Arabidopsis*. Resources for genetic and genomic analyses are well developed for *Arabidopsis* and the entire genome is scheduled to be sequenced this year (2000) (Meinke *et al.*, 1998). *Arabidopsis* is resistant to economically important oomycetes such as *P. infestans* and *P. sojae*. Following inoculation of *Arabidopsis* leaves with *Phytophthora* zoospores, a typical HR response is observed (Chapter 3). *Arabidopsis* genes, perhaps homologues of known *R* genes that direct this nonhost resistance may occur.

Transfer of R genes

Within Solanaceae, the transfer of disease resistance genes between different plant species has been successful for several resistance genes such as *Pto, Cf9, N* and *Bs2* (Rommens *et al.*, 1995; Thilmony *et al.*, 1995; Whitham *et al.*, 1996; Hammond-Kosack *et al.*, 1998; Tai *et al.*, 1999). In addition, the potential exists to isolate resistance genes from *Arabidopsis* and transfer them to potato to engineer nonhost resistance to *P. infestans*. However, to apply genetic engineering across the family boundary, may be more complicated because of restricted taxonomic functionality (RTF): the pepper gene *Bs2* functioned only in Solanaceae, and the *Arabidopsis RPS2* gene was nonfunctional in transgenic tomato (Tai *et al.*, 1999). Understanding the molecular basis of RTF will be necessary to overcome it, before those nonhost genes can be used. Till then, the tremendous diversity of *Solanum* and Solanaceae, with their enormous treasure of *R* genes, will provide a great source of resistances.

Future prospects

The traditional negative view that plant *R* genes would only confer race-specific non-durable resistance to late blight can be abandoned. Resistance breeders should now shift to a positive view, and make use of *R* genes in *Solanum* and possibly various other plant genera.

The ubiquitous association of the HR with *P. infestans* resistance in a diversity of plants, including plants without known *R* genes, suggests that an arsenal of *R* genes is waiting to be identified. This prompts us to expand beyond the *S. demissum* genes and search for *R* genes by novel approaches, for example by using putative *Avr* genes to identify the corresponding *R* genes. Biochemical screening approaches such as the identification of *P. infestans* elicitor molecules that interact with plant receptors are promising. Recent advances in the molecular genetic analysis of *P. infestans,* the development of tools such as routine DNA transformation (Judelson, 1996) and genetic manipulation using gene silencing (van West *et al.*, 1999), and the availability of detailed genetic maps (van der Lee *et al.*, 1997) and BAC libraries (Randall and Judelson, 1999; Whisson *et al.*, 2000) will facilitate cloning and allow functional analyses of oomycete genes involved in interactions with plants. As demonstrated recently, an EST gene discovery program is a powerful approach to identify numerous new candidate genes from this relatively unexplored organism (Kamoun *et al.*, 1999b). Viral systems for expression of *Avr*/elicitor genes in plants (Hammond-Kosack *et al.*, 1995; Laugé *et al.*, 1998; Kamoun *et al.*, 1999a) should facilitate the identification of elicitor activity. Unraveling the nature of oomycete *Avr* and elicitor molecules and their role in the various types of resistance will aid in understanding the molecular basis of race evolution and defining sustainable strategies for engineering durable genetic resistance.

The merits of genomics will provide new opportunities for understanding plant disease resistance and plant-pathogen interactions. Detailed information on *R* gene clusters can be obtained by large-scale sequencing. In the USA, a public *Solanum*/potato genomics program has been initiated, and it is expected that similar initiatives will follow in Europe. On the pathogen side, the *Phytophthora* Genome Initiative (PGI, at http://www.ncgr.org/pgi) involves several laboratories worldwide, and aims at fundraising and coordinating sequence projects on *Phytophthora* (Kamoun *et al.*, 1999b). Application of micro-arrays will reveal valuable gene expression profiles during the plant-pathogen interaction.

Concluding remarks

In plants, various types of resistance mechanisms occur, and these involve a complex set of defense responses (Dorey *et al.*, 1997; Thomma *et al.*, 1998; Broekaert *et al.*, 1999) (Figure 6-4). While the HR is recognized as a fast and rather drastic cell response, other mechanisms may function systemically and may obstruct the pathogen in a different aspect. The efficiency of all the mechanisms together will determine whether the plant is resistant. The frequent association of the HR with resistance responses in interactions between plants and oomycete

pathogens, particularly *Phytophthora* and downy mildews, illustrates the role of *R* genes in resistance.

A remaining challenge is the identification of *R* genes involved in specific, nonhost or partial resistance to the economically important late blight disease. With our improved understanding of the molecular nature of *R* genes, new methods such as PCR-based approaches to isolate analogs of *R* genes from resistant plants combined with reverse genetics and complementation into the host crop should facilitate the cloning and identification of novel *R* genes.
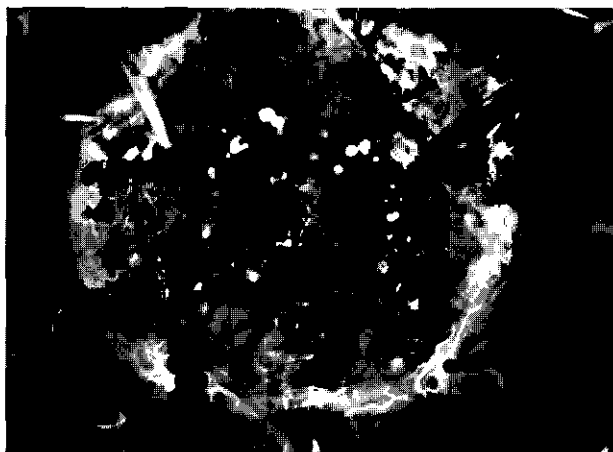

Figure 6-4

Various defense mechanisms are activated at a *P. infestans* penetration site. The HR was induced resulting in cell death of the initially penetrated cell and a few adjacent cells. The neighboring cells deposited a layer of callose on the adjacent cell wall to impede hyphal penetration. In addition, more localized callose depositions accumulated in papillae. Autofluorescence of more distant cell walls indicates the accumulation of phenolic compounds.

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Summary

Summary

Ever since the late blight epidemics of the mid-nineteenth century, man has endeavored to protect his potato crop. Every year, extensive chemical protection is applied, which is expensive and may be harmful to the natural environment. The use of resistant potato cultivars would provide an elegant alternative, however breeding for late blight resistance has not yet resulted in adequately resistant cultivars. In addition to the demand for a high level of resistance, the resistance should be durable. The causal agent of the late blight disease is *Phytophthora infestans*, a biotrophic oomycete pathogen. Oomycetes are often incorrectly referred to as fungi, but they evolved the ability to infect plants independently from fungi, and may therefore have distinct mechanisms for interacting with plants. Insight in the mechanisms of resistance to *P. infestans* may assist the breeders in their operation to achieve durable resistance.

Genetic resistance can be determined at the subspecies or variety level (race-specific resistance), or at the species or genus level (nonhost resistance). Nonhost resistance is full resistance, and is present in most plant species. In addition, resistance may be a quantitative trait (partial resistance). A rich pool of diverse resistances to *P. infestans* has been found in wild *Solanum* species, with levels ranging from full resistance to various levels of partial resistance. Some old potato cultivars also exhibit partial resistance, which proved to be durable. To achieve a durable late blight resistance, a better understanding of the molecular basis of the various types of resistances is essential. To this end, we compiled a set of *Solanum* species with various types and levels of resistance to *P. infestans*, and studied the cellular and molecular aspects of the resistance mechanisms present in these plants.

Laboratory studies at the cellular and molecular level require an experimental assay, which is both comparable to the natural situation, and assures a high percentage of successful infections. To this end, we developed a resistance assay with detached leaves under controlled conditions in the laboratory and compared this assay with a field trial. The tested growing conditions of the plants did not affect the resistance to *P. infestans*. Leaves on intact plants however, were more resistant than detached leaves. The incubation conditions of the detached leaves in the laboratory assay rather than the detachment itself appeared to affect the resistance expression. However, on intact plants the infection frequency was too low for molecular studies. Since the ranking of resistance levels within a set of twenty plant clones was similar under laboratory and field conditions, the laboratory assay proved adequate to study the *Solanum* - *P. infestans* interaction.

A cytological survey of the interaction between twenty *Solanum* clones and three *P. infestans* isolates provided the first impression of the nature of the resistance responses. Potato cultivars with race-specific resistance (*R*) genes displayed the hypersensitive response (HR), a programmed cell death of plant cells, upon inoculation with *P. infestans*. Through this rapid cell death, the biotrophic pathogen became localized between dead cells, and was prevented from further growth. Also durably resistant potato cultivars without known *R* genes, wild *Solanum* species, and nonhosts displayed the HR. Interestingly, in highly resistant *Solanum* species such as *S. berthaultii* and *S circaeifolium,* and nonhosts such as *Arabidopsis thaliana* and *S. nigrum* (black nightshade), the HR was extremely fast and effective, resulting in very localized cell death. In partially resistant plants, the HR was delayed, and resulted in larger HR lesions. Occasionally, hyphae were able to escape from these lesions and established a

biotrophic interaction with the host. The effectiveness of the HR in restricting growth of the pathogen differed considerably between clones, and correlated with resistance levels. In addition to the HR, local depositions of callose and phenolic compounds occurred, which may function as physical barriers. Although these responses did not correlate with resistance levels, they may influence the balance between growth of the pathogen and induction of the HR. Ultimately, this fine balance may determine resistance at the cellular level, and illustrates the quantitative nature of the resistance to *P. infestans* at the plant or field level.

The HR is initiated upon recognition of pathogen elicitors by plant cell receptors or *R* gene products as suggested by the gene-for-gene hypothesis. Several types of *R* genes are recognized in plants, including the nucleotide binding site leucine-rich repeat (NBS-LRR) type, and the Pto-like serine/threonine protein kinase type. The *Pto* gene was originally identified from wild relatives of tomato. We exploited our *Solanum* collection to identify *Pto*-like sequences, and studied evolutionary scenarios for *Pto*-like genes. Polymerase chain reaction (PCR) amplifications using primers based on conserved and variable regions of *Pto* yielded 32 intact *Pto*-like sequences from six *Solanum* species, and revealed an extensive *Pto* family. *Pto*-like transcripts were also detected in leaf tissue of all tested plants. The kinase consensus and autophosphorylation residues were highly conserved, in contrast to the kinase activation domain which is involved in ligand recognition in Pto. Phylogenetic analyses distinguished nine classes of *Pto*-like genes, and revealed that orthologues (homologues separated by a speciation process) were more similar than paralogues (homologues generated by a gene duplication event). This suggests that the *Pto* gene family evolved through a series of ancient gene duplication events prior to speciation in *Solanum*. The phylogenetic data are in line with recent results on the NBS-LRR class of *R* genes, and suggest that *Pto*-like genes are ancient, and highly diverse.

Various levels of nonspecific resistance were revealed in *Solanum* species after inoculation with five *P. infestans* isolates. In partially resistant plants where hyphal escape occurred, the lesions expanded often slower than in susceptible plants. Here, defense mechanisms other than HR are thought to operate; this might for example involve systemic acquired resistance (SAR). SAR can be induced by various signals, but also basal levels of SAR may vary between plants. When we monitored basal expression levels of SAR marker genes in healthy leaves, we found variation between the *Solanum* clones in constitutive mRNAs levels of the pathogenesis-related (*PR*) genes *PR-1*, *PR-2*, and *PR-5*. At the genus level, there was no correlation between basal *PR* mRNA levels and nonspecific resistance to *P. infestans*. In contrast, a positive correlation was found at the species level in *S. arnezii x hondelmannii*, *S. microdontum*, *S. sucrense* and *S. tuberosum*. In *S. tuberosum* cultivars, the levels of *PR* gene expression were the highest in resistant 'Robijn', intermediate in partially resistant 'Première', 'Estima' and 'Ehud', and the lowest in susceptible 'Bintje'. These results suggest that constitutive expression of *PR* genes may contribute to nonspecific resistance to *P. infestans* in *Solanum*. Therefore, *PR* mRNA levels could serve as molecular markers in potato breeding programs.

In conclusion, diverse resistance reactions to *P. infestans* operate at various levels in *Solanum* species, including specific and nonspecific mechanisms. The ubiquitous association

of the HR in all types of resistance suggests that numerous *R* genes are present in *Solanum* against the oomycete *P. infestans*. A remaining challenge is the identification and transfer of these *R* genes into commercially grown potato cultivars.

Samenvatting

Het is inmiddels al meer dan 150 jaar geleden dat in Europa de aardappelziekte uitbrak, en nog steeds kost het grote inspanningen om ons gewas te beschermen. Elk jaar worden enorme hoeveelheden dure chemische middelen toegediend, die een bedreiging kunnen vormen voor het milieu. Het gebruik van resistente aardappelrassen zou een elegante oplossing voor het probleem zijn. Naast het benodigde hoge niveau van resistentie is het ook belangrijk dat deze duurzaam is. Er zijn echter nog te weinig rassen beschikbaar die aan beide eisen voldoen. De veroorzaker van de aardappelziekte is *Phytophthora infestans.* Hoewel *P. infestans* morfologisch lijkt op een schimmel, is het een heel ander type organisme: een oömyceet. Oömyceten zijn onafhankelijk van schimmels geëvolueerd, hetgeen impliceert dat ze op een eigen manier de interactie met de plant aangaan. Naar verwachting zal een beter inzicht in resistentiemechanismen van de plant tegen *P. infestans* de veredelaars kunnen helpen om een duurzame vorm van resistentie te ontwikkelen.

Genetische resistentie kan worden bepaald op ras- of variëteitniveau (fysio-specifieke resistentie, is alleen werkzaam in bepaalde variëteiten en tegen bepaalde isolaten), of op soort- of geslachtniveau (niet-waard resistentie). De niet-waard resistentie is volledig en komt voor in de meeste plantensoorten. Daarnaast kan resistentie kwantitatief zijn (partiële resistentie). In wilde verwanten van de aardappel (*Solanum tuberosum*) komen diverse vormen en niveaus van resistentie voor, variërend van volledig resistent tot verschillende niveaus van partiële resistentie. Ook in een aantal oude aardappelrassen is partiële resistentie aanwezig, die bovendien duurzaam blijkt te zijn. Om duurzame resistentie tegen *P. infestans* in moderne aardappelrassen te bereiken, is een dieper inzicht in de moleculaire basis van deze verschillende resistenties essentieel. Hiertoe hebben we een set van *Solanum* soorten met verschillende vormen en niveaus van resistentie samengesteld, en de cellulaire en moleculaire aspecten van onderliggende resistentiemechanismen bestudeerd.

Voor cellulair en moleculair resistentie-onderzoek is een betrouwbaar experimenteel toetssysteem nodig, dat een hoge en reproduceerbare infectie garandeert, en dat bovendien vergelijkbaar is met de veldsituatie. Hiertoe hebben we onder gecontroleerde omstandigheden in het laboratorium een resistentietoets met afgesneden bladeren ontwikkeld. Deze laboratoriumtoets hebben we vervolgens vergeleken met een veldproef. De geteste condities waaronder de planten waren opgekweekt hadden geen invloed op hun resistentie tegen *P. infestans*. De bladeren aan intacte planten waren resistenter dan afgesneden bladeren, en het verschil in resistentie kon verklaard worden door de incubatiecondities die gunstiger zijn in de laboratoriumtoets. Op intacte planten was de infectiefrequentie echter erg laag, hetgeen ongewenst is in moleculair onderzoek. Omdat een set van twintig planten onder verschillende omstandigheden eenzelfde rangorde in resistentieniveau liet zien, konden we concluderen dat de laboratoriumtoets geschikt is om de interactie tussen *Solanum* en *P. infestans* te bestuderen.

Een cytologische overzichtstudie van de interactie tussen twintig *Solanum* genotypen en drie *P. infestans* isolaten gaf een eerste indruk van de aard van de resistentiereacties. Aardappelrassen met fysio-specifieke resistentiegenen (*R* genen) lieten de overgevoeligheids-reactie zien ('Hypersensitieve Response', HR). De HR is een vorm van geprogrammeerde celdood, waarbij het pathogeen in korte tijd geïsoleerd wordt te midden van dode plantencellen,

en daardoor niet verder kan uitgroeien. Ook in de duurzaam resistente aardappelrassen zonder bekende R genen, wilde Solanum soorten, en niet-waardplanten was de HR zichtbaar. In hoog-resistente Solanum soorten zoals S. berthaultii en S. circaeifolium, en in niet-waardplanten zoals Arabidopsis thaliana (zandraket) en S. nigrum (zwarte nachtschade), was de HR extreem snel en effectief; de reactie bleef beperkt tot een klein aantal cellen. In partieel resistente planten kwam de HR later op gang, wat resulteerde in grotere HR lesies voordat doorgroei van het pathogeen gestopt werd. Soms bleken hyfen in staat om uit deze HR lesies te ontsnappen, en een biotrofe interactie met hun waardplant aan te gaan. De effectiviteit waarmee de HR P. infestans kon indammen verschilde aanmerkelijk tussen de verschillende Solanum genotypen, en was gecorreleerd met de resistentieniveaus. Naast de HR werden ook nog andere reacties waargenomen, zoals locale afzettingen van callose en fenolische verbindingen, die zouden kunnen fungeren als fysische barrières. Deze reacties waren niet gecorreleerd met de resistentieniveaus. Uiteindelijk lijkt een balans tussen uitgroei van het pathogeen en inductie van de resistentiereacties te bepalen of infectie slaagt op celniveau; de variatie tussen de effectiviteit van reacties op meerdere infectieplaatsen illustreert het kwantitatieve karakter van de P. infestans resistentie op plant- of veldniveau.

De HR wordt in gang gezet wanneer specifieke receptoren van de plantencel elicitoren van pathogenen herkennen. Volgens de 'gen-om-gen' hypothese coderen specifieke resistentiegenen voor deze receptoren. Er is een aantal typen R genen bekend, waarbij het 'nucleotide binding site leucine-rich repeat' (NBS-LRR) type het meest bestudeerd is. Een ander type omvat de Pto-achtige serine/threonine kinasen, die oorspronkelijk zijn gevonden in wilde verwanten van tomaat. We hebben in het geslacht Solanum een aantal Pto-achtige sequenties geïdentificeerd, en met behulp hiervan de evolutie van Pto-achtige genen bestudeerd. Polymerase chain reaction (PCR) amplificaties met primers gebaseerd op geconserveerde en variabele domeinen van Pto resulteerden in 32 intacte Pto-achtige sequenties in zes Solanum soorten. Er komt dus een uitgebreide genenfamilie van Pto-achtigen voor in Solanum. De kinase consensus en de autofosforylatie-residuen bleken sterk geconserveerd, in tegenstelling tot het activeringsdomein, dat van belang is voor de herkenning van de ligand. In bladeren hebben we de aanwezigheid van Pto-achtige transcripten aangetoond. Fylogenetische analyses onderscheidden negen klassen van Pto-achtige genen, en hier leken de orthologen (homologen ontstaan door soortvorming) meer op elkaar dan de paralogen (homologen ontstaan door genduplicatie). Dit wekt de suggestie dat de Pto familie geëvolueerd is door middel van een serie genduplicaties die nog voor de soortvorming in Solanum zijn opgetreden. In lijn met recente bevindingen voor NBS-LRR R genen, blijken de Pto-achtige genen een grote diversiteit te bezitten en van oude oorsprong te zijn.

Verschillende niveaus van niet-specifieke resistentie in Solanum kwamen naar voren in resistentieproeven met vijf verschillende P. infestans isolaten. In partieel resistente planten waar hyfen uit HR lesies ontsnappen, breidden de lesies zich vaak langzamer uit dan in vatbare planten. Blijkbaar is hier een systemisch resistentiemechanisme actief; dit zou bijvoorbeeld op 'systemic acquired resistance' (SAR) kunnen berusten. SAR kan worden geïnduceerd door verschillende signalen, maar ook basale niveaus van SAR kunnen variëren tussen planten. Om te testen of basale niveaus van SAR correleren met niet-specifieke

resistentie hebben we in gezonde bladeren de basale expressie niveaus bepaald van een aantal SAR merker genen, de zogenaamde pathogenese-gerelateerde (*PR*) genen *PR-1, PR-2* en *PR-5*. Op geslachtsniveau was er geen correlatie tussen basale mRNA niveaus van *PR* genen en niet-specifieke resistentie tegen *P. infestans*. Binnen de soorten *S. amezii x hondelmannii, S. microdontum, S. sucrense* en *S. tuberosum* was er een positieve correlatie tussen niveaus van *PR* mRNA en niet-specifieke resistentie. Voor de aardappelrassen waren de *PR* expressieniveaus het hoogst in de resistente 'Robijn', middelmatig in de partieel resistente 'Ehud', 'Estima' en 'Première', en het laagst in de vatbare 'Bintje'. Dit wekt de suggestie dat constitutieve expressie van *PR* genen zou kunnen bijdragen aan niet-specifieke resistentie tegen *P. infestans*. *PR* mRNA niveaus zouden dus als moleculaire merkers gebruikt kunnen worden in de aardappelveredeling.

Resumerend, in *Solanum* soorten opereren diverse specifieke en niet-specifieke resistentiemechanismen tegen *P. infestans* op verschillende niveaus. De sterke associatie van de HR met alle vormen van resistentie geeft aan dat een groot aantal *R* genen tegen oömyceten aanwezig is in *Solanum*. Het is nu van belang om de *R* genen tegen *P. infestans* te identificeren en over te brengen naar commerciële aardappelrassen.

Nawoord

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Curriculum vitae

Vivianne Vleeshouwers werd geboren op 18 juli 1968 te Geleen. Na het VWO doorlopen te hebben op de Albert Schweitzer Scholengemeenschap te Geleen, begon zij in 1986 de studie Biologie aan de Landbouwuniversiteit Wageningen. Tijdens de doctoraalfase, concentreerde zij zich op plantkundige vakken binnen de oriëntatie 'individu'. Vervolgens liep zij in totaal vijf afstudeervakken en stages waarvan drie binnen Wageningen, namelijk bij de vakgroep Plantencytologie en -morfologie, de vakgroep Plantenfysiologie en de afdeling Ontwikkelingsbiologie van het Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO). Daarnaast ging ze op stage naar de Dipartimento di Biologia Ambientale, Università di Siena, Italië en naar de Department of Crop Science, University of Guelph, Canada. Na het afronden van haar voornamelijk plantenfysiologisch getinte studie, richtte zij zich op de moleculaire biologie tijdens een na-doctoraal onderzoeksproject bij de vakgroep Fytopathologie van de Landbouwuniversiteit. Vervolgens startte zij haar promotieonderzoek naar resistentie tegen *Phytophthora infestans* in *Solanum* soorten bij de afdeling Akkerbouw- en Groentegewassen van het (toenmalige) CPRO-DLO, in nauwe samenwerking met de vakgroep Fytopathologie. Hiervan staan de resultaten beschreven in dit proefschrift. Aansluitend werkte zij als 'Pre-Postdoc' bij het Laboratorium voor Fytopathologie aan elicitors van *P. infestans*. Momenteel is zij werkzaam bij het Laboratorium voor Plantenveredeling in Wageningen, waar zij onderzoek doet naar het verkrijgen van duurzame resistentie tegen *P. infestans* in aardappel.

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