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INVESTIGATING DURABILITY OF RESISTANCE TO *PHYTOPHTHORA INFESTANS* IN TRANSGENIC AND CISGENIC POTATO PLANTS

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Abstract

Potato (Solanum tuberosum L.) is leading commercial crop its production is largely constrained by devastating disease late blight. Stacking of two or more resistance genes into single plant proved a viable strategy for broad spectrum durable resistance. Transgenic and cisgenic potato plants were developed by team of potato breeders in Wageningen University under project Durable resistance to Phytophthora that contain multiple resistance (R) genes after successful stacking. The study was done during 2011 spring season in greenhouse, growth chamber and laboratory condition to verify resistance in transgenic and cisgenic plants with stacked R genes. Detached leaf assay (DLA) was performed to verify resistance. The cisgenic construct A26 (Rpi-blb3: Rpi-sto1) and transgenic constructs A19 (Rpi-vnt1.1: Rpi-chc1-long), A29 (Rpi-edn2) and A41 (Rpi-chc1-short) were tested against different isolates breaking one of both R genes. Screening of the A29 transformants with two isolates revealed that the Rpi-edn2 candidate gene was not active. Plants that were slightly resistant did not show specific recognition of selected Pi isolates. The resistance gene Rpi-chc1 (short promoter) in transgenic A41 plants was specific to isolate 90128 but not to IPO-C. We observed that Rpi-chc1 (long promoter) had transformation efficiency of 50% in A19 plants. Cisgenic A26 (Rpi-blb3: Rpi-sto1) plants evaluated with four Pi isolates (USA618, NL08797, 90128 and IPO-C) exhibited strong activity of both R genes in A26-1679, A26-1554, A26-1389, A26-1735 and A26-1065. The study identified few resistant transformants containing single and stacked R genes were active. The findings will be helpful in selecting the genotypes with broad spectrum resistance to Phytophthora in future breeding programs.

Keywords: Potato, Phytophthora infestans, Cisgenic, Transgenic, Durable Resistance. R genes, Avr genes

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important member of the Solanaceae family. Most potato cultivars are auto-tetraploid (2n=4x=48) and highly heterozygous ('Genome sequence and analysis of the tuber crop potato,' 2011). It ranks fourth position among major food crops worldwide after cereals (Foster *et al.*, 2009; Lokossou *et al.*, 2010; Verzaux, 2010) and achieved second prominent place after wheat in Europe (Haverkort *et al.*, 2008). It is a major staple food, has high yield potential and considered as highly nutritious food. The worldwide potato production is increasing trend however; it is decreasing in developed countries and increasing in developing countries. The production of potato was 325.30 million ton with productivity of 16.8 t/ha (FAO, 2008). The global potato production is an increasing trend especially because of increasing demand in developing countries in Asia, Africa and South America (Verzaux, 2010). Late blight is a devastating disease caused by an oomycete *Phytophthora infestans* (Mont.) de Bary, a major cause of an Irish famine (1845-1849), is a major biotic constraint to successful crop production (Park *et al.*, 2005; Foster *et al.*, 2009; Verzaux, 2010). The oomycete is a hemi-biotrophic and can establish intimate association at an early stage to initiate infection structures and colonizes by forming haustoria. Extensive efforts have been made in the management of this deadly pathogen. The only way to overcome significant crop damage is through application of fungicide timely. Repeated and haphazard use of the chemicals leads to evolution of new pathogen lineages with resistance to fungicides (Foster *et al.*, 2009; Park *et al.*, 2009). Presently development of new cultivars with high levels of broad spectrum resistance becomes a promising alternative to fungicides (Park *et al.*, 2005; van der Vossen *et al.*, 2005). The gene for gene hypothesis was first time reported in Melampsora lini (Flor, 1971), stated that for any particular resistance gene in host, there is a corresponding gene in pathogen for avirulence that has no pathogenicity. The resistance and avirulence genes are dominant. The plants become resistant only when it

possesses an *R* gene and pathogen carries *Avr* gene. The reaction is called as incompatible in which *R* gene recognizes *Avr* gene from the pathogen then plant becomes resistant and rest of other combinations are called compatible resulting in disease (Table 1). The *R* and *Avr* genes result from an evolutionary arms race between host and pathogen, in which the host tries to avoid the infection, while the pathogen tries to counteract the host defences to cause disease (Maor and Shirasu, 2005; van der Does and Rep, 2007).

Table 1. Gene for gene relationship as proposed by (Flor, 1971).

Pathogen genotypes		*Host genotypes*	
		RR or Rr (Resistance)	rr (Susceptible)
	AA or Aa (A-virulence)	Resistant	Disease
	aa (Virulence)	Disease	Disease

Two types of resistance explained in potato against late blight, mainly field and *R* gene mediated resistance. Field resistance is basically a quantitative resulting into partial resistance (Umaerus and Umaerus, 1994). The field resistance found to be more durable than *R* genes resistance (Turkensteen, 1993). Breeding for durable resistance is achieved through stacking of multiple broad spectrum resistance (*R*) genes in a single genotype (Douglas and Halpin, 2009). The wild potato cultivars possess several *R* loci that are active against late blight and valuable genetic resources for traditional breeding (Pankin *et al.*, 2010). The resistance genes derived from the different sources induce a hypersensitive reaction (HR) after interaction between *R* protein and corresponding avirulence (*Avr*) effectors. Introgression of single *R* genes turned out not to be durable. Currently, *R* gene stacking is hypothesised to enhance durability. Unfortunately, stacking of *R* genes through conventional breeding is time consuming and is hampered by linkage drag of undesired traits. Gene stacking by transformation overcomes the limitation of linkage drag during conventional breeding with precise incorporation of several *R* genes into one genotype (Zhu *et al.*, 2011). Three *R* genes were simultaneously introduced to obtain resistance to late blight (Zhu *et al.*, 2011). Initially the breeding programs emphasized on finding dominant resistant genes to get full resistance and become very efficient and cost effective against late blight disease. These dominant genes were identified in *Solanum demissum* and crossed to cultivated potato for introgression genes (Golas *et al.*, 2010). The *R* genes were monogenic and race specific and were broken down rapidly by *Pi*. It necessitates the investigation of new *R* genes from other wild species. In addition to *S. demissum* many wild species of genus *Solanum* are considered as valuable source of resistance. However, the introgression process is quite complicated and needs interspecific bridge crosses (Golas *et al.*, 2010). Breeding activities in the nineties of the previous century mainly focussed on improving the horizontal race non-specific resistance controlled by quantitative traits with many genes (Leonards-Schippers *et al.*, 1994). Besides hexaploid *S. demissum*, diploid and self–incompatible potato species like *Solanum bulbocastanum* were identified as promising sources of resistance against late blight (Park *et al.*, 2005). Recently many *R* genes obtained from different *Rpi* gene clusters and wild species have been cloned. Those are *R1*, *R2* and *R3a* originating from *Solanum demissum* (Lokossou *et al.*, 2009), *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (van der Vossen *et al.*, 2005; Lokossou *et al.*, 2009) and *Rpi-vnt1.1* from *S. venturii* (Foster *et al.*, 2009; Pel *et al.*, 2009). In addition to introgression breeding for *R* genes, some efforts were made in introgression of QTLs (Foolad *et al.*, 2008) that provides horizontal or partial resistance to many races of pathogen ((Bradshaw *et al.*, 2004). So in order to get the durable resistance against late blight, multiple stacking of broad spectrum *Rpi* genes is considered important. This is also naturally occurring in some resistant plants (Lokossou *et al.*, 2010; Verzaux, 2010). Studies revealed that the stacking of broad spectrum *Rpi* genes provide durable resistance of potato in the field (Zhu *et al.*, 2011). Currently, potato breeding efforts are concentrated on durable resistance through marker free transformation techniques that involve cisgenesis approach. Both methods employ *Agrobacterium*-mediated transformation.

A cisgene is defined as 'existing natural gene from the crop plant itself or from crossable species. It possesses the gene's own native promoter and terminator. The gene belongs to traditional breeder's gene pool and is the already existing result of natural evolution.' (Jacobsen and Schouten, 2009).

Transgene is defined as 'A synthetic gene with some or all regulatory sequences and coding sequences from donors other than crossable plants, including micro-organisms and animals. The gene belongs to new gene pool for plant breeding.' (Jacobsen and Schouten, 2009). The cisgene is possible to be transferred by other traditional breeding techniques. Hence, there is less risk of fitness and effect on target organisms because of application of existing plant genes is the basic difference between cisgenesis and transgenesis (Schouten *et al.*, 2006). Furthermore, cisgenesis rely on three fundamental premises namely cisgenes, marker free transformation and backbone free genes.

The cisgenes are very closely related to traditionally bred plant in all respects. The cisgenes have also avoided major drawbacks of conventional breeding where linkage drag offered a major constraint for efficient breeding. The cisgenesis only uses interesting genes isolated from the donor and introduced into recipient in single event that eliminates

linkage drag and accelerates the breeding program (Schouten *et al.*, 2006).

Therefore cisgenesis is a very promising technique for stacking the durable resistance into cultivated potato against late blight pathogen. It needs to transfer several genes from the resistant parents that are sources of *R* genes. The most commonly used wild relatives are *Solanum demissum*, *S. bulbocastanum* and *S. venturii*. The essential requirement for the cisgenesis is isolation and characterization of desired genes from the related crossable species (Schouten *et al.*, 2006).

Development of genotypes with two or more resistance genes is more economical from the viewpoint of resistance breeding. In multiple stacking of *R* genes into one genotype, it is not possible to get fully resistance in transgenic plant, however could be possible in cisgenics to some extent (Zhu *et al.*, 2011). In order to identify the biological activity of *R* genes into the transgenic and cisgenic plants, they should be tested against many isolates, specific effectors and *Avr* genes to confirm which plants actively expressed *R* genes and which plants didn't. The study was focused on validation of the transgenic and cisgenic plants for resistance against *Phytophthora infestans*, a major cause of late blight disease in potato, using complex races. The main aim of this project was to screen the potato genotypes that confer durable resistance against various isolates of *Phytophthora infestans*. The research objectives were to test transgenic plants A41 (*Rpi-chc1*-short), A29 (*Rpi-edn2*) and A19 (*Rpi-vnt1.1: Rpi-chc1*-long) and cisgenic plants A26 (*Rpi-blb3: Rpi-sto1*) with isolates carrying corresponding avirulence genes.

MATERIALS AND METHODS

The study was conducted in Wageningen University, the Netherlands during 2011 spring season under laboratory and greenhouse condition. In this study, transgenic plants were A41 (*Rpi-chc1*-short), A29 (*Rpi-edn2*) and A19 (*Rpi-vnt1.1: Rpi-chc1*-long) and A26 (*Rpi-blb3: Rpi-sto1*) were cisgenic plants. These plants possessed different resistance *R* genes in stacks. For example, *Rpi-blb* gene was derived from *S. bulbocastanum* accessions, *Rpi-chc1* from *S. chacoense*, *Rpi-vnt1.1* from *S. venturii*, *Rpi-edn2* from *S. edinense*, *Rpi-sto1* from *S. stoloniferum*. The broadened resistance genes were monitored with different pairs of isolates breaking *R* genes for evaluating resistance spectrum. The transformants were tested in detached leaf assay (DLA). The more information on plant materials and isolates spectrum is shown in Table 2.

DLA was carried out using a differential set of isolates of *Pi*. The details of DLA on transgenic and cisgenic plants and isolates used in those different constructs for the experiments are presented in Table 2.

The transformants were screened by means of DLA. DLA was carried out in two young and uniform sized leaves detached from five weeks old plants. The leaves were fixed upside down to the foam in a tray with wetted filter paper. The isolates were inoculated on the abaxial side of leaf. The number of spots depended on size of leaves. Ten microliter (10µl) droplets of inoculum with zoospore suspension (50000 spores/ml) of respective isolate were applied in the leaves. The two isolates were inoculated in the either side of the mid-vein. The trays covered with plastic and kept in 15^0C. After six days, scoring of inoculum spots was carried out.

Table 2. DLA experiments on different constructs with different isolates

S.N	*Transformants tested*	*Total Plants*	*Isolate spectrum*	*Control plants*
1.	A41 (*Rpi-chc1*-short)	42	IPO-C (*Avr-chc1*) 90128 (*Avr-chc1*)	Desiree
2.	A29 (*Rpi-edn2*)	41	IPO-C (*Avr-end2*) 90128 (*avr-edn2*)	Desiree
3.	A26 (*Rpi-blb3: Rpi-sto1*)	14	90128 (*Avr-blb3, Avr-sto1*) IPO-C (*avr-blb3, Avr-sto1*)	A10-43, A14-16, A03-142, A09-277, Desiree
4.	A26 (*Rpi-blb3: Rpi-sto1*)	14	USA618(*avr-blb3, Avr-sto1*) NL08797(*Avr-blb3,avr-sto1*)	A10-43, A14-16, A03-142, A09-277, Desiree
5.	A41-52 (*Rpi-chc*-short) A41-76 (*Rpi-chc*-short) A17-27 (*Rpi-chc*-long) 94-2034-01(*Rpi-ber*) CHC-544-5, Desiree CHC- 543-5, G254	8	91011, 99189, IPO-C, NL-7379, Katshaar, Dinteloord, NL07245, H30P04, 90128, IPO-O, 99183, 3928-A, 99177, USA618, 7135, UK98014	Desiree
6.	A19 (*Rpi-vnt1.1: Rpi-chc1)*	40	EC-1(*avr-vnt1.1, Avr-chc1*) NL07245(*Avr-vnt1.1,avr-chc1*)	A17-27, A13-13, Desiree
7.	A26 (*Rpi-blb3: Rpi-sto1*)	23	USA618 (*avr-blb3, Avr-sto1*) NL08797 (*Avr-blb3, avr-sto1*)	Desiree

Criteria for scoring of DLA was followed as per standard rule: R9; complete resistance (score 1), R8; hypersensitive response, HR (score 2), R7; bigger than HR (score 3), R6; spots much bigger than R7 (score 4), V5; similar to R6 (score 5), V6; water soaked lesion without spores (score 6), V7; spores in lower side of inoculation (score 7) and V8; spores on both sides of leaves (score 8). Based on the final score obtained for transformants, the scores were transformed other way around and indicated as higher score represented more resistance and lower score for more susceptibility. Plants were grouped as resistant or susceptible by comparison to resistant and susceptible controls. In some cases when there was no resistant control included in the experiment, we arbitrarily fixed the criteria to define the resistant or susceptible level as: plants scoring above 7 were regarded as highly resistant, in between 4 and 7 were grouped as resistant and those scored below 4 were defined as susceptible.

The *Pi* isolates were multiplied for the inoculation during detached leaf assay. Multiplication of the isolate was done at least two weeks before in petridishes with growing medium. After two weeks of growth at 15^0C, before inoculation, number of zoospores and sporangia were put into haemocytometer and viewed under microscope to count spores in three big squares. Based on the average number of spores, dilution was carried out. It should be about ten spores per big square to make 50000 spore densities.

It included screening of eight different transformants: A41-52 and A41-76 (*Rpi-chc1*-short), A17-27 (*Rpi-chc1*- long), 94-2034-01(*Rpi-ber*), CHC-544-5, Desiree, CHC-543-5, G254. Sixteen different isolates were used to identify the virulence spectrum on the single genotypes. The spectrum mainly consist the *Rpi-chc1* and *Rpi-ber* constructs. The resistance spectrum was compared for *chc1*-short and long promoters as well as with donor plants. The list of isolates used for this testing was presented in the **Table 2**. The data were also obtained from previous studies for comparison of isolate spectrum analysis of two genotypes 94-2034-01(*Rpi-ber*) and CHC-543-5 (*Rpi-chc1*- long) to same isolates used in this study.

RESULTS

Functional Analysis on Transgenic Plants:

DLA on A29 (Rpi-edn2) plants

Functional analysis of *R* genes in transgenic A29 (*Rpi-edn2)* construct was done with detached leaf assay against two isolates (90128 and IPO-C) of *Phytophthora infestans.* Forty one transformants were tested, Desiree was used as susceptible check but resistant check was not included. The resistance level of plants to isolates 90128 and IPO-C was presented in Figure 1, Table 3. The transformant A29-54 was found highly resistant to isolate 90128 but only resistant to IPO-C. We also observed that transformants A29-15 and A29-23 had higher degree of resistance to isolate 90128 but showed susceptibility to IPO-C. In addition to this, A29-42 and A29-45 showed almost similar resistance level to both isolates (Figure 1). Similarly, two transformants A29-5 and A29-25 were highly susceptible to both isolates 90128 and IPO-C.

Many transformants showed low levels of resistance to both isolates as compared to susceptible check variety Desiree (Figure 1). Resistance to 90128 was unexpected since previously it was shown that 90128 was virulent on plants containing the *Rpi-edn2* gene (Table 3). Since high levels of resistance to IPO-C was not found frequently, and if it was found, it was coinciding with resistance to 90128, it ensured that no plants expressed *Rpi-edn2* specific resistance.

Table 3. Degree of resistance of A29 (*Rpi-edn2*) transformants in DLA with two isolates 90128 and IPO-C.

Degree of resistance	*Isolates*	
	90128 (*avr-edn2*)	IPO-C (*Avr-edn2*)
Highly resistant	1	0
Resistant	33	10
Susceptible	7	31

DLA on A41 (Rpi-chc1) plants:

DLA was performed in 42 genotypes of A41 (*Rpi-chc1*-short) with two isolates 90128 and IPO-C that were expected to be a-virulent to *Rpi-chc1*. Since the *Rpi-chc1* donor plant was resistant to both isolates. The DLA showed that transgenic A41-52 was found highly resistant to isolate 90128 but susceptible to other isolate IPO-C. Additionally, we observed that A41-14, A41-16, A41-63, A41-74 and A41-76 were also resistant to isolate 90128 however, showed greater susceptibility to IPO-C. Three transformants A41-41, A41-45 and A41-66 were highly susceptible to both isolates. One transformant A41-15 showed similar resistance level to both the isolates (Figure 2). The isolate IPO-C was virulent to 41 transformants as compared to only 26 plant in case of isolate 90128 that showing susceptibility. The isolate 90128 was found less virulent that resulted into large number of resistant plants (Table 4, Figure 2).

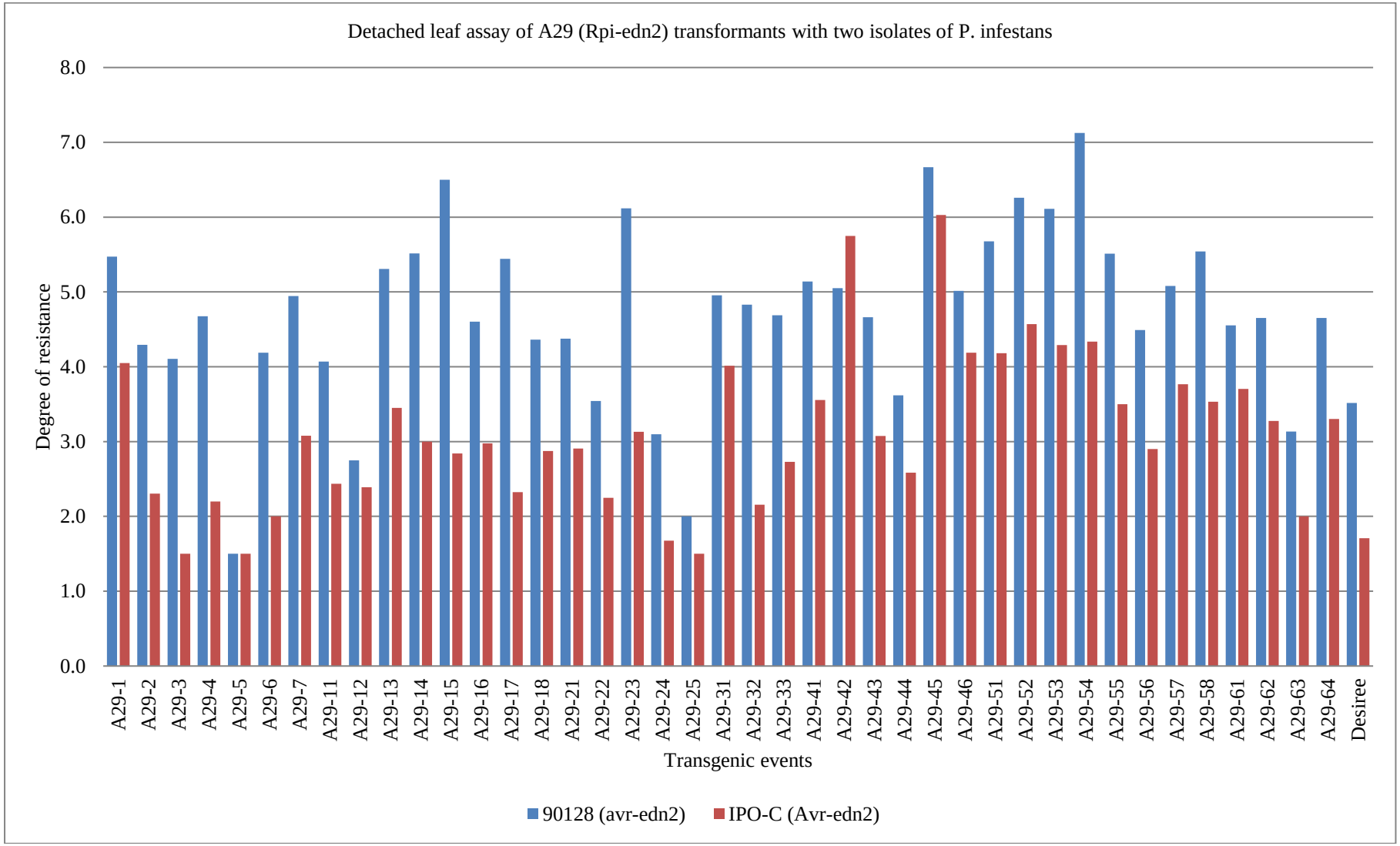

Figure 1. *Detached leaf assay in A29 (Rpi-end2) plants against two isolates 90128 and IPO-C of Phytophthora infestans. The values were obtained from average of two DLA and two days scoring.*

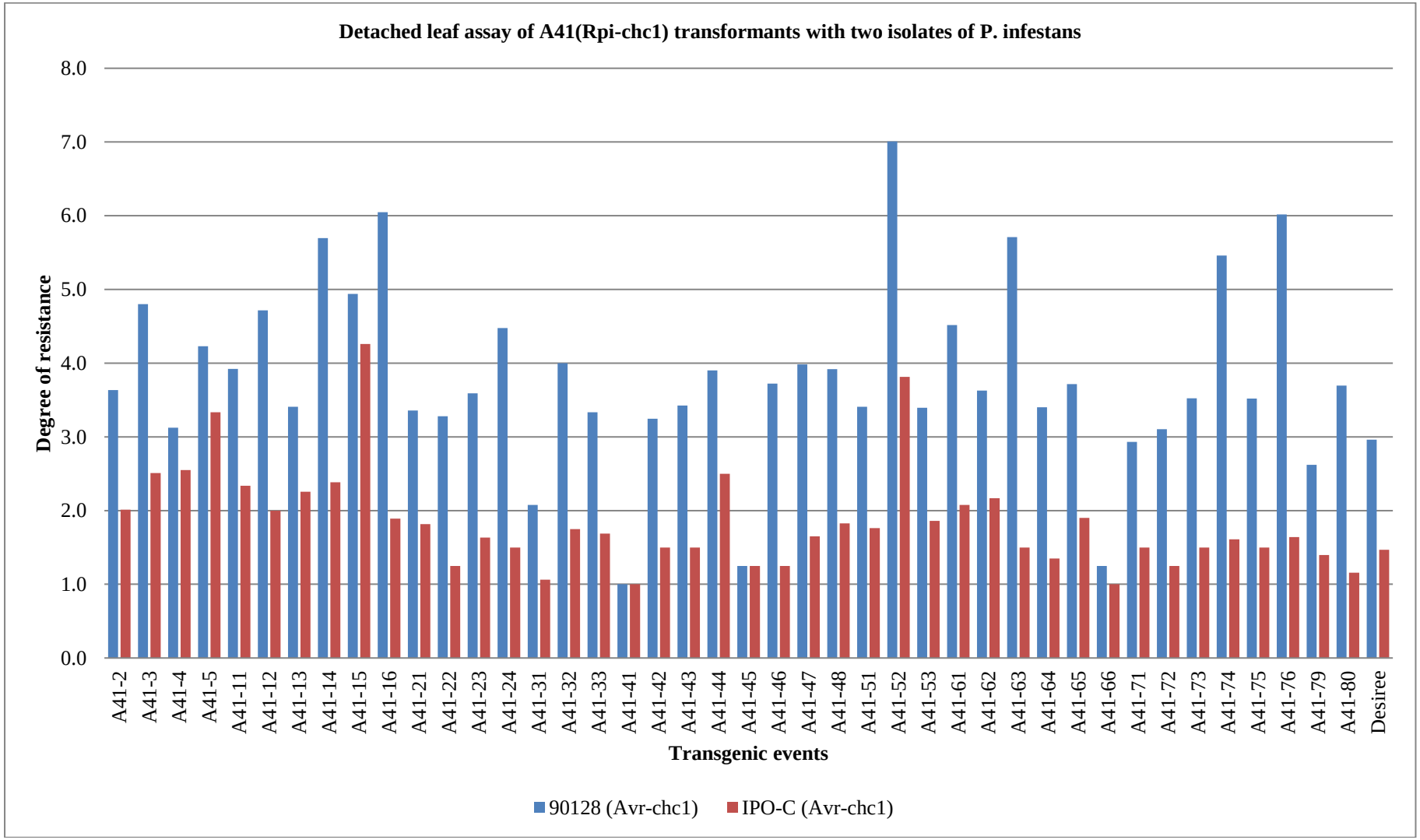

Figure 2. *Functional analysis of A41 (Rpi-chc1) transformants to isolates 90128 and IPO-C. The values were obtained from the average of two repeated DLAs and two days scoring per DLA.*

Table 4. Degree of resistance of A41 (*Rpi-chc1*) transformants in DLA with two isolates 90128 and IPO-C

Degree of resistance	*Isolates*	
	90128 (*Avr-chc1*)	IPO-C (*Avr-chc1*)
Highly resistant	1	0
Resistant	15	1
Susceptible	26	41

Multiple isolates disease spectrum: Multiple isolates DLA spectrum was done in plants containing resistance genes *Rpi-chc1* and *Rpi-ber*. Most of genotypes had given higher level of resistance to three isolates H30P04, IPO-O and 99177 (Table 5). Whereas isolates IPO-C, Katshaar, Dinteloord and NL07245 were breaking the resistance *R* genes present in majority of genotypes except CHC-543-5. When comparing the two years result, we obtained consistent results for both genotypes 94-2034-01(*Rpi-ber)* and CHC-543-5(*Rpi-chc1)* to all tested isolates. The wild genotypes 94-2034-01(*Rpi-ber)* and CHC-543-5(*Rpi-chc1)* had broader recognition spectrum to all the isolates as compared to transgenic plants. The construct with A17-27 (*chc1*-long promoter) had shown resistance to more isolates as compared to A41 (*chc1*-short promoter). The virulence level of different isolates to individual genotypes was presented in Table 5,

DLA on A19 (Rpi-vnt1.1: Rpi-chc1-long) construct

Functional resistance of A19 construct (*Rpi-vnt1.1: Rpi-chc1*-long) was evaluated with isolates EC-1 and NL07245. The genotypes were grouped based on comparison of Desiree with one of A13-13 (*Rpi-vnt1.1*) or A17-27 (*Rpi-chc1*-long) constructs to identify the activity of particular *R* gene. We found that isolate NL07245 was less virulent as compared to isolate EC-1 (Table 6, Figure 3). Transformants A19-2, A19-23, A19-48 and A19-120 were superior against isolate EC-1. The transgenics A19-46, A19-48, A19-120 had exhibited similar resistant pattern to both isolates and A19-3, A19-14, A19-51 and A19-73 werevery poor in expression to both isolates (Figure 3).

Functional analysis of cisgenic A26 (Rpi-blb3:Rpi-sto1) plants: Nine backbone free cisgenic transformants of constructs A26 (Rpi-blb3: Rpi-sto1) together with positive and negative controls were evaluated in detached leaf assay. The plants were tested against four isolates 90128, IPO-C, USA618 and NL08797 to identify the functionality of R genes stacked. The details on degree of resistance were shown in Figure 4 and Table 7. Among the tested genotypes; A26-1679 was found highly resistant comparable to positive controls A10-43 (nptII: blb3:sto1) and A14-16 (nptII: blb3:vnt1:sto1) to all four isolates. In the positive control A3-142 only one R gene Rpi-blb3 was active but other stacked R gene Rpi-sto1 was not functional. Two plants A26-1554 and A26-1735 had given more resistance to isolates USA618 and NL08797 as compared to 90128 and IPO-C. The A26-1263, A26-1369 and A26-1172 plants were susceptible to all the isolates tested (Figure 4). The isolate USA618 and NL08797 were less aggressive resulting in more resistance to tested plants and isolates IPO-C and 90128 were observed equally virulent to plants (Table 7). In the first screening with four isolates to 9 cisgenic plants, two isolates 90128 and IPO-C were more aggressive than USA618 and NL08797 (Table 7, Figure 4). We observed that USA618 was less aggressive that resulted in two highly resistant plants (A26-1679 and A26-1389) while other isolate NL08797 gave only resistant plants when compared to both positive and negative controls (Table 7).

In second screening, 23 cisgenic plants tested with two isolates USA618 and NL08797. We found that transformant A26-97 was strongly resistant to both isolates also similar resistance level observed in A26-1735 (Figure 5). Likewise, A26-528, A26-882, A26-915, A26-1065, A26-1206 and A26-1679 were highly resistant to isolate USA618. Eight transformants: A26- 941, A26- 946, A26-948, A26- 949, A26-1985, A26- 2351, A26- 1172, A26-1263 and A26- 2371were observed as highly susceptible to both isolates (Figure 5).

Table 5. Multiple isolate DLA spectrums in *Rpi-chc* and *Rpi-ber* constructs

	Genotypes									
	(chc-long)	(chc-short)	(chc-short)	94-2034-01 (*Rpi-ber*)	Previous	CHC 543-5 (*Rpi-chc1*)	CHC543-5 (*Rpi-chc1)*	(*Rpi-chc1*)	(*Rpi-chc1*)	
Isolates	A17-27	A41-52	A41-76	Current	94-2034-01	Current	Previous	CHC 544-5	G254	Desiree
91011	S	S	S	R	R	R	R	S	S	S
99189	R	S	R	R	R	R	R	S	S	S
IPO-C	S	S	S	S		R		S	S	S
NL7379	S	S	S	S		R		S	S	S
Katshaar	S	S	S	R		R		S	S	S
Dinteloord	S	S	S	S		S			S	S	S
7245	S	S	S	S		R		S	R	S
H30P04	R	R	R	R	R	R	R	R	R	R
90128	R	S	R	R	R	R	R	S	R	S
IPO-O	R	R	R	R	S	R	R	S	R	R
99183	R	R	R	R	S	R	R	S	S	S
3928-A	R	R	S	R	R	R	R	S	S	S
99177	R	R	R	R	R	R	R		R	S
USA618	R	R	S	R		R			S	S
7135	R	S	S	R		R			S	S
UK98014	R	S	S	R		R			S	S

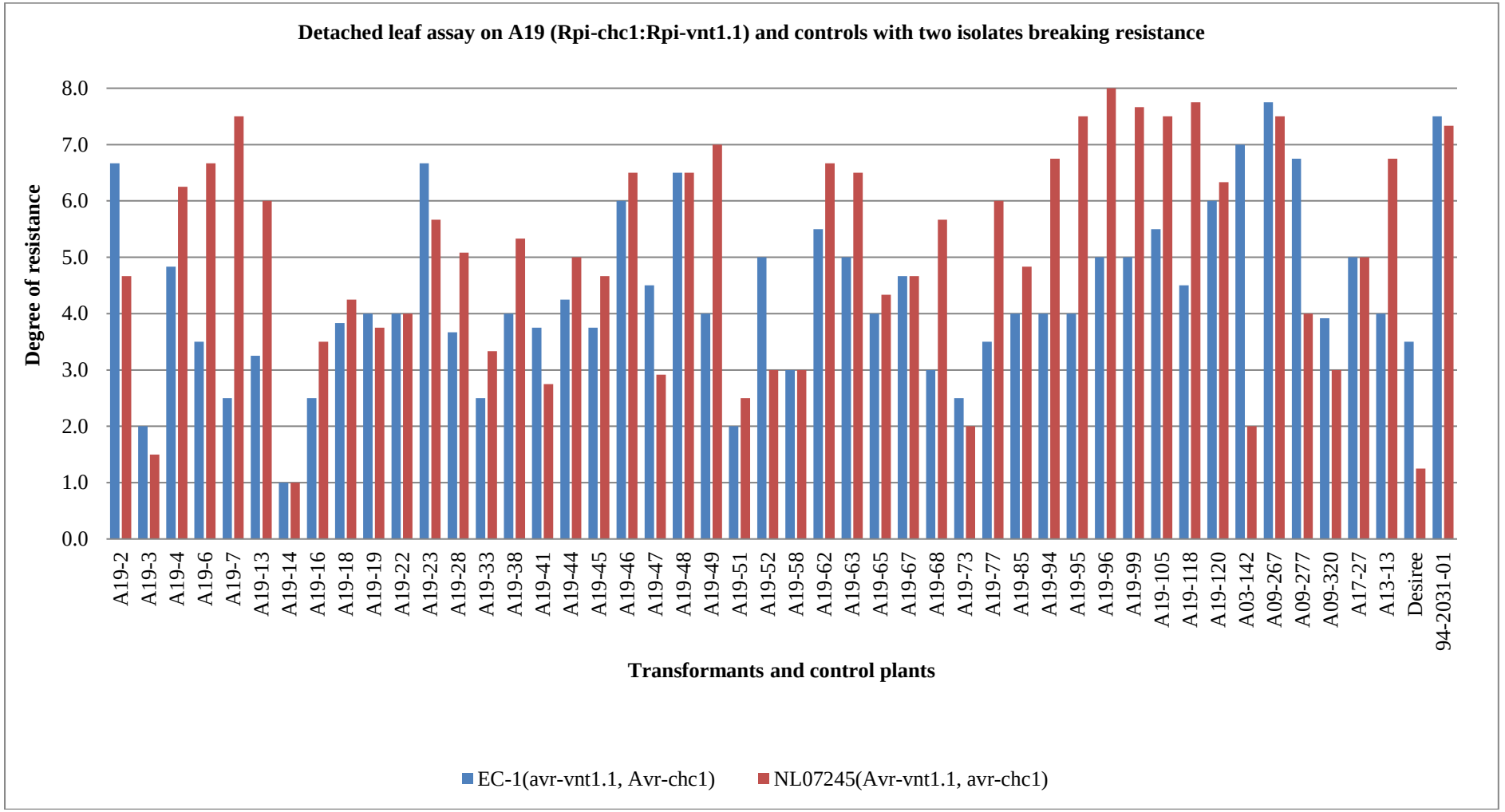

Figure 3. Functional analysis of A19 (Rpi-vnt1.1: Rpi-chc1) transformant plants to isolates EC-1 and NL07245. The result is from only one DLA and one time scoring.

Table 6. Degree of resistance of A19 (*Rpi-vnt1.1: Rpi-chc1*-long) transformants and control plants with two isolates EC-1 and NL07245

Genotypes	Isolates		
A19(*Rpi-vnt1.1:Rpi-chc1*-long)		EC-1 (*avr-vnt1.1, Avr-chc1*)	NL07245 (*Avr-vnt1.1, avr-chc1*)
(40 transformants)	HR	7	10
	R	22	29
	S	11	1

Note: HR denotes highly resistant that were above A13-13 or A17-27 for given isolates, R refers to resistant that lies between Desiree and one of A13-13 or A17-27 and S refers to susceptible below Desiree. The values were obtained from the average of two consecutive scoring of only one DLA screening.

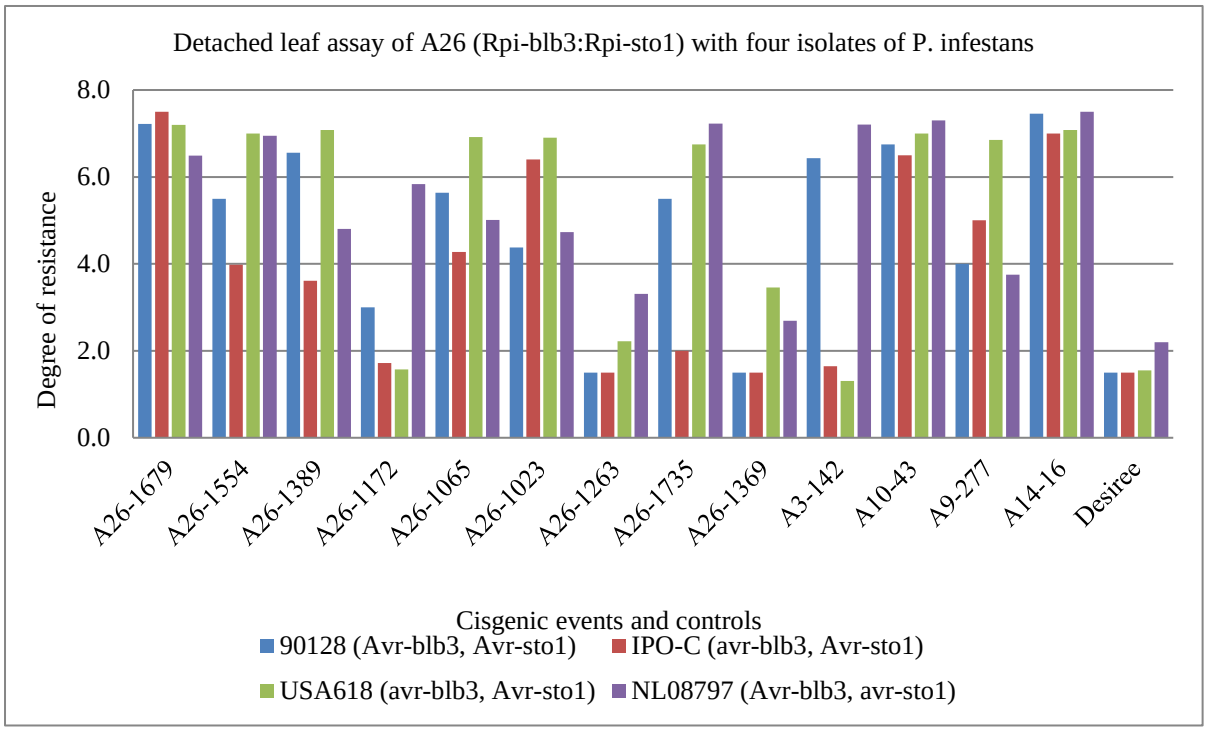

Figure 4. Functional analysis of A26 (Rpi-blb3: Rpi-sto1) transformants to isolates 90128, IPO-C, USA618 and NL08797. The values are average of one DLA with two days scoring in case of NL90128 and IPO-C. For isolates USA618 and NL08797 the values are obtained from average of two DLAs in two consecutive scoring days (6 DPI)

Table 7. Degree of resistance of different cisgenic transformants of A26 (*Rpi-blb3: Rpi-sto1*) in two different DLA screenings with isolates 90128, IPO-C, USA618 and NL08797. The results were compared differently in two screenings as indicated in footnote.

Isolates	**Degree of resistance**		
First screening	Highly Resistant	Resistant	Susceptible
90128 (*Avr-blb3, Avr-sto1*)	1	6	2
IPO-C (*avr-blb3, Avr-sto1*)	1	6	2
USA618 (*avr-blb3, Avr-sto1*)	2	7	
NL08797 (*Avr-blb3, avr-sto1*)		9	
Second screening			
NL08797 (*Avr-blb3, avr-sto1)*	1	1	21
USA618 (*avr-blb3, Avr-sto1*)	5	7	11

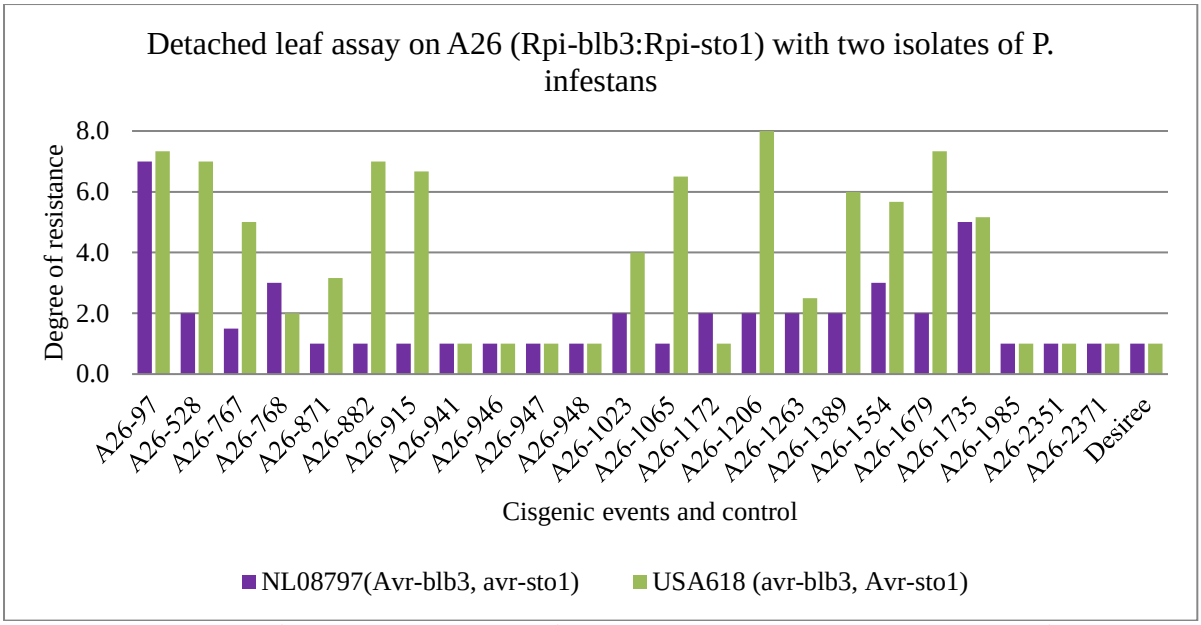

Figure 5. Functional analysis of A26 (Rpi-blb3: Rpi-sto1) transformants to isolates USA618 and NL08797. The result is from only one DLA and one time scoring. For repeated DLA plants were severely damaged by thrips in the greenhouse and leaves were very ugly in appearance thus not possible to score due to insect sucking.

DISCUSSION

Stacking of resistance *R* genes into a single genotype was an alternative to achieve durable resistance in the plants against *Phytophthora infestans.* The transformants were evaluated for presence of resistance *R* genes that have been stacked from transformation process. DLA was applied to screen for the transgenic (A41, A29 and A19) and cisgenic (A26) constructs with *R* genes *Rpi-chc1* (short), *Rpi-edn2*, *Rpi-chc1*(long)*:Rpi-vnt1.1* and *Rpi-blb3:Rpi-sto1* respectively. Corresponding isolates with a/virulence genes were used for breaking the resistance conferred by them. The isolates used for breaking resistance were 90128, IPO-C, USA618, NL08797, NL07245 and EC-1.

Rpi-edn2 functionality: The transgenic plants of construct A29 (*Rpi-edn2)* showed unexpected results to the isolates 90128 and IPO-C. The *Rpi-edn2* gene was previously conformed as conferring resistance to IPO-C (Verzaux, 2010) due to an a-virulent gene, however it showed contradictory response in this study. Isolate 90128 supposedly virulent, gave less virulence to many transformants. It indicated that *Rpi-edn2* gene recognition was not specific to the isolates 90128 and IPO-C (Figure 1, Table 3). The resistance spectrum of *Rpi-end2* was very narrow (Verzaux, 2010) was consistent with our result.

Rpi-chc1 gene activity: In order to explore the functionality of *Rpi-chc1*, the *chc1* gene containing plants were tested in different screens. In the A41 plants, carrying the *chc1*-short promoter, we did find specific resistance against isolate 90128, although at a very low frequency. The frequency of finding 90128 resistant plants was much higher (50%) when the *Rpi-chc1*- long promoter was used (J. Vossen Personal communication, WUR). Furthermore, in our analyses of the A19 plants (*vnt1.1* and *chc1*-long) in DLA, we found that plants with an active *Rpi-chc1* gene were found at frequency of 69% (Table 6). However, we did not observed resistance against IPO-C. This could in retrospect be explained by the observation that IPO-C was compatible with the *Rpi-chc1* gene as identified using spectrum analysis (Table 4). This was in contrast to the previous assumption that both 90128 and IPO-C carried the a-virulent gene *Avr-chc1* (Figure 2). The transformants showed wide variation in resistance spectrum. *Rpi-chc1*-(short) showed much stronger degree of resistance to the isolate 90128 as it was expected normally and but caused susceptibility to IPO-C despite of presence of a-virulence gene *(Avr-chc1).* This indicated that resistance gene *Rpi-chc1* recognition was not very specific with respect to isolates used (Figure 2). In addition, we obtained comparatively high frequency of resistant transformants with *Rpi-chc1* than Desiree (Figure 2), was in agreement with earlier study in *S. chacoense* genotypes (Micheletto *et al.*, 2000). Our finding was also very similar to previous studies on *S. chacoense* accessions, challenged against complex races of *Phytophthora* resulting to low frequency of incompatible reaction (Micheletto *et al.*, 1999).

Functional analysis of construct A19 (*Rpi-vnt1.1: Rpi-chc1*-long) was carried out against isolates EC-1(*avr-vnt1.1: Avr-chc1*) and NL07245 (*Avr-vnt1.1: avr-chc1*) for breaking resistance. More than 97% showed higher resistance to isolate NL07245 because the plants expressed *Rpi-vnt1.1* (Figure 3), was already identified as wide spectrum resistant gene (Zhu *et al.*, 2011). EC-1 was observed more virulent as compared to NL07245 resulting into more susceptibility because gene for a-virulence (*Avr-chc1*) was not recognized by *Rpi-chc1.* However, other virulent gene *avr-vnt1.1* became more pronounced that induced breaking resistance caused by

Rpi-vnt1.1 (Figure 3, Table 6). It was in agreement with (Foster *et al.*, 2009; Pel *et al.*, 2009) that explained virulence of EC-1 to *Rpi-vnt1.1*. It designates that *Rpi-chc1* gene was not very active against this isolate, thus virulence gene *avr-vnt1.1* caused more susceptibility. Higher level of resistance was observed in A19-23, A19-46, A19-48, A19-62, A19-118 and A19-120 to both isolates EC-1 and NL07245. It indicated that *Rpi-chc1* gene was more strongly expressed in them. It could be due to insertion site dependent. Furthermore, we also observed that some plants showed greater resistance to EC-1(A19-2, A19-23 and A19-48) reflected stronger *Rpi-chc1* recognition than *Rpi-vnt1.1* (Figure 3).

In multiple isolate spectrums to identify promising genotypes, the resistance level of A17-27 (*chc1*-long promotor) was much broader than A41 (*chc1*-short promotor) (Table 6). The reason could be due to long promoter was more active than short promoter. The genotypes 94-2034-01(*Rpi-ber*) and CHC-543-5(*Rpi-chc1*) had also expressed very high level of resistance to many isolates than other transgenics. It was caused by presence of additional resistance genes in the wild genotypes than transgenics. It was supported by previous findings that level of infection of *Phytophthora* was reduced to 5% and was delayed for more than three weeks for infection in construct with *Rpi-ber* gene (Tan *et al.*, 2010). The genotype CHC-543-5 showed resistance to all the isolates except Dinteloord indicated that this wild donor harboured extra resistance genes (Table 6).

Functionality of cisgenic plants: Detached leaf assay of cisgenic construct with two resistance genes A26 (*Rpi-blb3: Rpi-sto1*) showed wide variation in the resistance level against four different isolates. The transformant *A26-1679* showed highest level of resistance to all the isolates comparable to positive control cisgenic genotypes A10-43 (*Rpi-blb3: Rpi-sto1*) and A14-16 (*Rpi-blb3: Rpi-sto1: Rpi-vnt1.1)* (Figure 4). It was likely because of presence of both the resistance genes *Rpi-blb3* and *Rpi-sto1* that conferred full resistance. These two *Rpi* genes *Rpi-blb3* and *Rpi-sto1* were broad spectrum resistance genes widely exploited in the Netherlands (Zhu *et al.*, 2011). Both *Rpi* genes were biologically active in DLA. Similarly, two transformants A26-1554 and A26-1735 had both resistance genes that conferred full resistance to isolates USA618 (*avr-blb3*, *Avr-sto1*) and NL08797 (*Avr-blb3, avr-sto1*) that carried one corresponding avirulence gene recognised by the plants (Figure 4). In case of two resistance genes stacked, pathogen carrying one a/virulence gene corresponding to *R* gene, that's why one virulent gene breaking the resistance but other a-virulent gene recognized by plant *R* gene led to resistance in the plants. The genotypes A26-1263 and A26-1369 were found more susceptible to all the isolates could be due to biological activity of *R* genes.

In the second screening of A26 (*Rpi-blb3: Rpi-sto1*) confirmed that genotype A26-1735 was equally resistant to both isolates due to presence of both functional *R* genes (Figure 5). The plants showed higher level of resistance to isolate USA618 than the other isolate NL08797 (Table 7, Figure 5). It was caused by stronger expression of *Rpi-blb3* in the transformant. A26-97 showed high resistance to both isolates might express both the resistant genes that were not broken down by isolates carrying virulent genes *avr-blb3* and *avr-sto1*. It was revealed that the transformants A26-1023, A26-1065, A26-1389, A26-1554, A26-1679 and A26-1735 were identified as resistant because they expressed high level of resistance in both the screenings. Both screenings revealed that isolate USA618 was less virulent resulting into a highly resistance in five plants (Figure 4, 5 and Table 7).

CONCLUSIONS

The major findings of the study were summarized as follows:

- A29 disease spectrum result was unexpected and *Rpi-edn2* gene recognition was not specific to isolates 90128 and IPO-C
- In A41, *Rpi-chc1*-short promoter had non-specific recognition
  - *Rpi-chc1* recognition specific to 90128 but not to IPO-C as was expected
  - A17-27 (*Rpi- chc1*-long) had higher resistance over A41(*Rpi-chc1*-short)
  - Wild relatives of *Rpi-chc1* donor plants had broader resistance spectrum
- *Rpi-blb3* and *Rpi-sto1* genes are more active to isolates USA618 and NL08797
- *Rpi-vnt1.1* gene was more active in A19 than *Rpi-chc1*(long)
- *Rpi-chc1*(long) had active resistance in 50% transformants

Some points were highlighted as recommendations to future resistance breeding.

- Include both positive and negative controls in all assays for better comparison
- Selected resistant genotypes can be further screened to confirm the durability of *R* gene (stack)

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