An Accurate In Vitro Assay for High-Throughput Disease Testing of *Phytophthora infestans* in Potato

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ABSTRACT

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An in vitro inoculation assay was developed as a routine disease testing method to study gene-for-gene interactions in the potato (*Solanum tuberosum*)–*Phytophthora infestans* pathosystem. The specificity and reliability of the new method was compared with the well-established detached-leaf assay. Four *P. infestans* isolates were tested for avirulence on a set of *R* gene differentials using tissue cultured plantlets and detached leaves. Both methods gave identical conclusions on avirulence profiles of all isolates. A population of 93 clones was phenotyped for segregation of two closely linked and functionally distinct genes—*R3a* and *R3b*—in the *R3* locus. Both methods resulted in phenotypic scorings that were in perfect agreement for all clones. Furthermore, the phenotyping of the population was fully consistent with the genotyping obtained from analysis of molecular markers that flank each gene. This new assay is quick, space-effective, and accurate and can be used for investigation of the qualitative interaction between potato and *P. infestans*.

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, remains the most devastating disease of potato (*Solanum tuberosum* L.) (4). Many resistance (*R*) genes from wild relatives have been introgressed into the cultivated potato via sexual or somatic hybridization (2,6,25). Despite the fact that some *R* genes have been overcome in monoculture in the field by virulent races of *P. infestans* (13), *R* genes are still sought by breeders with the hope that the combination of several *R* genes by pyramiding or polyculture strategies (14) could offer better protection against late blight. The interaction between potato and *P. infestans* follows the gene-for-gene model (2,3,21). The molecular isolation of *R* genes is a prerequisite to understand the biochemical mechanism underlying this interaction and to unveil their coevolutionary pattern that may have implications on the strategy of *R* gene deployment in dis-

ease management. Several major *R* genes derived from wild species of potato have been mapped molecularly (5). Recently, three *R* genes, namely *R1* (1), *R3a* (8), and *RB/Rpi-blb1* (15,22), have been cloned. Several labs plan to isolate more late blight *R* genes from potato using a map-based cloning strategy. To apply this strategy, a large segregating population must be reliably phenotyped to create a high-resolution genetic map. For this purpose, an accurate and high-throughput disease testing method is needed.

Several methods have been described to evaluate foliar late blight resistance, including field tests, whole-plant greenhouse assays (18), and laboratory tests on detached leaves (10,24), leaflets (11,20), or leaf disks (7). The detached leaf assay is considered a reliable method for quantification of both major- and minor-gene resistances and is extensively used in laboratory studies (24). However, to monitor only major genes, such extensive quantitative assays are not necessary, and therefore, a quicker test should be feasible. Plants for detached leaf assay have to be grown in a greenhouse 6 to 8 weeks prior to inoculation. In addition, the response of potato leaves from greenhouse-grown plants to *P. infestans* can be affected by other diseases that may occur in the greenhouse. Especially in studies with high-throughput genomics research, a faster testing assay is desirable.

Potato is very amenable to tissue culture. In an in vitro environment, humidity and temperature can be manipulated to favor disease development of *P. infestans* (19). To date, no in vitro experiment on intact plantlets has been reported to study the specificity involved in the gene-for-gene interaction of potato and *P. infestans*. In this study, we describe the development of an in vitro inoculation assay to study the interaction of *R* genes from potato and *Avr* genes from *P. infestans*. Using the detached-leaf assay as a control, we assessed the specificity and reliability of in vitro inoculation in two experimental designs: (i) avirulence profiling of several *P. infestans* isolates using a set of *R* gene differentials; and (ii) phenotyping of a population that segregates for the *R3* locus and confirmation by molecular marker analysis. The results from both experiments demonstrate that in vitro inoculation could serve as an efficient and reliable alternative for the detached-leaf assay when major resistance genes and their cognate avirulence genes are concerned.

MATERIALS AND METHODS

Plant material. Plant material used in this study is listed in Table 1. F1 seeds from SH83-92-488 (SH) × RH89-039-16 (RH) were sterilized with 70% alcohol (2 min) and 1.5% sodium hypochlorite (15 min), washed with sterilized water (3 × 10 min), and sown in vitro on Murashige and Skoog (MS) medium supplemented with 30 g of sucrose per liter (MS30). The SH × RH population segregates at the *R3* locus, which consists of two functionally distinct genes, *R3a* and *R3b* (9). All plant material was maintained on MS30 at 24°C and 16 h light/8 h dark. Shoots or single node cuttings were transferred to fresh MS30 medium every 3 to 4 months for maintenance.

The preparation of plant material for the detached leaf assay was previously described (24). Briefly, for propagation, shoots or single nodes were cut and transferred to fresh MS30 medium. After 1 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 plants were then grown in the green-

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house. Leaves from plants 6 to 8 weeks old were cut for inoculation.

To prepare plantlets for in vitro inoculation, fresh shoots or single nodes were cut and transplanted to glass jars (diameter 10 cm) or plastic jars (diameter 15 cm) with MS30. Each jar contained five cuttings. A 2- to 3-cm space was left between the cuttings and the inner wall of the jar. In vitro plantlets were grown at 24°C and 16 h light/8 h dark. Plantlets with three to four fully developed leaves were used for in vitro inoculation. Depending on the potato genotype, it took 2 to 4 weeks from trans-

planting to inoculation. Generally, it took about 4 to 5 weeks for in vitro sown seedlings to develop enough leaf material for inoculation.

***P. infestans* isolates, maintenance, and inoculum preparation.** *P. infestans* isolates GER8601 (race 1), 99018 (race 1.4), 91001 (race 7), 89148-27 (race 3a.3b.7), 89148-09 (race 0), H30P04 (race 3a.7), and IPO-0 (race 3b) were used in the study. The avirulence profiles of the latter three isolates were determined previously (9). The isolate IPO-0 was kindly provided by W. Flier, Plant Research International,

the Netherlands; the other isolates were kindly provided by F. Govers, Laboratory of Phytopathology, Wageningen University, the Netherlands. The maintenance and preparation of inoculum was performed according to previous protocols (24).

Inoculation and resistance scoring. For the detached leaf assay, the third to fifth fully developed leaves (counted from the top) were cut from greenhouse-grown plants and placed in water-saturated florists foam (Oasis, Grünstadt, Germany) in a tray. Five leaflets per compound leaf were inoculated (one spot per leaflet) by pipetting 10- μ l droplets of a zoospore suspension of 5×10^4 spores per ml on the abaxial side (24). The trays were then covered with transparent lids (covered trays), transferred into a climate chamber, and incubated at a 16-h/8-h day/night photoperiod at 16°C.

For the in vitro assay, the three largest leaves of each in vitro plantlet were inoculated (1 spot per leaf) by pipetting 10- μ l droplets of a zoospore suspension of 2.5×10^4 spores per ml on the adaxial side. Inoculum preparation and inoculation were performed in sterilized conditions. Leaves touching the inner wall or the lid of the jars or the medium were excluded because we found these leaves to be more susceptible than others of the same plants. The jars with inoculated in vitro plantlets were incubated in the same climate chamber as the trays with inoculated detached leaves.

Inoculated detached leaves were scored at 6 to 8 days postinoculation (dpi). The observation unit was compound leaf (with five leaflets). Depending on the level of susceptibility of the potato genotype and the aggressiveness of the *P. infestans* isolate, compatible interactions were scored as interaction class 1 (spreading lesion with massive sporulation) or class 2 (spreading lesion with no or little sporulation). Incompatible interactions were scored as class 5 (no symptom or localized HR-like necrosis) or class 4 (trailing HR necrosis). Rarely, intermediate phenotypes (class 3) between compatible and incompatible ones were observed, such as sporulation on two to three leaflets per compound leaf and localized HR on the other leaflets. Generally, two compound leaves were examined for each isolate-potato genotype combination.

The scoring of in vitro inoculation was similar to that of detached-leaf assay except that the unit of observation was jar with five plantlets instead of compound leaf with five leaflets. For each interaction, two jars with 10 plantlets were scored on the same 1 to 5 scale as the detached leaf assay.

In the duplicated experiments, the average of the interaction classes (on the 1 to 5 scale) scored from four observations resulted in a quantitative disease index (DI) for each genotype-isolate combination. The DIs were converted into qualitative

Table 1. Plant material used in this study and their known *R* genes to *Phytophthora infestans*

Clone	Known <i>R</i> gene	Identity
MaR0 ^a	None	Potato cv. Bintje
MaR1	<i>R1</i>	CEBECO 43154-5
MaR2	<i>R2</i>	CEBECO 44158-4
MaR3	<i>R3a</i> , <i>R3b</i> ^b	CEBECO 4642-1
MaR4	<i>R4</i>	CEBECO 4431-5
MaR5	<i>R5</i>	Black 3053-18
MaR6	<i>R6</i>	Black XD2-21
MaR7	<i>R7</i>	Black 2182 ef (7)
MaR8	<i>R8</i>	Black 2424 a (5)
MaR9	<i>R9</i> or <i>R1R2R3R9</i> ^c	Black 2573 (2)
MaR10	<i>R10</i>	Black 3681 ad (1)
MaR11	<i>R11</i>	Black 5008 ab (6)
RH89-039-16 (RH)	None	Paternal clone of the RH $\times$ SH <i>R3</i> mapping population
SH83-92-488 (SH)	<i>R3a</i> , <i>R3b</i>	Maternal clone of the RH $\times$ SH <i>R3</i> mapping population

^a The Dutch differential set for profiling virulence of *P. infestans* isolates is designated as MaR0-R11. MaR1-MaR4 were developed by C. Mastenbroek, the other *R* differentials are identical to the Scottish differential set, developed by Black and colleagues (2,12).

^b Huang et al. (9).

^c Malcolmson and Black (12).

Fig. 1. Compatible and incompatible interaction observed on in vitro plantlets. The in vitro plantlets of MaR0 and MaR7 inoculated with *Phytophthora infestans* isolates 89148-9 (race 0) and H30P04 (race 3a.7) at 6 days postinoculation. A race-specific resistance response occurred on MaR7 leaves upon inoculation with isolate 89148-9 (HR), which was scored as interaction class 5. A small plug of mycelium (M) is growing on the surface of the medium. Extensive sporulation (S, interaction class 1) or massive lesion (L, interaction class 2) was noted on the three compatible interactions.

scores: susceptible (S, $DI \leq 2.0$), questionable (Q, $3.0 < DI < 4.0$), and resistant (R, $DI \geq 4.0$).

Paired sample *t* tests were conducted to examine whether there was a difference between disease indexes (DIs) obtained from the detached leaf assay and the in vitro assay.

Molecular marker analysis. DNA was extracted from in vitro plantlets using a high-throughput method (9). Two cleaved amplified polymorphic sequence (CAPS) markers, cLET5E4 and GP185, which define a 0.25 cM interval that spans *R3a*, and two amplified fragment length polymorphism (AFLP) markers, PCAMATA_4 and EATCMAGC_15, which define a 0.25 cM interval spanning *R3b*, were used for genotyping of the *R3* locus. Polymerase chain reaction (PCR) conditions were described previously (9).

RESULTS

Determination of inoculum concentration in vitro. Inoculum concentration is an important factor that can influence disease development (16). An optimal inoculum concentration for in vitro conditions had to be determined. In a concentration serial experiment with *P. infestans* isolate IPO-0 in vitro, we inoculated zoospore suspensions of 0.5, 1, 2.5, 5, and 10×10^4 spores per ml on the resistant SH83-92-488 (SH, Table 1) and susceptible Bintje and RH89-039-16 (RH, Table 1). The inoculum concentration of 0.5×10^4 spores per ml resulted in suboptimal disease development for Bintje and RH. At a concentration of 10×10^4 spores per ml, intensive sporulation was observed on inoculated leaves of SH due to saprophytic growth on the HR-caused necrotic tissue, and lesions spread to noninoculated leaves. Occasionally, spores dropped and mycelium started growing on the medium (Fig. 1). The intermediate concentration gave a clear phe-

notypic separation between resistant and susceptible genotypes. We thereafter chose 2.5×10^4 spores per ml as the standard inoculum concentration for in vitro inoculation, half the amount as for detached leaves.

Observation of compatible and incompatible interaction on in vitro plantlets. In the compatible interactions (interaction class 1 and 2), the inoculated leaves first bent to the abaxial side, and lesions could be observed on the adaxial side of the inoculated leaves at 3 to 5 dpi. The lesions gradually spread across the entire inoculated leaves, to their petioles, to stems, to uninoculated leaves, and the whole plantlets. The degradation of stems often caused the upper part of the plantlets to collapse, and the spreading lesions eventually caused the death of the inoculated plantlets within 2 weeks (Fig. 1). The phenotypes of the incompatible interactions (interaction class 5 and 4) often appeared at 2 to 4 dpi, displayed as localized HR necrosis (Fig. 1) or trailing HR necrosis but limited to the inoculated leaf.

A typical differential interaction between *P. infestans* and potato was observed on in vitro plantlets (Fig. 1). The combination of *R7* and *Avr7* displayed a resistance phenotype with localized HR necrosis (interaction class 5), while the combinations of *R0/Avr7*, *R0/avr7*, and *R7/avr7* showed susceptible phenotypes, either as massive sporulation (interaction class 1) or as a spreading lesion with little sporulation (interaction class 2, Fig. 1). This result was consistent with that obtained by using the detached-leaf assay with the same potato clones and *P. infestans* isolates (data not shown).

Avirulence profiling. The avirulence profiles of four *P. infestans* isolates, GER8601, 99018, 91001, and 89148-27, were determined on both detached leaves and in vitro plantlets of the differential set

(Table 2). For qualitative resistance scoring, the in vitro and the detached leaf results were in full agreement. The data were also analyzed quantitatively. Identical scores were obtained for each isolate with the two methods for most differentials. Minor quantitative differences in DIs were found only in a few combinations. Of these, five gave a higher DI on detached leaves than on in vitro plantlets and four were the opposite, suggesting no directional bias between the methods. Statistical analysis on DIs revealed that there was no significant difference between the two methods ($P = 0.7$ for two-tailed *t* test).

Phenotyping and genotyping of the *R3* locus on a segregating population. The detached leaves and in vitro plantlets of SH and RH, the parental line of the *R3* segregating population, were inoculated more than 10 times with two diagnostic *P. infestans* isolates, IPO-0 (3b) and H30P04 (3a.7). Consistently, SH displayed a typical localized HR reaction that was visible at 3 dpi, while RH displayed intensive sporulation at 5 dpi, confirming our previous observation that SH carries both *R3a* and *R3b* and RH is susceptible (9). This clearly demonstrated that the *R3a* and *R3b* genes in SH are active in both detached leaves and in vitro plantlets.

We then investigated the reliability of the in vitro assay by phenotyping the *R3a* and *R3b* resistance using an SH $\times$ RH population of 93 plants. In vitro plantlets of the entire population and a subset of 33 plants were inoculated with *P. infestans* isolate IPO-0 and H30P04, respectively. For comparison, the entire population was phenotyped for both isolates with the detached leaf assay. To simplify the scoring, only qualitative scoring was done on the population using the same criteria as with avirulence profiling. In the population, 59 individuals were susceptible and 33 were resistant to both isolates. One

Table 2. Avirulence profiling of *Phytophthora infestans* isolates GER8601, 99018, 91001, and 89148-27 with both in vitro (I) and detached leaf (D) assays

Differential	GER8601		99018		91001		89148-27 ^a	
	I	D	I	D	I	D	I	D
MaR0	S ^b (1 ^c)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)
MaR1	S (1)	S (1)	S (1)	S (1)	R (5)	R (5)	R (5)	R (5)
MaR2	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR3	R (5)	R (5)	R (5)	R (4)	R (5)	R (5)	S (1)	S (1)
MaR4	R (5)	R (5)	S (1)	S (1)	R (5)	R (5)	R (4.5)	R (5)
MaR5	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR6	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR7	R (5)	R (5)	R (5)	R (4)	S (2)	S (1)	S (1)	S (1)
MaR8	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR9	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR10	R (5)	R (5)	R (5)	R (4)	R (4)	R (5)	R (4.5)	R (5)
MaR11	R (4.5)	R (5)	nd ^d	R (4)	R (5)	R (5)	R (4)	R (5)
Conclusion	Race 1	Race 1	Race 1.4	Race 1.4	Race 7	Race 7	Race 3a.3b.7	Race 3a.3b.7

^a Besides MaR3 and MaR7, SH (*R3a*, *R3b*) was also susceptible to the isolate 89148-27 in both detached leaf assay and in vitro assay (data not shown); therefore 89148-27 was defined as race 3a.3b.7.

^b Phenotypes were determined based on the disease indexes (DIs): susceptible (S, $DI \leq 2.0$), questionable (Q, $3.0 < DI < 4.0$), and resistant (R, $DI \geq 4.0$).

^c Average of the interaction classes (on the 1 to 5 scale) scored from the four observations (four compound leaves for the detached leaf assay and four jars with five in vitro plantlets in each jar for the in vitro assay) for each genotype-isolate combination.

^d nd = no data.

plant (Table 3, progeny type 2) displayed a differential response both on detached leaves and in vitro plantlets. In all 126 observations, none gave a difference between the two methods, indicating that in vitro inoculation is reliable in phenotyping a large number of individuals (Table 3).

To confirm the result of phenotyping, we genotyped the whole population with the molecular markers cLET5E4 and GP185 flanking *R3a*, and PCAMATA_4 and EATCMAGC_15 flanking *R3b* (9). No recombination was found between cLET5E4 and GP185 or between PCAMATA_4 and EATCMAGC_15 in the population, which indicated cLET5E4 and GP185 were fully diagnostic to the presence of *R3a*, as were PCAMATA_4 and EATCMAGC_15 to *R3b*. All of the 59 plants that were susceptible to the two isolates lacked the resistance alleles of the

four markers, while these resistance alleles were present in all 33 plants that were resistant to the two isolates (Fig. 2). Marker analysis also confirmed the differential response of the single recombinant plant as being associated with the absence of the resistance alleles on the *R3a* flanking markers and the presence of the resistance alleles on the *R3b* flanking markers (Table 3, Fig. 2). These data clearly demonstrated that the in vitro assay could reliably phenotype a huge segregating population and accurately display recognition specificities of potato *R* genes to the interacting pathogen.

DISCUSSION

In this study, we developed an in vitro assay as a routine method for monitoring major *R* genes in potato and their corresponding *Avr* genes in *P. infestans*. We

observed perfect correspondence between the new method and the control detached leaf assay and concluded that the in vitro assay can be a new tool to study the gene-for-gene interaction between potato and *P. infestans*.

Compared with the detached-leaf assay, in vitro inoculation has several advantages. First, healthy and uniform in vitro plantlets are used, minimizing the influence of physiological condition of leaves and interference of other pathogens in assessment of the resistance of the genotypes tested. Second, experiments can be planned more flexibly because of the immediate availability of plant materials and minimal space needed for testing. Third, for high-throughput phenotyping, seeds of a segregation population can be sterilized and sown directly in vitro. However, the in vitro assay is not suitable for scoring quantitative resistance where the parameters such as lesion growth rate and infection efficiency are used (24).

Individual *R* genes respond with their own specific effectiveness (23). Strong *R* genes, such as *R1* (17), always show full resistance to avirulent *P. infestans* isolates, whereas weak *R* genes, such as *R10* and *R11*, often confer less complete resistance to avirulent isolates (17,21,23). The level of effectiveness of individual *R* genes in the differentials is related to their behavior toward the four isolates used in the study (Table 2). The weak *R* genes were more difficult to phenotype than the strong ones both on detached leaves and on in vitro plantlets. This might account for the slight quantitative difference in the replicated experiments between the two methods on phenotyping *R10* and *R11* in this study.

We concluded that the in vitro assay is a reliable alternative to the detached leaf assay to phenotype a large population segregating in the *R3* locus (Table 3). It was later applied to high-throughput phenotyping of the enlarged *R3* segregating population (~3,000 in vitro sown progeny) and facilitated the accurate delimitation of the *R3a* gene into a 0.07-cM interval (8). The in vitro assay has been used in our lab to phenotype the primary transformants of the *R3a* gene candidates (8), which resulted in the identification of the *R3a* gene at least 2 months earlier than with the detached leaf assay. It has also been applied to screen a wide spectrum of *Solanum* spp. germ plasm for new resistance genes against *P. infestans* (V. G. A. A. Vleeshouwers, data not shown). It is a versatile new tool for potato and *P. infestans* researchers.

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Table 3. Reaction of the SH × RH F1 population segregating at the *R3* locus to inoculation with *Phytophthora infestans* isolates IPO-0 and H30P04

Plant	<i>P. infestans</i> isolate				Number of individuals
	IPO-0 (3b)		H30P04 (3a.7)		
	D ^a	I ^a	D	I	
RH ^b	S	S	S	S	
SH ^b	R	R	R	R	
PT1 ^c	S	S	S	S	3
PT1 ^c	S	S	S	nd ^d	56
PT2 ^e	S	S	R	R	1
PT3 ^f	R	R	R	R	29
PT3 ^f	R	R	R	nd	4
Total					93

^a Inoculation with the detached-leaf assay (D) or the in vitro assay (I).

^b Parental clones RH and SH were included in the analysis.

^c Progeny that lack the *R3a* and *R3b* genes and are susceptible to both isolates.

^d nd = not done. Only 33 individuals were inoculated with *P. infestans* isolate H30P04.

^e A recombinant between the *R3a* and *R3b* genes, which carries the *R3b* gene and is susceptible to the isolate IPO-0 but resistant to H30P04.

^f Progeny that carry both genes and are resistant to both isolates.

Fig. 2. Genotyping of the three progeny types (PT, ref. to Table 3) in the SH × RH *R3* mapping population and the parental clones RH and SH with four molecular markers. cLET5E4 and GP185 are flanking *R3a*, and PCAMATA_4 and EATCMAGC_15 are flanking *R3b*. The markers are indicated at the right with an arrow pointed to the polymorphic band. The phenotypes of plants after inoculation with *Phytophthora infestans* isolates IPO (race 3b) and H30P04 (race 3a.7) are indicated.

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