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Agroinfection-based high-throughput screening reveals specific recognition of INF elicitins in *Solanum*

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SUMMARY

We adapted and optimized the use of the *Agrobacterium tumefaciens* binary PVX expression system (PVX agroinfection) to screen *Solanum* plants for response to pathogen elicitors and applied the assay to identify a total of 11 clones of *Solanum huancabambense* and *Solanum microdontum*, out of 31 species tested, that respond to the elicitins INF1, INF2A and INF2B of *Phytophthora infestans*. Prior to this study, response to INF elicitins was only known in *Nicotiana* spp. within the Solanaceae. The identified *S. huancabambense* and *S. microdontum* clones also exhibited hypersensitivity-like cell death following infiltration with purified recombinant INF1, INF2A and INF2B, thereby validating the screening protocol. Comparison of INF elicitin activity revealed that *Nicotiana* plants responded to significantly lower concentrations than *Solanum*, suggesting variable levels of sensitivity to INF elicitins. We exploited natural variation in response to INF elicitins in the identified *Solanum* accessions to evaluate the relationship between INF recognition and late blight resistance. Interestingly, several INF-responsive *Solanum* plants were susceptible to *P. infestans*. Also, an *S. microdontum* × *Solanum tuberosum* (potato) population that segregates for INF response was generated but failed to identify a measurable contribution of INF response to resistance. These results suggest that in *Solanum*, INF elicitins are recognized as general elicitors and do not have a measurable contribution to disease resistance.

INTRODUCTION

The ability to perceive a diverse range of pathogen elicitor molecules is a basic defence strategy in plants (Dangl and Jones, 2001; Staskawicz *et al.*, 1995). In some cases, such as in interactions that follow the gene-for-gene model, there is a robust correlation between elicitor perception and resistance to pathogens, with recognition only occurring in resistant host genotypes. Elicitors of this class are termed specific elicitors and are often the direct products of avirulence genes (Bonas and Lahaye, 2002; Botella *et al.*, 1998; Bryan *et al.*, 2000; Laugé and De Wit, 1998; Mackey *et al.*, 2002). In other cases, perception of elicitors occurs in both susceptible and resistant plants. The contribution to the infection process of this class of elicitors, known as general elicitors, is often unclear owing to a lack of genetic evidence. Nevertheless, general elicitors can contribute quantitatively to the defence response in a similar fashion as pathogen-associated molecular patterns (PAMPs) that induce the expression of defence response genes and the production of antimicrobial compounds in animal cells undergoing innate immunity (Brunner *et al.*, 2002; Gomez-Gomez and Boller, 2002; Nurnberger *et al.*, 2004). Recently, Zipfel *et al.* (2004) showed that perception of the PAMP flagellin restricts bacterial invasion of host plants and contributes to disease resistance. Therefore, the study of both specific (avirulence) and general (PAMP) elicitors of plant pathogens and their perception by plants is critical to understanding the molecular basis of disease resistance in a given pathosystem. The identification and characterization of pathogen elicitor–plant receptor interactions could also lead to novel strategies for engineering or breeding for disease resistance.

One class of pathogen elicitors of the oomycete *Phytophthora* consists of elicitins, a superfamily of extracellular proteins that share a 98-amino-acid domain (InterPro IPR002200) (Baillieul *et al.*, 2003; Ponchet *et al.*, 1999; Qutob *et al.*, 2003). Elicitins are lipid-binding proteins that induce the hypersensitive response (HR) and other biochemical changes associated with plant defence responses in a narrow range of plants, such as *Nicotiana* species in the Solanaceae and some radish and rape varieties in the Brassicacae (Blein *et al.*, 2002; Kamoun *et al.*, 1993, 1997; Pernollet *et al.*, 1993; Ponchet *et al.*, 1999; Ricci *et al.*, 1989; Sasabe *et al.*, 2000). Recognition of elicitins is thought to be one component of resistance of *Nicotiana* spp. to a multitude of *Phytophthora* species, such as the potato and tomato late blight pathogen *Phytophthora infestans* and the tobacco black shank

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pathogen *Phytophthora parasitica* var. *nicotianae*. Evidence for this model was obtained using *P. infestans* strains engineered to be deficient in the elicitin INF1 by gene silencing. These strains induce disease lesions on the wild tobacco species *Nicotiana benthamiana*, suggesting that INF1 conditions avirulence to this plant species (Kamoun *et al.*, 1998). Other evidence originated from work with *Phytophthora parasitica,* a species that produces the elicitin PARA1. Some strains of *P. parasitica*, named *P. parasitica* var. *nicotianae*, naturally infect tobacco and cause the black shank disease. Remarkably, the great majority of these isolates do not produce PARA1 (Colas *et al.*, 2001; Kamoun *et al.*, 1994; Ricci *et al.*, 1992), and those that do exhibit down-regulation of *parA1* gene expression *in planta* (Colas *et al.*, 2001). Altogether, these findings suggest that elicitins are species-specific avirulence factors. However, a recent report by Becktell *et al.* (2006) describes INF1-producing isolates of *P. infestans* that can colonize *N. benthamiana* even though this plant responds to INF1 elicitins. Therefore, elicitins may not always function as strict avirulence factors and perhaps function as general elicitors in some pathosystems.

The signalling pathways that are activated in tobacco during response to elicitins have been well characterized and involve the typical biochemical hallmarks of the HR (Ponchet *et al.*, 1999; Sasabe *et al.*, 2000). Elicitins interact with a high-affinity binding site on the plasma membrane of tobacco, which appears to consist of a receptor complex of two glycoproteins of about 170 and 60 kDa in mass (Lebrun-Garcia *et al.*, 1999). However, the molecular genetic basis of elicitin perception remains unknown. Molecular cloning of the receptor gene(s) has been hampered by limitations of the *Nicotiana* system, namely little genetic variation in response to elicitins and the difficulty of map-based cloning in these species. To facilitate the genetic dissection of elicitin response in plants, we undertook to screen *Solanum* germplasm for response to elicitins from *P. infestans* (INF elicitins). Many species in the highly diverse *Solanum* genus are known to exhibit resistance to a variety of pathogens, including *P. infestans* (Hawkes, 1990). Different types of resistance to *P. infestans* in *Solanum*, including partial and non-host resistance, have been associated with the HR, suggesting that recognition of elicitors may form the basis of resistance and that identification of the corresponding receptors could help improve cultivated potatoes (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000).

RESULTS

Optimization of the PVX agroinfection assay in *Solanum*

PVX agroinfection enables high-throughput screening of elicitor activity *in planta* as performed in *Nicotiana benthamiana*, tobacco and tomato (Huitema *et al.*, 2004; Qutob *et al.*, 2002; Takken *et al.*, 2000; Torto *et al.*, 2003). To optimize the use of the binary PVX system in *Solanum*, we took advantage of CRN2, a general necrosis-inducing elicitor of *P. infestans* (Torto *et al.*, 2003). We wound-inoculated 3–4-week-old potato plants (MaR3) with an *A. tumefaciens* strain carrying the binary PVX construct pGR106-CRN2 on both sides of the mid-vein. Nine days post inoculation (dpi), local necrosis became visible around the inoculation sites. These necrotic lesions slowly expanded over time to become particularly obvious around 16 dpi (Fig. 1A,B). No systemic PVX symptoms became visible, suggesting that the recombinant PVX-CRN2 may have become restricted to the necrotic lesions. As a negative control, we inoculated potatoes with the same *A. tumefaciens* strain carrying the empty pGR106 vector. In these plants no local responses were observed, and systemic mosaic symptoms became visible after 2–3 weeks, indicating that the virus spread throughout the plant.

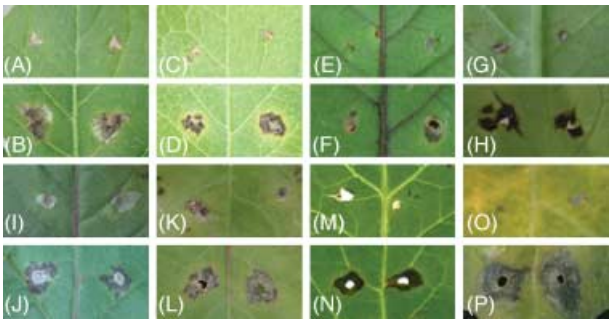

Fig. 1 Suitability of *Solanum* species for PVX agroinfection assay. *Solanum tuberosum* MaR3 (A, B), *S. tuberosum* MaR7 (C, D), *S. microdontum* 17596-1 (E, F), *S. huancabambense* 17719-1 (G, H), *S. bulbocastanum* 17693–5 (I, J), *S. fendleri* 17717-8 (K, L), *S. stoloniferum* 17605–4 (M, N) and *S. polytrichon* 17750-4 (O, P) were wound-inoculated with *Agrobacterium tumefaciens* strains carrying pGR106 (A–G, I–O) and pGR106-CRN2 (B–H, J–P). Pictures were taken at 13–19 dpi.

The agroinfection assay in *Solanum* poses a number of challenges compared with tobacco and tomato because plants are cultured *in vitro* rather than from seeds and a large variation in plant size and leaf morphology occurs. To determine the effect of plant maturity and size, we compared potato plants of different ages (time after transfer from *in vitro* culture to soil) for their ability to show local and systemic symptoms following PVX inoculation. Three- to 4-week-old plants showed the most consistent results and offered the best balance in terms of age vs. number of leaves available for inoculation. Inoculation of 10-day-old plants with the pGR106-CRN2 strain revealed local necrosis starting from 9 dpi. However, at 13–16 dpi, the inoculated leaves senesced and dropped, leaving a limited time range for scoring although visualization of systemic symptoms remained possible to some extent. In 5-day-old plants, systemic symptoms were not observed, possibly because the root system was not yet fully

adjusted after transfer to the soil. Inoculation of plants older than 5 weeks resulted in a limited window for observing both local and systemic responses, as leaves started senescing before symptoms became clear.

In conclusion, our optimized protocol consists of inoculating at least three leaves from 3–4-week-old plants. A minimum of 12 toothpick-inoculations per leaflet can be performed, suggesting that at least 90 *A. tumefaciens* strains can be screened per plant and in duplicate for localized necrotic responses.

Is the PVX agroinfection assay applicable to many *Solanum* species?

Resistance to PVX is known to occur in *Solanum* and would interfere with large-scale screenings with the binary PVX vector. To determine the extent to which the assay is applicable to the diverse *Solanum* germplasm, we inoculated plants corresponding to 80 *Solanum* clones from 31 species with *A. tumefaciens* carrying pGR106 and pGR106-CRN2. Treatment with the pGR106-CRN2 strain consistently caused necrosis around the inoculation sites on 50 plant clones (63%), whereas the empty vector strain caused no symptoms. We concluded that these plants are suitable for PVX agroinfection assays (Fig. 1, Table 1). The remainder of the *Solanum* clones tested were not suitable for the assay. Responses to both the positive and the negative controls occurred in 20 clones and were regarded as non-specific reactions to PVX. Ten other clones showed no necrotic response to pGR106-CRN2 possibly because the gene insert was not expressed *in planta*. In summary, it appears that the PVX assay is suitable for about 80% of the species examined and 60% of the clones.

Table 1 Suitability of *Solanum* germplasm for PVX agroinfection assay. A selection of 80 *Solanum* clones belonging to 31 different species were tested with an *A. tumefaciens* strain carrying the empty binary PVX vector pGR106 and a strain carrying pGR106-CRN2 that expresses the non-specific cell death elicitor CRN2. Plants that responded to the pGR106-CRN2 but not the vector strain were deemed suitable for the assay.

Solanum species	No. of plant clones tested	*A. tumefaciens* strain	
		pGR106	CRN2
S. agrimonifolium	2	–	+
S. agrimonifolium	1	+	+
S. andreanum	2	–	+
S. berthaultii	1	–	–
S. brachistotrichum	2	+	+
S. brachycarpum	2	–	+
S. bulbocastanum	2	–	+
S. bulbocastanum	2	+	+
S. canense	1	–	+
S. cardiophyllum	1	–	+
S. cardiophyllum	1	+	+
S. chacoense	1	–	+
S. chacoense	1	–	–
S. chaparense	1	–	+
S. circaeifolium	1	–	–
S. demissum	8	–	+
S. demissum	3	+	+
S. demissum	1	–	–
S. fendleri	2	–	+
S. guerreroense	2	–	+
S. hjertingii	2	–	+
S. huancabambense	2	–	+
S. jamesii	1	–	+
S. leptophyes	1	–	+
S. microdontum	1	–	+
S. microdontum	2	+	+
S. multiinterruptum	1	–	+
S. multiinterruptum	2	+	+
S. neorossii	1	–	+
S. okadae	1	–	+
S. okadae	2	+	+
S. papita	1	–	+
S. papita	1	+	+
S. pinnatisectum	2	–	+
S. polyadenium	2	–	+
S. polytrichon	1	–	+
S. raphanifolium	1	+	+
S. sparsipilum	1	+	+
S. sparsipilum	1	–	–
S. stoloniferum	1	–	+
S. sucrense	2	–	–
S. tuberosum	9	–	+
S. tuberosum	2	+	+
S. tuberosum	3	–	–

Screening for response to INF elicitins in *Solanum*

We selected 43 different *Solanum* clones representing 21 species for screening for response to INF elicitins. The majority of the clones were identified from an initial set of 518 clones from 78 accessions of 46 *Solanum* species as showing high levels of resistance to *P. infestans* in both detached leaves and *in vitro* plant assays (Huang *et al*., 2005b; Vleeshouwers *et al*., 1999). In addition, we also complemented the set with various partially resistant and susceptible genotypes that are currently used in potato breeding programmes (Table 2). All 43 clones showed a necrotic response to *A. tumefaciens* (pGR106-CRN2) but not to the vector strain, suggesting that they are suitable for the PVX agroinfection assay.

Inoculation of the 43 *Solanum* clones with *A. tumefaciens* strains carrying pGR106-INF1, pGR106-INF2A and pGR106-INF2B revealed that two clones, i.e. *S. huancabambense* (hcb) 17719-1 (Fig. 2A) and *S. microdontum* (mcd) 17596-1, show reproducible localized necrosis, suggesting that these plants respond to INF elicitins (Table 2).

Table 2 Functional screening for response to INF elicitins in *Solanum*. The taxonomic series within the *Solanum* sect. *Petota* (Hawkes, 1990), species, origin of *Solanum* material, and *Solanum* clone numbers (CGN accession-clone number) are presented. Plants were inoculated with pGR106-INF1, -INF2A and –INF2B *A. tumefaciens* strains; pGR106-CRN2 and -empty vector were included as positive and negative control for the PVX method, respectively. *Nicotiana* plants were included as positive controls for INF responses. Inoculated plants were examined for local necrotic responses (+) or no symptoms (–) at inoculation sites.

Solanum taxonomic series	*Solanum* species	Origin*	Accession-clone number	*A. tumefaciens* pGR106 insert				
				empty	INF1	INF2A	INF2B	CRN2
NA†	*S. chaparense*	Bol	18060-1	–	–	–	–	+
Bulbocastana	*S. bulbocastanum*	Mex	21306-1	–	–	–	–	+
Canensa	*S. canense*	Bol	18062-1	–	–	–	–	+
Demissa	*S. brachycarpum*	Mex	17721-2, -3	–	–	–	–	+
	S. demissum	Mex	17810-01, -06, -14	–	–	–	–	+
		Mex	17820-01, -21	–	–	–	–	+
		Gtm	20571-01, -10, -12	–	–	–	–	+
	S. guerreroense	Mex	18290-1, -2	–	–	–	–	+
Longipedicellata	*S. fendleri*	Mex	17717-3	–	–	–	–	+
	S. hjertingii	Mex	17718-1, -2	–	–	–	–	+
	S. papita	Mex	17830-1	–	–	–	–	+
	S. polytrichon	Mex	17750-4	–	–	–	–	+
	S. stoloniferum	Mex	17605-4	–	–	–	–	+
Pinnatisecta	*S. cardiophyllum*	Mex	18326-1	–	–	–	–	+
	S. jamesii	Usa	18349-1	–	–	–	–	+
	S. pinnatisectum	Mex	17743-1, -4	–	–	–	–	+
Polyadenia	*S. polyadenium*	Mex	17749-1, -4	–	–	–	–	+
Tuberosa	*S. andreanum*	Col	18344-1, -6	–	–	–	–	+
	S. leptophyes	Bol	18174-8	–	–	–	–	+
	S. microdontum	Arg	17596-1	–	+	+	+	+
	S. neorossii	Arg	18000-1	–	–	–	–	+
	S. tuberosum	Bre	MaR2, MaR3, MaR4, MaR6, MaR7, MaR10, MaR11, RH89-039-16, RH90-038-21	–	–	–	–	+
Yungasensa	*S. huancabambense*	Per	17719-1	–	+	+	+	+
		Per	18306-1	–	–	–	–	+
	N. benthamiana		ben	–	+	+	–	+
	N. tabacum		SR1	–	+	+	+	+

*Bol, Bolivia; Mex, Mexico; Per, Peru; Arg, Argentina; USA; Gua, Guatemala; Bre, Breeding clone.
†NA, not available; *S. chaparense* is a non-tuberbearing *Solanum* that does not belong to section *Petota*.

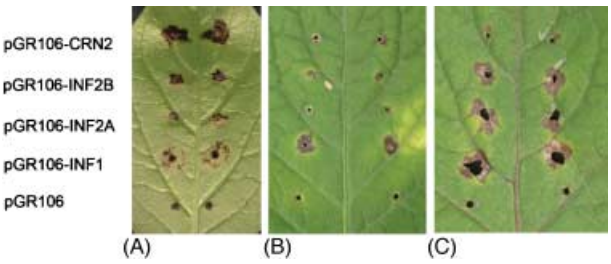

Fig. 2 PVX agroinfection assay reveals response to INF elicitins in *Solanum huancabambense* and *S. microdontum*. *S. huancabambense* 17719-1 (A), *S. microdontum* 17596-7 (B) and *S. microdontum* 17596-9 (C) were wound-inoculated with *Agrobacterium tumefaciens* strains carrying pGR106, pGR106-INF1, -INF2A, -INF2B and -CRN2 at both sides of the vein and scored for occurrence of local necrosis at the inoculation site. Pictures were taken at 14 dpi (A, B) and 17 dpi (C).

To determine the extent of INF elicitin response within *S. huancabambense* and *S. microdontum*, we assayed 23 additional accessions and clones representing these two species (Table 3). All six clones from hcb17719 (*S. huancabambense*, CGN accession no. 17719) and one of five from hcb18306 showed a necrotic response following inoculation with the pGR106-INF1, pGR106-INF2A and pGR106-INF2B strains. Also, four of seven clones of *S. microdontum* mcd17596 responded to the INF constructs (Fig. 2B,C), but all tested clones from mcd18083 were negative. We also tested eight clones of *S. microdontum* mcd18046 but most of them showed responses to *A. tumefaciens* (pGR106) and could not be unambiguously evaluated for INF response (data not shown). In summary, variation in INF response was evident within the two *Solanum* species. In total, seven *S. huancabambense* and four *S. microdontum*

Table 3 Validation of INF response in *S. huancabambense* and *S. microdontum* using infiltration of purified INF proteins. Leaves of *Solanum* plants responding to pGR106-INF1, -INF2A and -INF2B were infiltrated with 100 nM purified INF1, INF2A and INF2B and examined for local necrosis (+) vs. no response (–). *S. tuberosum* cv. Bintje and *N. tabacum* cv. Petite Havana SR1 were included as negative and positive controls for INF recognition, respectively. Infiltrations of equimolar concentrations of BSA and 4 nM AVR2 from *C. fulvum* were used as a negative control for *P. pastoris*-produced proteins containing a His-Flag tag (Luderer *et al.*, 2002) and did not cause a necrotic response.

	Inoculation with *A. tumefaciens* pGR106 derivatives					Infiltration with recombinant proteins			
Solanum accession-clone	empty	CRN2	INF1	INF2A	INF2B	AVR2	INF1	INF2A	INF2B
hcb17719-1	–	+	+	+	+	–	+	+	+
hcb17719-2	–	+	+	+	+	–	+	+	+
hcb17719-4	–	+	+	+	+	–	+	+	+
hcb17719-5	–	+	+	+	+	–	+	+	nd
hcb17719-8	–	+	+	+	+	–	+	+	+
hcb17719-10	–	+	+	+	+	–	+	+	+
hcb18306-1	–	+	–	–	–	–	–	–	–
hcb18306-2	–	+	–	–	–	–	–	–	–
hcb18306-6	–	+	+	+	+	–	+	+	+
hcb18306-8	–	+	–	–	–	–	–	–	–
hcb18306-9	–	+	–	–	–	–	–	–	–
mcd17596-1	–	+	+	+	+	–	+	+	+
mcd17596-2	–	+	+	+	+	–	+	+	+
mcd17596-5	–	+	–	–	–	–	–	–	–
mcd17596-6	–	+	–	–	–	–	–	–	–
mcd17596-7	–	+	+	+	+	–	+	+	+
mcd17596-8	–	+	–	–	–	–	–	–	–
mcd17596-9	–	+	+	+	+	–	+	+	+
mcd18046-6	–	+	–	–	–	–	–	–	–
mcd18046-7	–	+	–	–	–	–	–	–	–
mcd18083-3	–	+	–	–	–	–	–	–	–
mcd18083-4	–	+	–	–	–	–	–	–	–
mcd18083-6	–	+	–	–	–	–	–	–	–
tbr-Bintje	–	+	–	–	–	–	–	–	–
N. tabacum-SR1	–	+	+	+	+	–	+	+	+

clones were identified to respond to INF elicitins. In general the response of all plants to pGR106-INF1 and pGR106-INF2B resulted in more extensive necrotic lesions than to pGR106-INF2A, similar to what was described for tobacco (Huitema *et al.*, 2005).

Validation of *Solanum* response to INF elicitins using infiltration of purified recombinant proteins

To validate and confirm the observed responses to the PVX-INF strains, we infiltrated purified recombinant INF proteins into leaves of the 23 clones of *S. huancabambense* and *S. microdontum* (Table 3, Fig. 3). Recombinant INF1, INF2A and INF2B fused to the His and Flag epitope tags were expressed and purified from cultures of *Pichia pastoris.* All of the 11 INF-responding *Solanum* clones showed an HR-like necrotic response in the areas infiltrated with 100 nM purified INF1, INF2A and INF2B. The necrosis usually started with a patchy phenotype at 1 or 2 dpi for most *Solanum* genotypes, and developed to confluent necrosis by 3 dpi (Table 3). By contrast, no response was observed following infiltration with a similarly expressed and purified Avr2 protein of the fungus *Cladosporium fulvum*. There was no variation in response between the different members of the elicitin family. These results confirm that some clones of *S. huancabambense* and *S. microdontum* specifically respond to INF elicitins.

Quantitative evaluation of INF response in *Solanum* and tobacco

To compare the sensitivity of INF response in *Solanum* and *Nicotiana*, we infiltrated a dilution series of 0.3–300 nM purified recombinant INF1 protein in hcb17719-1, hcb17719-4, mcd17596-1, mcd17596-9 and *N. tabacum* cv. Samsung and cv. SR1 and observed the severity of the necrosis at 1, 2 and 3 dpi (Fig. 4). In *Nicotiana*, necrotic responses to 0.3 nM INF1 were observed for both cultivars at 2 dpi. Confluent necrosis was detected 2–3 days after infiltration with 3 nM INF1, and within 1 day after infiltration with 30 nM INF1. The EC50 values for confluent necrosis after 3 days in *Nicotiana* were 2 and 3 nM INF1 for cv. SR1 and cv. Samsung, respectively.

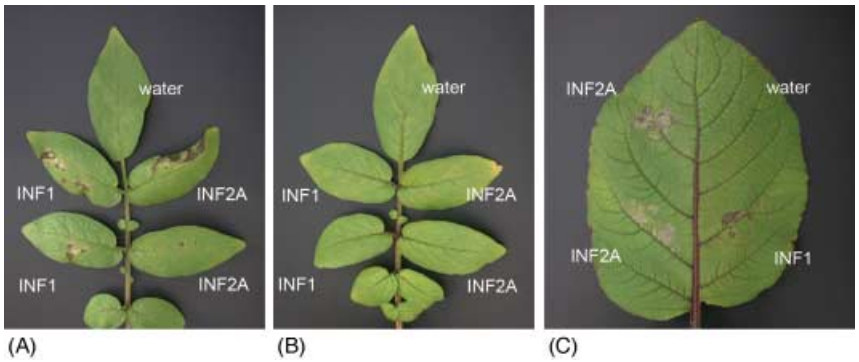

Fig. 3 Infiltration of purified INF proteins triggers cell death in *Solanum huancabambense and Solanum microdontum*. *S. huancabambense* 17719-10 (A), *S. huancabambense* 18306-1 (B) and *S. microdontum* 17596-2 (C) were infiltrated with INF1, INF2A and water as indicated. Pictures were taken at 3 dpi. The *S. huancabambense* plants (A,B) were infiltrated with 300 nM solutions of the INF proteins while the *S. microdontum* plant (C) was infiltrated with 300 nM INF1 (leaf basis right), 300 nM INF2A (leaf basis left) or 1000 nM INF2A (leaf tip left) solutions. Contrast the response observed with *S. huancabambense* 17719-10 and *S. microdontum* 17596-2 with the lack of response of *S. huancabambense* 18306-1.

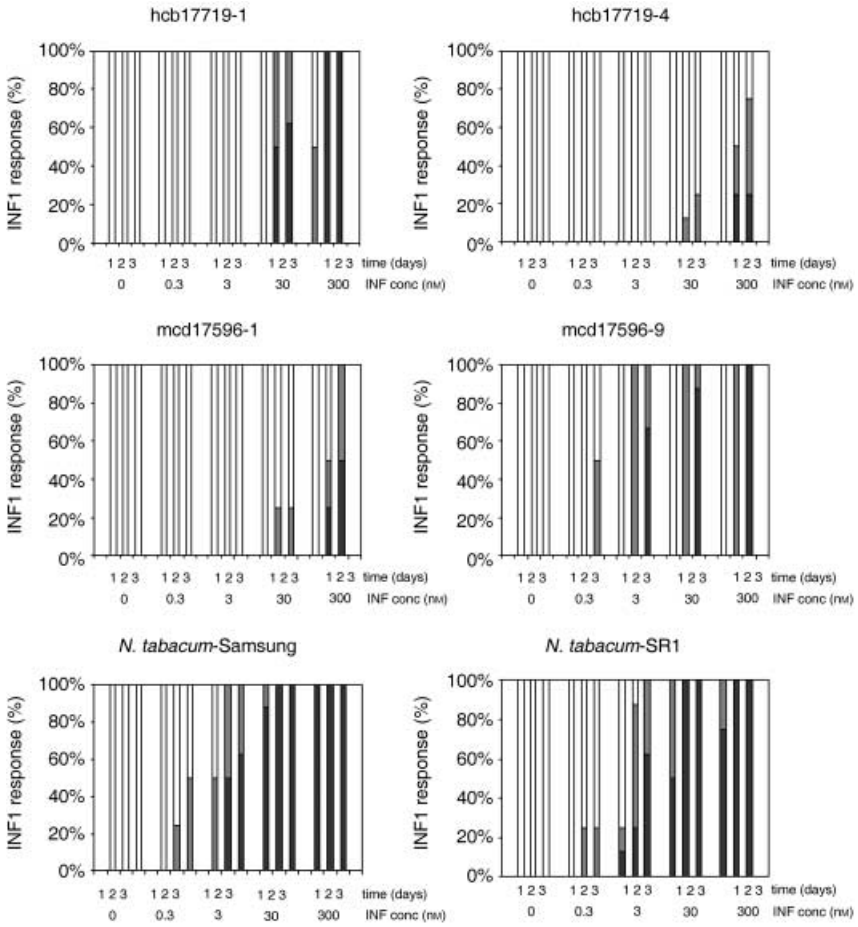

Fig. 4 Quantitative evaluation of INF response in *Solanum* and *Nicotiana* plants. A concentration range of 0, 0.3, 3, 30 and 300 nM INF1 was infiltrated in two clones of *S. huancabambense*, *S. microdontum* and *N. tabacum* and plants were examined for response to INF1 at 1, 2 and 3 dpi. Severity of the response was visually scored, i.e. no response (white), patchy necrosis (grey) and confluent necrosis (black). Four or eight replicates were tested in this experiment and the percentage of INF1-responding leaves was calculated.

Table 4 Resistance levels to *P. infestans* of *S. huancabambense* and *S. microdontum* accessions that show variation for response to INF, and *S. tuberosum* cv. Robijn and *N. benthamiana* control plants. Lesion sizes caused by *P. infestans* isolate 90128 and PY23 at 5 dpi were calculated for 48 replicates. Two independent experiments were performed and combined for analyses. LSD_{LS} for the *S. huancabambense* and the *S. microdontum* experiment were 19.2 and 40.1, respectively. Lesion sizes of *Solanum* clones that are significantly more susceptible for one isolate compared to the other are indicated with asterisks, lesion sizes below 50 mm are considered as resistance.

Solanum accession-clone	INF response	Lesion size (mm)	
		90128	PY23
hcb17719-1	+	7	1
hcb17719-2	+	163*	64
hcb17719-4	+	102*	17
hcb17719-5	+	0	1
hcb17719-10	+	135*	87
hcb18306-6	+	25	0
hcb18306-1	–	127*	39
hcb18306-2	–	23	3
hcb18306-8	–	6	2
hcb18306-9	–	10	0
mcd17596-1	+	25	40
mcd17596-2	+	95*	34
mcd17596-7	+	6	1
mcd17596-5	–	89	210*
mcd17596-6	–	29	22
mcd17596-8	–	0	1
tbr-Robijn	–	84*	32
N. benthamiana	+	8	63*

In *Solanum*, there was significant variation between the clones, and EC50 values for confluent necrosis at 3 dpi ranged from 3 nM to 3000 nM INF1. Overall, response to INF1 in *Solanum* was less severe and slower; necrosis was never detected earlier than 2 days after infiltration when INF1 concentration was lower than 300 nM. The most sensitive *Solanum* clone was mcd17596-9, but even in this case confluent necrosis to 300 nM INF1 was not observed until 3 days after infiltration. In summary, the response of *Solanum* to INF elicitins was less sensitive and slower compared with *Nicotiana.*

Late blight resistance in INF responding *S. huancabambense* and *S. microdontum*

To evaluate the effect of INF response on resistance to *P. infestans*, we determined resistance levels of the *S. huancabambense* and *S. microdontum* clones using a detached leaf assay (Table 4). Putative contributions of response of INF elicitins to resistance were determined in two ways: first, by weighting the infection levels of INF responding against non-responding *Solanum* plants, and second, by comparing the infection levels caused by an INF1-producing *P. infestans* isolate vs. PY23, an INF1-non-producing derivative of wild-type *P. infestans* isolate 88069 (Kamoun *et al.*, 1998; van West *et al.*, 1999). Preliminary assays determined that 88069 and other INF1-producing derivatives of this isolate are hardly virulent on the examined *Solanum* plants. Therefore, we used the INF1-producing and aggressive *P. infestans* isolate 90128 for comparisons with PY23, although we have to realize that other effectors than INF1 can interfere with the resistance response.

Late blight disease levels were scored using standard procedures (Vleeshouwers *et al.*, 1999) by measuring lesion sizes at 5 dpi (LS), infection efficiencies (IE) and lesion growth rates (LGR). The partially resistant potato cultivar Robijn (no response to INF elicitins) was used for comparison. In both *Solanum* species, there was a large variation between clones of the same accessions and between clones from different accessions in LS (Table 4), IE and LGR (data not shown). This variation could not be explained by the difference in response to INF elicitins. Indeed, four INF-responding clones, hcb17719-2, hcb17719-4, hcb17719-10 and mcd17596-2, were susceptible to the INF1-producing strain 90128 resulting in lesions of 100–160 mm at 5 dpi. By contrast, INF-non-responding clones from accessions hcb18306 and mcd17596 were not more susceptible than INF-responding clones from the same accessions. These results suggest that response to INF elicitins in *S. huancabambense* and *S. microdontum* does not correlate with enhanced resistance to INF1-producing *P. infestans*.

The late blight assay data did not indicate that INF1-non-producing strain PY23 is more virulent than 90128 on plants that respond to INF elicitins, suggesting that INF1 does not have a measurable effect on virulence on the examined *Solanum* plants. *P. infestans* strain PY23 was more virulent than 90128 on *S. microdontum* mcd17596-5 but this clone does not respond to INF elicitins. Also, PY23 exhibited significantly higher LS, IE and LGR than 90128 on *N. benthamiana* (Table 4) (data not shown) in line with previous experiments (Kamoun *et al.*, 1998).

Evaluation of a segregating population for INF response and resistance

To assess the relationship between response to INF elicitins and resistance to *P. infestans* in genetically more uniform material, we generated a population that segregates for both phenotypes (Fig. 5). The INF-responding *S. microdontum* clone mcd17596-1 was crossed with the *S. tuberosum* clone RH89-039-16. A population of 15 plants was inoculated with *A. tumefaciens* carrying pGR106-INF1 and scored for INF1 response. A necrotic response to the pGR106-INF1 strain was observed in eight of the 15 plants, suggesting a possible 1 : 1 Mendelian segregation. All 15 plants responded to the pGR106-CRN2 strain but none responded to the empty vector strain. Next, we determined quantitative levels of

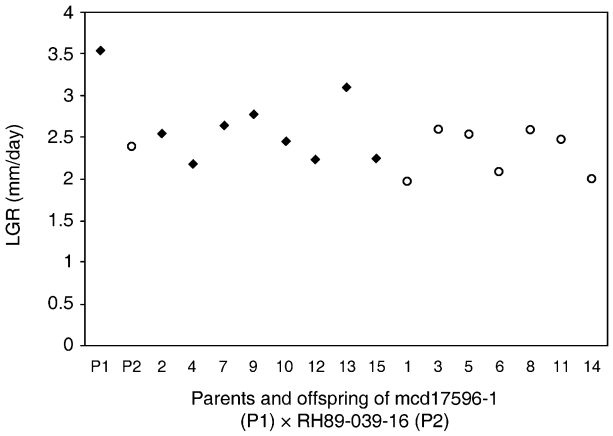

Fig. 5 Lesion growth rates of the segregating population mcd17596-1 (P1) × RH89-039-16 (P2) inoculated with *P. infestans* isolate 90128. In the progeny, eight vs. seven plants recognize (◆) or do not (○) recognize INF. ANOVA analysis revealed that there is no correlation between INF1 recognition and resistance level to *P. infestans* isolate 90128, $LSD_{LGR} = 0.35$.

resistance to *P. infestans* strains 90128 (Fig. 5) and PY23 (data not shown). Overall infection levels of both strains were similar between INF-responding and INF-non-responding plants and no significant effect of INF response on resistance levels to *P. infestans* isolate 90128 was detected (ANOVA, $P = 0.20$ for both LS and LGR).

DISCUSSION

In this study, we adapted and optimized the use of the binary PVX expression system to screen *Solanum* plants for response to pathogen elicitors and applied the assay to identify a total of 11 clones of *S. huancabambense* and *S. microdontum* that respond to the elicitins INF1, INF2A and INF2B of *P. infestans*. The identified *S. huancabambense* and *S. microdontum* clones also exhibited HR-like cell death following infiltration with purified recombinant INF1, INF2A and INF2B, thereby validating the screening protocol. Prior to this study, response to INF elicitins was only known in *Nicotiana* spp. within the Solanaceae (Kamoun *et al.*, 1997). Previous screenings by infiltration of INF1 recombinant protein failed to identify responsive plants among 17 clones from seven *Solanum* spp. (Kamoun *et al.*, 1997). In recent PVX-based experiments we have identified additional *Solanum* clones responding to INF (data not shown), yet interestingly all identified species belong to Yungasensa or species that are known to cross-hybridize with Yungasensa species (Hawkes, 1990). This suggests that response to INF is conferred by an ancient gene in *Solanum*.

In the past, genetic analysis of the contribution of INF elicitin recognition to resistance has been hampered by the low levels of variation in *Nicotiana* in elicitin response and the sexual incompatibility between *Nicotiana* spp. and hosts of *P. infestans*, such as tomato and potato. We exploited natural variation in response to INF elicitins in the identified *Solanum* accessions to evaluate the relationship between INF recognition and late blight resistance. Interestingly, several INF-responsive *Solanum* plants were susceptible to *P. infestans*. Also, an *S. microdontum* × *S. tuberosum* (potato) population that segregates for INF response and partial resistance was generated but failed to identify a measurable contribution of INF response to resistance. These findings are in line with the recent report that INF1-producing isolates of *P. infestans* can infect *N. benthamiana* (Becktell *et al.*, 2006) and suggest that a strict correlation between INF response and resistance does not occur in *Nicotiana* and *Solanum*.

It is well established that INF1 and other elicitins induce hypersensitivity and several biochemical hallmarks of plant defence responses. In some interactions there is evidence for a role of elicitin perception in restricting pathogen colonization (Colas *et al.*, 2001; Kamoun *et al.*, 1998), suggesting that INFs act as specific elicitors. Why is it then that some INF-responding *Solanum* plants are highly susceptible to *P. infestans*? We propose several explanations. First, although the identified *Solanum* plants consistently respond to INF elictins they are about 100–1000-fold less sensitive than tobacco and often a patchy non-confluent necrosis is observed following infiltration with INF elicitins. Thus, one possibility is that the levels that INF1 and other elicitins reach *in planta* are not sufficient to trigger an effective response. Indeed, the expression of the *inf1* gene is down-regulated during the colonization of potato (Kamoun *et al.*, 1997). Future experiments need to evaluate the stability and levels of the various INF elicitins during *P. infestans* colonization of different plant species. Identifying more sensitive *Solanums*, or cloning and engineering the *inf*-responding gene under the control of a different promoter might reveal whether INF could act as an avirulence factor and confer resistance upon recognition. A second explanation is that *P. infestans* may secrete effectors that suppress the cell death induced by INF1 and therefore evade plant response to this elicitor. In line with this hypothesis, the *P. infestans* effector AVR3a was recently shown to suppress the HR triggered by INF1 in a coexpression assay in *N. benthamiana* (Bos *et al.*, 2006). Also, many bacterial plant pathogens are known to carry masked avirulence genes whose action is suppressed by epistatic effectors (Espinosa and Alfano, 2004).

Together with previous studies, our findings suggest that INF elicitins function as general elicitors in *Solanum*. The response to INF1 in susceptible *Solanum* plants is reminiscent of the response of tomato to the necrosis-inducing elicitors CRN1 and CRN2 of *P. infestans* (Torto *et al.*, 2003) or the response of soybean to PsojNIP of *P. sojae* (Qutob *et al.*, 2002). To date, the contribution of perception of such general elicitors to resistance is often unclear. Some authors have likened the perception of general elicitors to PAMP response and assume a quantitative effect of

this perception on innate immunity as occurs in animal cells (Nurnberger *et al.*, 2004). Other authors are more skeptical and argue that because plants remain susceptible, the contribution of general elicitor perception is negligible (Parker, 2003). These two views are combined in an arms race model that depicts PAMP-triggered immunity (PTI) as the most ancient active response triggered by invading plant pathogens (Chisholm *et al.*, 2005). Pathogens then evolved effectors to suppress PTI, and these effectors were in turn recognized by plant R proteins (Chisholm *et al.*, 2005). Elicitins fulfil a number of the criteria ascribed to PAMPs: (1) they do not occur in plants and thus can be viewed as non-self molecules; (2) as sterol scavengers, they fulfil an important function for the microbe; and (3) they are expressed during host interaction. Also, unlike AVR3a and other oomycete RXLR effectors, or the small cysteine-rich protein SCR74 (Armstrong *et al.*, 2005; Kamoun, 2006; Liu *et al.*, 2005), elicitins are structurally conserved and under purifying selection in *Phytophthora* (Jiang *et al.*, 2006). Perhaps, INF elicitins function as oomycete PAMPs (Nurnberger *et al.*, 2004) and are recognized by ancient genes in *Solanum*, but evolved to act as specific elicitors in some plant genera such as *Nicotiana*.

The molecular genetic basis of elicitin perception remains unknown. The observed segregation in the F1 population we examined suggests that a single dominant gene may confer response to INF1 in *S. microdontum*. Heritable INF response was also reported in radish (Keizer *et al.*, 1998) and *Brassica rapa* (Takemoto *et al.*, 2005). In *Brassica*, variation for visible elicitin response was due to the extent of cell death, microscopic cell death in 'non-responsive' plants vs. confluent necrosis in responsive plants, but in Arabidopsis no response could be identified (Takemoto *et al.*, 2005). It remains to be determined whether recognition of elicitins in *Solanum* acts as in *Brassica.* The availability of *Solanum* germplasm that varies in response to INF elicitins may facilitate the genetic dissection of elicitin response in plants and will ultimately allow molecular cloning of the genes that determine INF response.

The genus *Solanum* is highly rich in late blight resistance genes, yet only four *R* genes have been cloned to date: *R1* (Ballvora *et al.*, 2002) and *R3a* (Huang *et al.*, 2005a) from *S. demissum*, as well as *Rb/Rpi-blb1* (Song *et al.*, 2003; van der Vossen *et al.*, 2003) and *Rpi-blb2* (van der Vossen *et al.*, 2005) from *S. bulbocastanum*. The PVX agroinfection assays brings a new dimension to the search for late blight *R* genes in *Solanum* (Torto *et al.*, 2003). For example, the discovery that all known avirulence proteins of oomycetes, including *P. infestans* AVR3a, carry a conserved motif (RXLR) following a signal peptide enabled the use of sequence pattern search tools to discover additional candidate effectors of this family (Kamoun, 2006; Rehmany *et al.*, 2005). We have already exploited this finding and embarked upon large-scale screening of *Solanum* germplasm for response to *P. infestans* RXLR effectors. This approach complements traditional screenings of plant germplasm with pathogen isolates and provides a powerful new tool to identify novel *R* genes that target cloned pathogen effectors.

EXPERIMENTAL PROCEDURES

Plant material and cultivation

Various breeding clones and wild *Solanum* plants were used for the optimization of the binary PVX assay. Experiments were carried out with *S. tuberosum* breeding genotypes RH89-039-16, RH90-038-21, the differential *R* gene set (Mastenbroek), and wild *Solanum* accessions which were obtained from CGN, the Center of Genetic Resources, Wageningen, The Netherlands (http://www.cgn.wur.nl/uk/). Considering that each accession consists of genetically diverse seeds, we propagated single-seed individual plants clonally *in vitro*. Each plant is referred to as a clone and is labelled with the CGN accession number followed by the clone number (e.g. 17719-1). Seeds were surface-sterilized and sown *in vitro* at 25 °C. The collection of individual *Solanum* clones was maintained *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog, 1962) at 18 °C. For the PVX and infiltration assays, top shoots were cut and transferred to fresh MS medium supplemented with 30% sucrose at 25 °C. After allowing 1–2 weeks for root formation, plants were transferred to sterilized soil and grown in regulated greenhouse compartments on a 22 °C/18 °C day/night regime.

Binary PVX assays

Recombinant *A. tumefaciens* GV3101 strains carrying pGR106-INF2A, pGR106-INF2B, pGR106-CRN2 or the pGR106 empty vector (Jones *et al.*, 1999; Takken *et al.*, 2000; Torto *et al.*, 2003) were used in this study. Cultures were grown for 2 days at 28 °C on solid agar LB medium supplemented with antibiotics. Excess bacteria was inoculated by piercing the leaf at both sides of the mid-vein. Local and systemic symptoms were visually scored every 2–4 days. For mature plant inoculations, the leaf age was rotated for replicates of the various treatments.

Preparation of purified INF proteins

The INF1, INF2A and INF2B proteins were produced using a heterologous expression system in *Pichia pastoris* (Invitrogen, Carlsbad, CA). Fermentation was performed in a Bioflow 3000 bioreactor (New Brunswick Scientific) as described by Stratton *et al.* (1998) with modifications. Fusion proteins were purified by affinity chromatography under native conditions using a Ni^{2+}-NTA agarose column (Qiagen). The purified AVR2 protein from *C. fulvum* (Luderer *et al.*, 2002) was kindly provided by J. van 't Klooster. Protein infiltrations were performed in attached leaves of mature plants in the greenhouse, 5–7 weeks after transplanting to soil.

P. infestans strains and culture conditions

P. infestans isolates 90128 (race 1.3.4.7.8.11), IPO-0 (race 0) and PY23 (race 1.3.4.7) were used throughout this study. Isolate 90128 and IPO-0 are aggressive isolates which are routinely used in our studies, and PY23 is a transformant which is silenced for the production of INF1 (Kamoun *et al.*, 1998; van West *et al.*, 1999). The isolates were grown on rye sucrose agar supplemented with 2% sucrose (Caten and Jinks, 1968) at 15 °C. To isolate zoospores, sporulating mycelium was flooded with cold water, and the sporangiospore suspension was gently poured off in a beaker and incubated at 4 °C. After *c.* 1–2 h, the zoospores were released, and the inoculum was adjusted to a concentration of 50 000 spores/mL.

Resistance assessment

For resistance screening of the wild Solanum accessions a high-throughput inoculation assay *in vitro* was applied (Huang *et al.*, 2005b) to obtain qualitative resistance data. Quantitative data on resistance level were obtained by a routine detached leaf assay as described previously (Vleeshouwers *et al.*, 1999).

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