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

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Abstract. 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 of the 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.

Key words: Hypersensitive response – Nonhost resistance – Phenolic compound – *Phytophthora* – Resistance (*R*) genes – *Solanum* (disease resistance)

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Abbreviations: DIC = differential interference contrast; hai = hours after inoculation; IE = infection efficiency; HR = hypersensitive response; LGR = lesion growth rate; *R* gene = resistance gene

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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 with accumulated callose have been found (Wilson and Coffey 1980; Gees and Hohl 1988; Cuypers and Hahlbrock 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). *Phytophthora 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 the mechanisms involved in durable resistance.

Materials and methods

Plant material. The plant material used in this study is listed in Table 1. The *Solanum* plants and *Mirabilis jalapa* were propagated in vitro. Plants of the other species were obtained from seeds.

Table 1. Plant material used in this study: individual clones, the origin, and known *P. infestans* resistances

Plants species	Family	Clone	Origin	Resistance previously observed	Reference
S. berthaultii	Solanaceae	9	BGRC[a] 10063	Complete	Colon et al. 1995a
S. berthaultii	Solanaceae	11	BGRC 10063	Partial	Colon et al. 1995a
S. arnezii × *hondelmannii*	Solanaceae	63	BGRC 27308	Partial	Colon and Budding 1988
S. arnezii × *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. 1995a
S. microdontum	Solanaceae	178	BGRC 24981	Partial	Colon et al. 1995a
S. microdontum var. *gigantophyllum*	Solanaceae	265	BGRC 18570	Susceptible	Colon et al. 1995a
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. 1995a
ABPT[b]	Solanaceae	(30 × 33)-44	WU	Partial	
S. tuberosum	Solanaceae	cv. Bintje	Plant Res. Int.	Susceptible	
S. tuberosum	Solanaceae	cv. Bildtstar	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. 1995b
S. nigrum-SN18	Solanaceae	SN18	Plant Res. Int.	Nonhost	
S. nigrum × *tuberosum* hybrid[c]	Solanaceae	SN18 × Désirée	Plant Res. Int.	Complete	Colon et al. 1993
Nicotiana rustica	Solanaceae	var. WAU	WU	Nonhost	
Nicotiana tabacum	Solanaceae	cv. Xanthi	WU	Nonhost	
Raphanus sativa	Cruciferae	cv. Daikon	WU	Nonhost	
Arabidopsis thaliana	Brassicaceae	ecotype Colombia	Plant Res. Int.	Nonhost	
Mirabilis jalapa	Nyctaginaceae	unknown	Commercial	Nonhost	

[a]Genetic Resource Centre at Braunschweig, Germany
[b]Double-bridge hybrid of *S. acaule*, *S. bulbocastanum*, *S. phureja*, *S. tuberosum* (Hermsen and Ramanna 1973), WU, Wageningen Agricultural University, The Netherlands
[c]A sexual hybrid of *S. nigrum*-SN18 × potato cv. Désirée (Eijlander and Stiekema 1994)

Plants were grown in climate chambers under controlled conditions (Vleeshouwers et al. 1999).

Phytophthora infestans isolates and inoculation. The *P. infestans* isolates IPO-0 (race 0), Bonn Complex (race 1.3.4.7.10.11, both kindly provided by L.J. Turkensteen, Plant Research Int., Wageningen, The Netherlands), 90128 (complex race 1.3.4.6.7.8.10.11), and *P. mirabilis* isolate CBS 136.86 (obtained from Central 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 ($A = 1/4 \times \pi \times \text{length} \times \text{width}$) were calculated and divided into 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).

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 by phase contrast and differential interference contrast (DIC) microscopy in a Zeiss Axiophot (Zeiss) microscope which was equipped with a high-pressure mercury-vapor lamp (HBO 50 W) for fluorescence analyses. 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 35-mm 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 17 *Solanum* clones inoculated with *P. infestans* isolates IPO-0 and 90128. Per stage, i.e. 22, 46 and 70 h 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 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* × *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.

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. *Solanum 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.

Microscopical overview: potato. Inoculation of the 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 (Fig. 1A,B). 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, Fig. 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

Table 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				Microscopy[a]			
	IE[b]			LGR (mm/day)	22 hai		46 hai	
	No symptoms (%)	Arrested lesions (%)	Growing lesions (%)		Number of HR cells (n)	Main observations	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(30 × 33)-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[c]	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 × *hondelmannii*-63	15	20	65	1.9	1.6 (35)	HR (100%), biotrophy	3.7 (33)	Confined & expanding HR lesions
S. arnezii × *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[c]	Expanding lesion
S. microdontum var. *gigantophyllum*-265	0	0	100	3.9	0.3 (20)	Biotrophy, HR (30%)	nd[c]	Expanding lesion
S. tuberosum-Bintje	0	0	100	4.0	0.6 (22)	Biotrophy, HR (28%)	nd[c]	Expanding lesion
LSD	21	21	16	0.9				

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

displayed the HR when inoculated with isolate IPO-0, as expected (Fig. 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 possess 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 (Fig. 3) and established a biotrophic interaction with the mesophyll cells. These escaping hyphae bore one or two haustoria per encountered plant cell, similar to the 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 2, Figs. 4–7). 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 the 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 a 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* (Fig. 1) and *M. jalapa-P. mirabilis* resulted in typical biotrophic growth of the pathogen. In contrast, inoculation of *M. jalapa* and *S. nigrum* (Fig. 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* (Fig. 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 2).

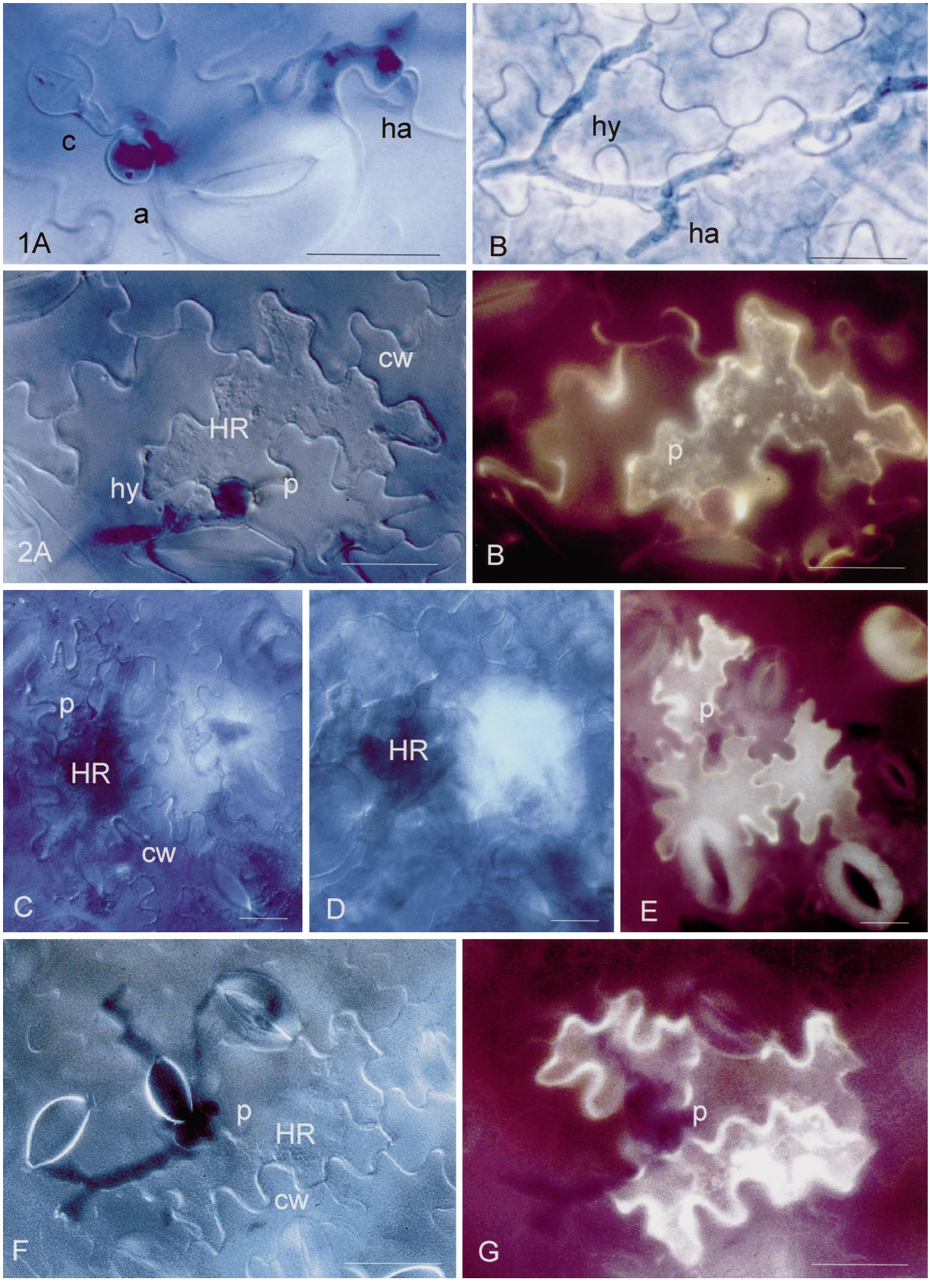
The relation between the HR and the cessation of pathogen growth. Although all *Solanum* clones reacted with a similar type of response to *P. infestans* isolate IPO-0, major differences were observed in severity and timing of the HR between clones with different resistance levels (Table 2).

In the nonhost *S. nigrum*-SN18, the HR was induced extremely fast. One to three cells displayed the HR at 22 hai (Fig. 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. arnezii* × *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 (Tables 2, 3). No increase in the number of cells exhibiting the HR was observed in the resistant *S. nigrum*-SN18 by 22 hai (Table 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 more slowly 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 (Fig. 4). Phenotypically, these cells looked completely different from dead cells. In contrast to dead mesophyll cells (Fig. 3) they retained an organized structure, and cytoplasmic strands could be discerned. In some cases,

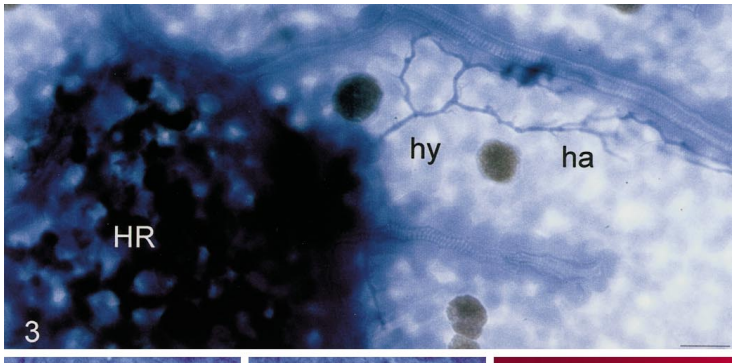
Fig. 1A,B. Compatible interaction between susceptible potato cultivars and *P. infestans*. No plant response is visible during early stages of infection. **A** Penetration of an epidermal cell (22 hai with *P. infestans* isolate IPO-0 on potato cultivar Bintje; DIC). **B** Branching hyphae with haustoria in mesophyll cells (31 hai with *P. infestans* isolate Bonn Complex on potato cultivar Bildtstar; phase-contrast optics). *c*, cyst; *a*, appressorium; *ha*, haustorium; *hy*, hypha. Bar = 35 µm

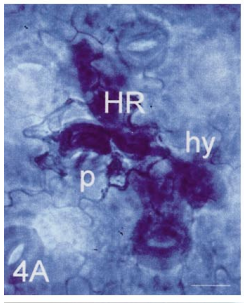
Fig. 2A–G. Hypersensitive response as the major defense response in the *Solanum–P. infestans* interaction. Characteristics of HR cells are the granular structure of the cytoplasm (visible with DIC optics), thickened cell walls and autofluorescence (under UV illumination). The HR occurs in susceptible (**A**, **B**), race-specific (**C–E**) and nonhost (**F**, **G**) interactions at 22 hai with *P. infestans* isolate IPO-0. **A**,**B** An HR epidermal cell of potato cultivar Bintje viewed with DIC optics (**A**) and UV light (**B**). **C–E** Three HR epidermal cells (**C**) of potato cultivar Ehud (*R1*), and the adjacent HR mesophyll cell (**D**), viewed with DIC optics (**C**,**D**) and UV light (**E**). **F**, **G** Three HR epidermal cells of *S. nigrum*-SN18, viewed with DIC optics (**F**) and UV light (**G**). *hy*, hyphae; *p*, penetration site; *HR*, HR-responding cell; *cw*, thickened cell wall. Condensed nuclei are not in focus (not indicated). Bar = 30 µm

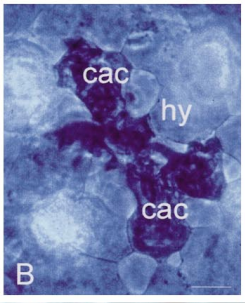
these cells appeared to restore their normal appearance. This reversible response was noted in all *Solanum* clones, particularly in highly resistant clones. Although the specimens sampled at 46 hai were different from those sampled at 22 hai, these observations were made in several independently repeated experiments, and suggest true reversibility of the early stages of the HR.

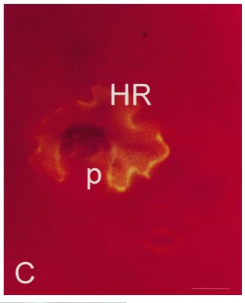
Hyphal growth in veins. After penetration and haustoria formation in epidermal cells that covered vascular tissue (rib cells), hyphae sometimes grew into the xylem vessels in the veins. This was observed in several clones, including the completely resistant *S. nigrum*-SN18 (Fig. 7). Since xylem vessels 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 (Fig. 5). Lesions following an 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 deposi-









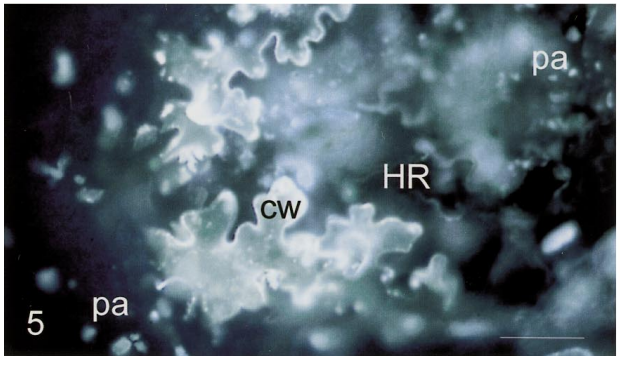
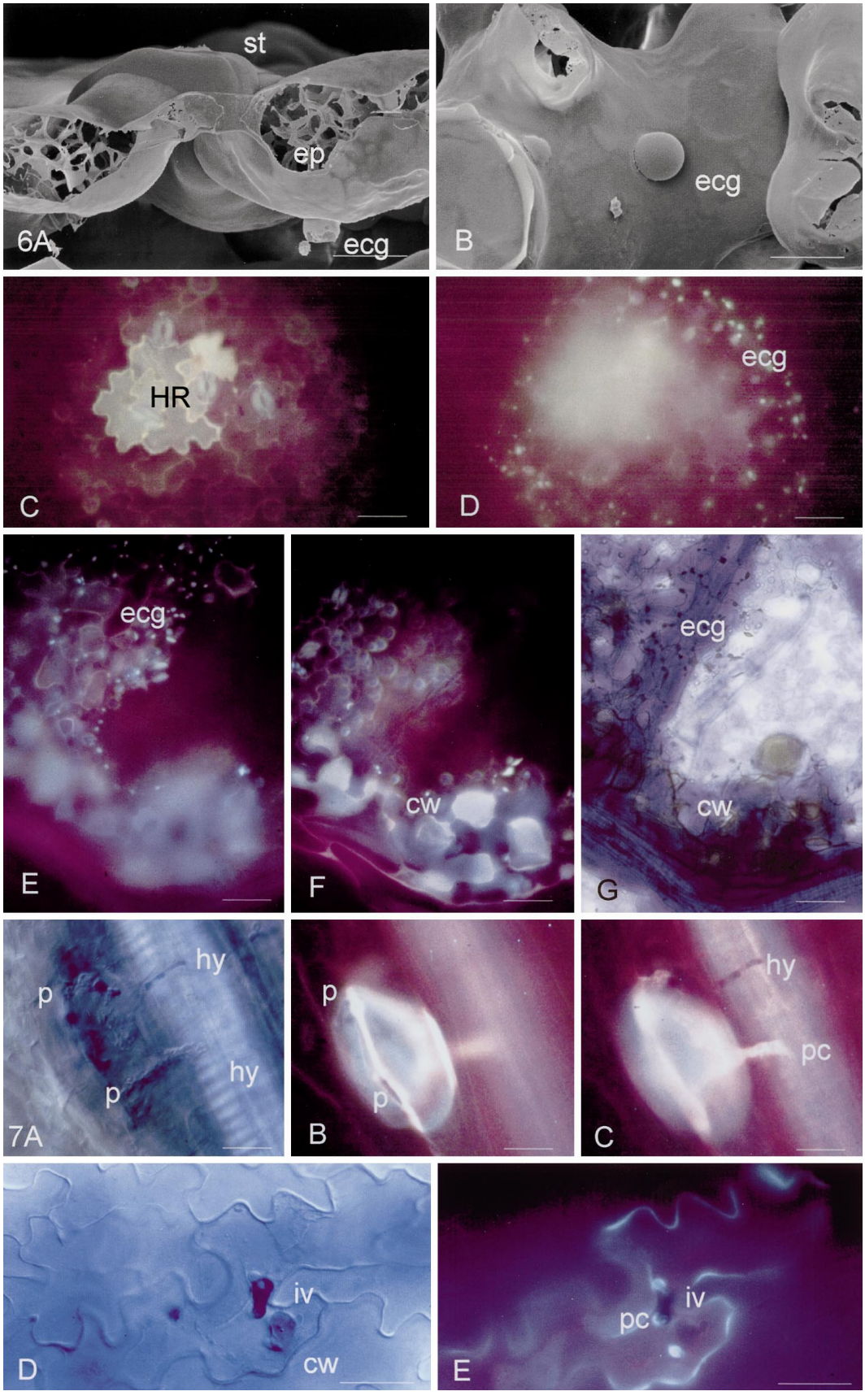


Fig. 3. Lesion caused by the HR, with escaping hyphae in cultivar Robijn, 46 hai with *P. infestans* isolate IPO-0, (bright field) *HR*, HR lesion; *hy*, hypha; *ha*, haustorium. Bar = 40 µm

Fig. 4A–C. 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 blue 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

Fig. 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



tion around HR cells. Partially resistant clones showed an intermediate phenotype.

Solanum nigrum × Désirée displayed callose deposition that was not associated with the inoculated area.

Fig. 6A,B. Scanning electron microsocopy of extracellular globules deposited on the epidermis (**A**) or mesophyll cells (**B**) near hypersensitively responding cells of potato cultivar Robijn 70 hai with *P. infestans* isolate IPO-0. **C**,**D** Hypersensitive response in the epidermis (**C**) and, in the same spot, extracellular globules in the mesophyll (**D**) in *S. arnezii* × *hondelmannii*-63 46 hai with IPO-0 (UV). **E** Extracellular globules in the mesophyll in *S. arnezii* × *hondelmannii*-63 at 70 hai with IPO-0 (UV). **F** At the same spot as **E**, but a lower focal plane, autofluorescing cell walls are visible. **G** The same spot as **F**, but 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**), 50 μm (**C**–**G**)

Fig. 7A–E. 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 hypha appears to be able to grow into the vein, whereas the other hypha is enwrapped in phenolic compounds (**C**). **D**, **E** An (HR) epidermal 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

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 scanning electron microscopy in frozen leaf discs and by light microscopy in trypan-blue-stained leaf discs (Fig. 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 oomycetal 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* × *hondelmannii*, abundant deposition of globules was observed on the cell walls of the mesophyll around HR lesions and near adjacent veins (Fig. 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. arnezii* × *hondelmannii*. In general, there was no indication of a correlation between the amount of globule formation and resistance levels of the different *Solanum* clones.

Table 3. Effectiveness of the HR in *S. nigrum*, Ehud, Robijn, Estima and Bintje, ranked according to decreasing resistance levels (see Table 2), after inoculation with *P. infestans* isolate IPO-0. Cells showing an HR 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 and 22 hai and 22 and 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

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 (Fig. 7). Similar features were found in *Arabidopsis*, where the primary and secondary hyphae in the HR epidermal cell were coated with autofluorescent material (Fig. 7). In addition to this lignification-like response (Mauch-Mani and Slusarenko 1996), the penetrated epidermal cell also showed the characteristics of the HR.

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 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* (E.E. Ewing, Cornell University, Ithaca, New York, USA, personal communication), *S. microdontum* (J.M. Sandbrink, Plant Research Int. Wageningen, the Netherlands, personal communication) 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 (Lauge 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 to be 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 of 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 to be mainly associated with the occurrence of the HR in the tissue, i.e. where the HR occurred, callose was deposited surrounding HR lesions, whereas in the absence of the HR callose was deposited in collars and papillae near the expanding mycelium. Although Hohl and Stossel (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; Cuypers and Hahlbrock 1988). In addition, the overall deposition of callose that was not associated with *P. infestans* infection in *S. nigrum* × 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). 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.

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