

Functional stacking of three resistance genes against *Phytophthora infestans* in potato

Suxian Zhu · Ying Li · Jack H. Vossen ·
Richard G. F. Visser · Evert Jacobsen

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Abstract Functional stacking of broad spectrum resistance (*R*) genes could potentially be an effective strategy for more durable disease resistance, for example, to potato late blight caused by *Phytophthora infestans* (*Pi*). For this reason, three broad spectrum potato *R* genes (*Rpi*), *Rpi-sto1* (*Solanum stoloniferum*), *Rpi-vnt1.1* (*S. venturii*) and *Rpi-blb3* (*S. bulbocastanum*) were selected, combined into a single binary vector pBINPLUS and transformed into the susceptible cultivar Desiree. Among the 550 kanamycin resistant regenerants, 28 were further investigated by gene specific PCRs. All regenerants were positive for the *nptII* gene and 23 of them contained the three *Rpi* genes, referred to as triple *Rpi* gene transformants. Detached leaf assay and agro-infiltration of avirulence (*Avr*) genes showed that the 23 triple *Rpi* gene transformants were resistant to the selected isolates and showed HR with the three *Avr* effectors indicating functional stacking of all the

three *Rpi* genes. It is concluded that *Avr* genes, corresponding to the *R* genes to be stacked, must be available in order to assay for functionality of each stack component. No indications were found for silencing or any other negative effects affecting the function of the inserted *Rpi* genes. The resistance spectrum of these 23 triple *Rpi* gene transformants was, as expected, a sum of the spectra from the three individual *Rpi* genes. This is the first example of a one-step approach for the simultaneous domestication of three natural *R* genes against a single disease by genetic transformation.

Keywords Potato · Gene stacking · Durable resistance · Functionality

Introduction

Many devastating diseases are threatening plants, such as potato late blight, apple scab and wheat yellow rust. In contrast to higher animals, which have an advanced adaptive immune system, plants mainly rely on innate immunity to resist invading pathogens (Staskawicz et al. 1995; Jones and Dangl 2006). As a consequence of competition, plant resistance and pathogen virulence have been co-evolving up till now. Co-evolution, on the one hand, is diversifying the species; on the other hand, it is also making the competitive species more adapted to the required environment (Rosenthal et al. 1976; Rausher 2001).

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S. Zhu (✉) · J. H. Vossen · R. G. F. Visser · E. Jacobsen
Wageningen UR Plant Breeding, Wageningen University
and Research Center, 6708 PB Wageningen,
The Netherlands
e-mail: suxian.zhu@wur.nl

Y. Li
Institute of Vegetables and Flowers, Chinese Academy
of Agricultural Sciences, 100081 Beijing, China

A good example, is the late blight pathogen *Phytophthora infestans* (*Pi*), whose genome has been recently sequenced (Haas et al. 2009). This pathogen evolved by mutation of effectors frequently and rapidly. Consequently, individual resistance genes (*Rpi*) were overcome relatively fast after their introduction into the potato crop (Black et al. 1953).

A promising strategy for breeding more durable resistance is to stack multiple, broad spectrum, resistance genes in one genotype (Halpin 2005; Douglas and Halpin 2010), especially with resistance genes originating from different gene clusters, representing different HR interactions between *R* genes and their cognate *Avr* effectors. In comparison to traditional breeding, with the increasing problem of linkage drag during stacking, genetic transformation is a fast and efficient way to introduce and domesticate multiple *R* genes into an existing cultivar. For potato, three different transformation methods can be used for *R* gene stacking: (1) transformation of one or two *R* genes followed by re-transformation of a selected, well performing, resistant transformant with additional *R* genes; (2) in one step via co-transformation, by using mixed *Agrobacterium* strains containing unlinked *R* genes in two or more vectors; (3) with multiple *R* genes in one vector (Douglas and Halpin 2010).

Multiple transgene introduction has been practiced to combine different traits using various methods, such as particle bombardment or *Agrobacterium*-mediated transformation (Altpeter et al. 2005; Sharma et al. 2005; Halpin 2005; Lacorte 2006). Until now, they were transgenes or pathway genes, corresponding to different agronomic traits, and the individual functionality of which could be easily detected in the transformed plants (Campbell et al. 2000; Beyer et al. 2002; Anand et al. 2003; Agrawal et al. 2005; Cao et al. 2005; Chan et al. 2005; Schmidt et al. 2008). The application of gene stacking using GM technology is increasing. In 2010, worldwide 148.1 million hectare of GMO crops were grown, of which 32.3 million hectares with two or three stacked traits. Double traits stacking was mainly restricted to insect and herbicide resistance. Triple traits stacking was restricted to herbicide resistance and two different insect pests (ISAAA 2010). Our approach differs from these examples as we are introducing three natural *R* genes to render immunity to one disease, potato late blight.

To date, several *R* genes against *Pi*, originating from different *Rpi* gene clusters and various wild species, have been cloned. For example, *R1*, *R2*, and *R3a* from *Solanum demissum* (Ballvora et al. 2002; Lokossou et al. 2009; Huang et al. 2005), *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003; 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). Some of them showed a broader resistance spectrum than others with a selected set of late blight isolates. It is expected that stacking of at least three broad spectrum *Rpi* genes is needed to obtain durable resistance in the field. Important reasons are the observation that multiple *Rpi* genes are frequently found in individual resistant plants in nature (Huang et al. 2005; Lokossou et al. 2010; Verzaux 2010). The *Rpi* gene differentials *R8* and *R9*, which have been reported to exhibit durable resistance (Zhang and Kim 2007; Lehtinen et al. 2008; Dr. G. Kessel, Plant Research International, Wageningen, The Netherlands, unpublished; YL, unpublished), are known to contain multiple *R* genes (Trognitz and Trognitz 2004; JV, unpublished). These observations show that stacking of broad spectrum *Rpi* genes in plants could contribute to the durability of resistance for potato in the field.

Therefore, it is important to know how to stack three or more *Rpi* genes into a potato variety efficiently and how to test the biological functionality of each gene individually. One way to answer this question is to investigate the relationship between the resistance reaction of individual *Rpi* genes and the HR reactions in response to cognate *Avr* effectors (Vleeshouwers et al. 2008). The consensus RXLR pattern of *Avr* genes and the recent genomic sequence of *Phytophthora infestans* promoted the research on *Avr* gene(s) considerably. *Avr1* (Dr. F. Govers, Phytopathology, Wageningen University and Research Center, Wageningen, The Netherlands), *Avr2* (Dr. P. Birch, Plant Pathology, SCRI, United Kingdom), *Avr3a* (Armstrong et al. 2005), *Avr4* (van Poppel et al. 2008), *Avrblb2* (Oh et al. 2009), *Avrvnt1* (Pel 2010), and *IpiO1* (Vleeshouwers et al. 2008) were cloned, and they are recognized by *Rpi* genes *R1*, *Rpi-blb3*, *R3a*, *R4*, *Rpi-blb2*, *Rpi-vnt1.1*, and *Rpi-blb1*, respectively.

In this paper, three broad spectrum resistance genes, *Rpi-sto1* (Vleeshouwers et al. 2008),

Rpi-vnt1.1 (Pel et al. 2009) and *Rpi-blb3* (Lokossou et al. 2009), were selected and cloned into a binary vector pBINPLUS, which was used to transfer these three *Rpi* genes simultaneously into the susceptible potato cultivar Desiree by *Agrobacterium tumefaciens*-mediated transformation. Co-integration and co-functioning were confirmed by molecular analyses, disease tests and agro-infiltration using corresponding avirulence (*Avr*) effectors. This approach provides a new foundation to distinguish the functionality of each stack component. Finally, it is concluded that the expected broadened resistance spectrum based on the functional combination of the three individual *Rpi* genes, was indeed achieved in the triple *Rpi* gene transformants.

Materials and methods

Materials

Susceptible potato cultivar Desiree was used for *Rpi* gene transformation. Single *Rpi* genes were transformed earlier in house and resulted in Desiree transformants harboring *R3a* (A04-22), *Rpi-blb3* (A03-142) and *Rpi-vnt1.1* (SF3) and *Rpi-sto1* (A09-6). These single *Rpi* gene transformants were tested for their resistance spectrum. Three plasmids, pBINPLUS:*Rpi-blb3*, pBIN19:*Rpi-vnt1.1* and pBINPLUS:*Rpi-sto1* were used as a basis for designing the triple *Rpi* gene construct. Supplementary Table 1 lists the effectors or avirulence genes used in the agro-infiltration. Sixteen late blight isolates, derived from multiple locations in Europe and America, used for *Rpi* gene spectrum analysis were maintained at Wageningen UR Plant Breeding, Wageningen. This in house set of isolates was partly selected based on the compatibility to particular *Rpi* genes such as isolate EC1 to *Rpi-vnt1.1*; IPO-0, Pic99177, Pic99189 and Pic99183 to *Rpi-sto1*; and USA618 to *Rpi-blb3*. Twenty-eight additional isolates (Table 1), from four different provinces in China, were provided by CAAS in Beijing, China.

Construction of triple *Rpi* gene vector

Three *Rpi* genes *Rpi-sto1* (Vleeshouwers et al. 2008), *Rpi-vnt1.1* (Pel et al. 2009) and *Rpi-blb3* (Lokossou et al. 2009) had been cloned. In order to design the

triple *Rpi* gene vector, first the two *Rpi* gene construct pBINPLUS:*Rpi-blb3*:*Rpi-sto1* was created by using the unique enzyme sites *Sma*I and *Sbf*I flanking *Rpi-blb3* in the donor vector pBINPLUS:*Rpi-blb3* and thus *Rpi-blb3* was transferred into the recipient pBINPLUS:*Rpi-sto1* vector. *Rpi-vnt1.1* was inserted into vector pBINPLUS:*Rpi-blb3*:*Rpi-sto1*, using the unique enzyme site *Sbf*I, located between *Rpi-blb3* and *Rpi-sto1*. Since the *Sbf*I site was not available in pBIN19:*Rpi-vnt1.1*, the *Rpi-vnt1.1* fragment flanked by *Eco*RI was cloned into a modified pGEM-T vector, with *Sbf*I-*Eco*RI-*Sbf*I enzyme sites in the multiple cloning site (MCS). Subsequently, the *Rpi-vnt1.1* fragment flanked by *Sbf*I was subcloned into pBINPLUS:*Rpi-blb3*:*Rpi-sto1*. Both anticipated orientations of the *Rpi-vnt1.1* gene were found and the clone with the orientation as depicted in Fig. 1 was selected. The resulting pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1* construct had a total size of 31.8 Kb and harbored a 22 Kb T-DNA insert (Fig. 1).

Stability and functionality tests of triple *Rpi* gene vector

After the vector had been made, we tested if it could replicate stably in *Agrobacterium* strains. Therefore, the stability of this construct was tested.

DNA was extracted from *E. coli* and transformed into three *Agrobacterium* strains (AGL1, AGL1 + VirG and Cor308) using electroporation, and transformants were cultured in three different media: LB (LB Broth: 20 g/l), LB-selected (Pepton-select: Gibco 10 g, yeast extract select Gibco 5 g, NaCl 5 g and agar 8 g per liter) and YEB (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract and 2 ml 1 M MgSO₄ per liter). Two colonies obtained from each of 9 combinations (3 *Agrobacterium* strains-3 culture media) were cultured for DNA isolation. These 18 DNA samples were transformed back into *E. coli*. Two colonies from each transformation were selected and DNA from 36 samples (two *E. coli* colonies were picked up from each of 18 DNA transformations) was extracted and digested with *Hind*III. The products were loaded on 1% agarose gel to check whether the pattern was the same with the original DNA isolated from *E. coli*.

Agrobacteria with stable plasmid insertions were used to check the functionality of the genes in the construct. Vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:

Table 1 DL-A on transgenic Desiree plants harboring single *Rpi* genes using 44 isolates

Isolate Plant	IPO-0	Pic99177	Pic99189	Pic99183	EC1	USA618	NL00228	VK98014	F95573	89148-09	NO01096	IPO-C	Set 1	Set 2	Set 3	Set 4
Desiree: <i>Rpi-sto1</i> ^a	S	S	SQ	SQ	R	R	R	R	R	R	R	Q	4 R	10 R	12 R	6 R
Desiree: <i>Rpi-blb3</i> ^a	R	Q	S	R	R	S	Q	RQ	R	R	R	S	4 R	9 R;1S	12 R	3 R; 3 S
Desiree: <i>R3d</i> ^a	R	S	R	S	S	S	R	RQ	S	R	Q	S	4 S	10 S	12 S	6 S
Desiree: <i>Rpi-vnt1.1</i> ^{ab}	R	R	R	R	S	R	R	R	R	R	R	R	4 R	10 R	12 R	6 R
Desiree	S	S	S	S	S	S	S	S	SQ	S	S	S	S	S	S	S

Set 1 In house isolates 88069, H3PO4, 90128 and 428-2 gave the same resistance patterns for these four, different *Rpi* gene containing, transformants

Set 2 Ten isolates from province Inner Mongolia of China

Set 3 Eight isolates from province Hei Longjiang and four isolates from province Fu Jian of China

Set 4 Six isolates from province Yunnan of China

R all of the 8 inoculated spots on each leaf showed resistance

RQ 6–7 out of 8 spots on each leaf showed resistance

Q 3–5 out of 8 spots on each leaf showed resistance

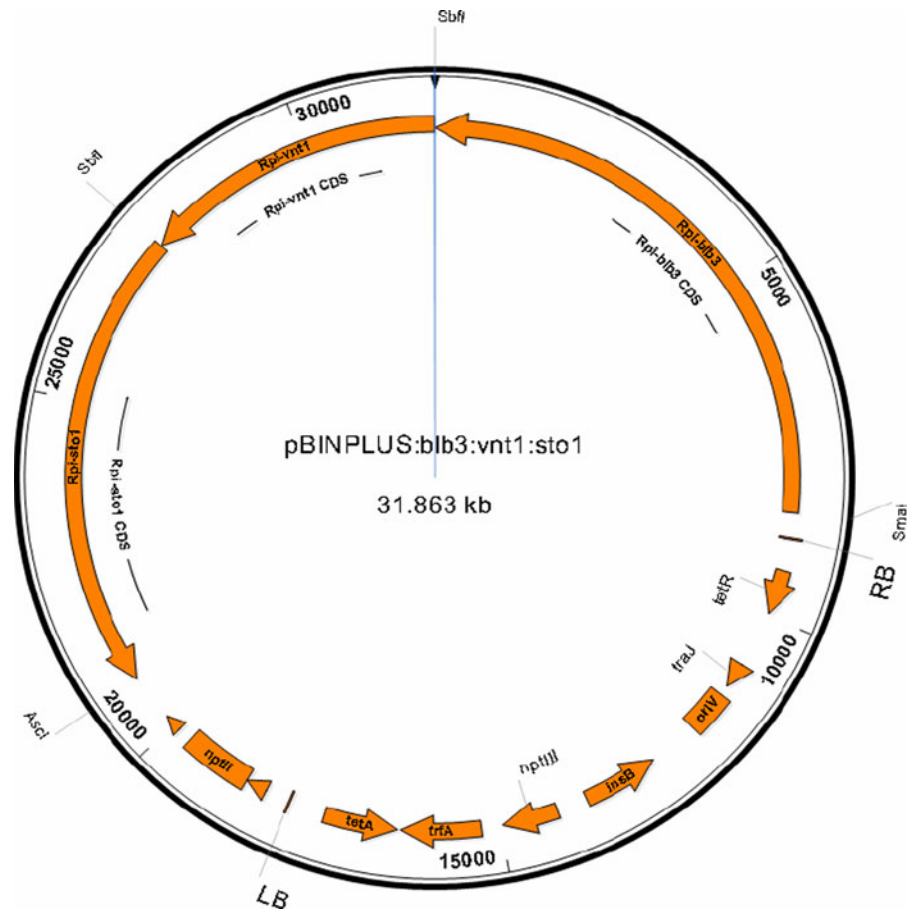
SQ 1–2 out of 8 spots on each leaf showed resistance

S all of the 8 spots on each leaf showed susceptibility

^a Desiree:*Rpi-xxx*: cv. Desiree transformant harbouring *Rpi-xxx*

^b Part of the data of *Rpi-vnt1.1* was adopted from Pei (2010)

Fig. 1 Graphical view of binary vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1*. Restriction enzyme cleavage sites, used for cloning of the *Rpi* genes into pBINPLUS have been indicated



Rpi-sto1 was co-infiltrated with *Avr* effectors *Avr2*, *Avrvnt1* and *IpiO1*, corresponding to *Rpi* gene *Rpi-blb3*, *Rpi-vnt1.1* and *Rpi-sto1*, respectively. Three clones (clone 2–2: vector in COR308 cultured in LB-select media, 5–1: vector in AGL1 cultured in LB-select, and 7–2: vector in AGL1 + *virG* cultured in LB) showed positive results in both tests, and they could be used for the transformation experiments.

Plant transformation

Stable insertion of the triple *Rpi* gene cassette into susceptible potato cultivar Desiree was carried out using *Agrobacterium*-mediated transformation (Filati et al. 1987; Hoekema et al. 1989), as adapted by Pel et al. (2009). Bacterial colony 5–1, selected from the stability and functionality tests, was used for this transformation. The transformation efficiency was calculated as the number of rooted shoots regenerated on kanamycin containing medium divided by the

number of explants used within the time course of the experiment.

Molecular analysis of transformants

Presence or absence of specific genes in the regenerated plants was screened by PCR. Primers were listed in Supplementary Table 2. PCR was performed in a 25 μ l reaction, including 0.5 μ l of forward primer (10 μ M), 0.5 μ l of reverse primer (10 μ M), 0.5 μ l of dNTPs (5 mM each), 2.5 μ l of 10 \times buffer, and 2.5 units of Dream Taq polymerase (Fermentas).

Functional analysis of transformants

Rpi gene transformants were characterized using a detached leaf assay (DLA), in which eight 10 μ l droplets of a zoospore suspension (50,000 spores/ml) of a specific *Pi* isolate (Vleeshouwers et al. 1999) were applied to the abaxial side of the leaf. Criteria

for scoring (DLA): R, all of the inoculation spots on a detached leaf showed resistance; RQ, 6–7 out of eight spots on a leaf showed resistance; Q, 3–5 out of eight spots on a leaf showed resistance; SQ, 1–2 out of eight spots on a leaf showed resistance; S, all spots on a leaf showed susceptibility.

Agro-infiltration, which is referred as *Agrobacterium tumefaciens* transient assay (ATTA), was used to assay for hypersensitive response (HR) to the cognate *Avr* effectors (Bendahmane et al. 2000; Vleeshouwers et al. 2008). Nine replicates were performed (three plants per clone, with infiltration of three leaves per plant) using each effector at three different concentrations, with optical density at wavelength 600 nm: 0.2,

0.05 and 0.0125 (Fig. 2). OD600 nm is successively used throughout this paper.

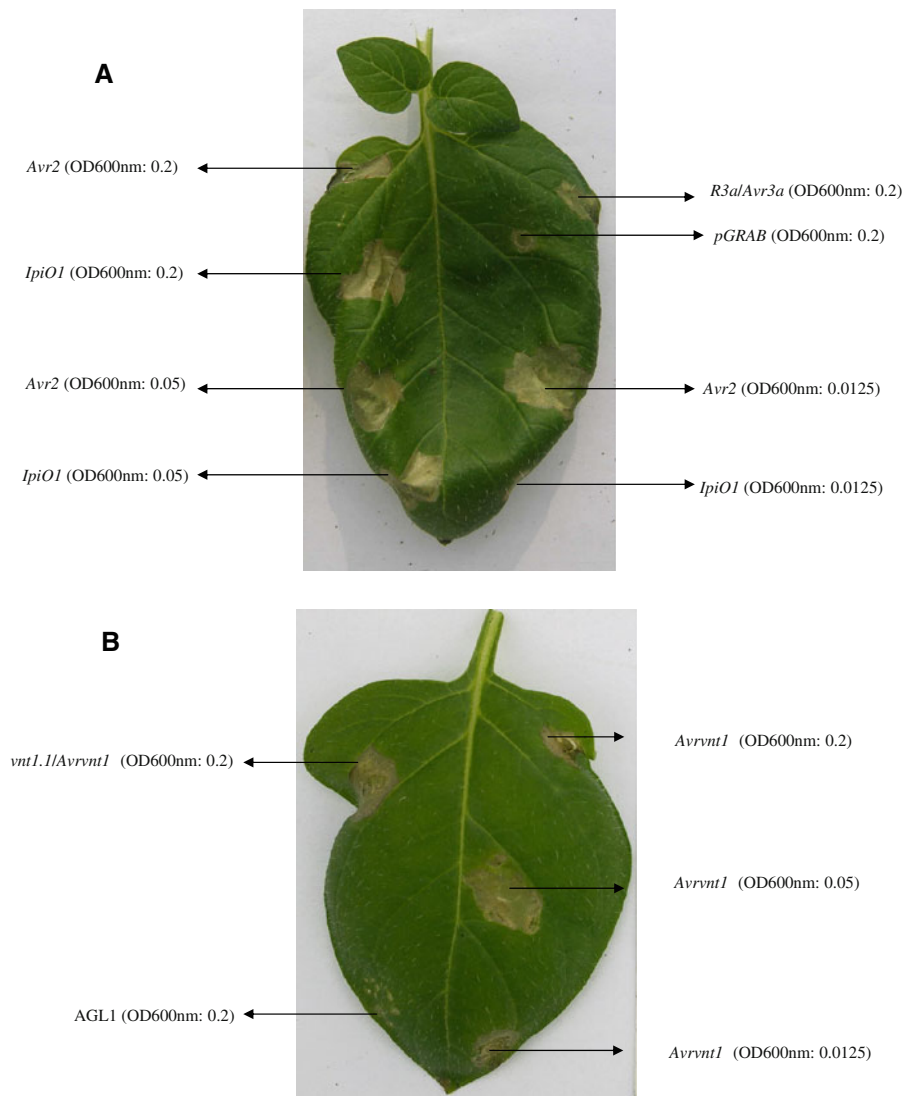
The functionality of each inserted gene was tested by isolates test and agro-infiltration using the scheme in Supplementary Table 3.

Results

Selection of cloned *Rpi* genes for broad spectrum resistance

In order to achieve more durable resistance to potato late blight by stacking genes with different

Fig. 2 Agro-infiltration of *Avr* effectors in triple *Rpi* gene regenerant A14 Z-22 using different densities of inoculum. Clear HR reactions were observed at all densities for *Avr2*, *IpiO1* and *Avrvnt1*. Co-infiltration of *R3a/Avr3a*, and *vnt1.1/Avrvnt-1* served as a positive controls. Empty vector pGRAB, and *Agrobacterium* strain AGL1 were negative controls. **a** Showed the infiltration with *Avr2* and *IpiO1*. **b** Showed the infiltration with *Avrvnt1*



resistances, the individual resistance spectra of four cloned *Rpi* genes (*Rpi-sto1*, *Rpi-blb3*, *R3a* and *Rpi-vnt1.1*) were compared with transformants of cv. Desiree using a set of 44 isolates, consisting of 16 in house and 28 Chinese isolates.

Table 1 shows the resistance pattern of the four *Rpi* genes against these 44 *Pi* isolates. Forty isolates were incompatible to *Rpi-sto1*, 37 to *Rpi-blb3*, 5 to *R3a* and 43 to *Rpi-vnt1.1*. Although it is known that *R3a* is overcome frequently, it is remarkable that *R3a* plants were susceptible to all the 28 Chinese isolates. In contrast to this, the *Rpi-vnt1.1* and *Rpi-sto1* plants were completely resistant to all Chinese isolates and *Rpi-blb3* showed resistance to all isolates from two provinces of China and in a third province to nine out of ten isolates. In Yunnan province, only 3 out of 6 isolates were incompatible on *Rpi-blb3* plants. Consequently, *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3* were selected as broad spectrum *Rpi* genes, and combined into a binary vector pBINPLUS, which included the *nptII* transgene as a selection marker (Fig. 1).

Transformation efficiency of a triple *Rpi* gene vector

Vector pBINPLUS:*Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1*, containing native promoters and terminators of each *Rpi* gene, was used to transform the late blight susceptible cv. Desiree. After co-cultivation with *A. tumefaciens* using 850 stem explants, 121 and 550 rooting regenerants were produced at the fifth and ninth month after the transformation, which resulted in a transformation efficiency of 14 and 59%, respectively.

Characterization of individual regenerants by PCR, isolates test and agro-infiltration

The first obtained 28 kanamycin resistant regenerants were selected for further characterization. Due to the broad spectrum of *Rpi* genes used, no isolates were available, which can directly distinguish the functionality of each *Rpi* gene without the interference from the other *Rpi* genes. The testing scheme, illustrated in Supplementary Table 3, was developed to determine the presence of individual *Rpi* genes and their simultaneous biological functionality. This specific way of functional gene testing is needed for plants harboring multiple broad spectrum *R* genes

against the same disease. These regenerants were characterized using *Rpi* gene specific primers (Supplementary Table 2), selected isolates (EC1 and Pic99189), and the corresponding *Avr* effectors (Fig. 2). Both isolates were selected due to the fact that (1) isolate EC1 is compatible on *Rpi-vnt1.1* plants but incompatible on *Rpi-sto1* and *Rpi-blb3* plants, and (2) isolate Pic99189 is incompatible to *Rpi-vnt1.1*, whereas, it is compatible to *Rpi-sto1* and *Rpi-blb3* plants.

Together with cv. Desiree and the single *Rpi* gene transformants harboring *Rpi-sto1*, *Rpi-blb3* or *Rpi-vnt1.1* in cv. Desiree, the 28 kanamycin resistant regenerants were investigated. PCR analysis showed that all 28 regenerants (Table 2) contained *nptII*, among which, 23 harbored all three *Rpi* genes. All five kanamycin resistant plants (18%), without *Pi* resistance, turned out to lack the three *Rpi* genes (as evidenced by PCR analysis). DLA and agro-infiltration tests indicated that the 23 triple *Rpi* gene transformants (82%), also functionally expressed all three *Rpi* genes. Remarkably, no silencing or other interfering effects were observed for any of the transformed *Rpi* genes.

Discussion

Generally, *R* gene cloning from donor wild species, followed by one-step transformation into cultivated plants can be considered as an efficient method for domestication of natural *R* genes into the agricultural environment. Stacking of several *R* genes is expected to provide more durable and broadened resistance as compared to the introduction of a single *R* gene, which was proven to be ineffective in the past. In this paper, a study was performed to explore the strategy of functional stacking broad spectrum *Rpi* genes. *Rpi-blb3*, *Rpi-vnt1.1* and *Rpi-sto1* were selected as broad spectrum *Rpi* genes based on virulence tests in the laboratory and greenhouse studies. It was observed that *Rpi-sto1* and *Rpi-vnt1.1* were potentially useful in all Chinese provinces and *Rpi-blb3* in three provinces and to a lesser extent in Yunnan province of China. Another observation was that not a single *Pi* isolate from China was avirulent to *R3a*. This could be confirmed with the molecular data of an overlapping and bigger set of 49 Chinese isolates (Li et al. 2009). Li et al. detected only the presence of the

Table 2 Presence and activity of *Rpi* genes in transgenic Desiree plants

l (# of) plant	PCR				<i>Pi</i> inoculation		Effector agro-infiltration		
	<i>nptII</i>	<i>sto1</i>	<i>vnt1.1</i>	<i>blb3</i>	EC1	Pic99189	<i>IpiO1</i>	<i>Avrvnt1</i>	<i>Avr2</i>
<i>Regenerants (28)</i>									
23	+	+	+	+	R	R	HR	HR	HR
5	+	–	–	–	S	S	–	–	–
<i>Control</i>									
Desiree: <i>Rpi-sto1</i> ^a	+	+	–	–	R	S	HR	–	–
Desiree: <i>Rpi-vnt1</i>	+	–	+	–	S	R	–	HR	–
Desiree: <i>Rpi-blb3</i>	+	–	–	+	R	S	–	–	HR
Desiree	–	–	–	–	S	S	–	–	–

The presence of the *Rpi* genes was determined by PCR. Biological activity of the *Rpi* genes was determined by *Pi* inoculation in detached leaf assays or by agro-infiltration using *Avr* effectors matching the introduced *Rpi* genes

HR Hypersensitive response

R Resistant

S Susceptible

“–” in PCR means no PCR product using gene specific primer pairs, and “–” in agro-infiltration means no HR symptom

^a Desiree:*Rpi-sto1* stands for one cv. Desiree transformant harbouring *Rpi-sto1*

virulence allele for *avr3a* in these isolates, indicating the general breakage of *R3a*. In contrast to the results from in house isolates, *Rpi-vnt1.1* was still a broad spectrum *Rpi* gene in Europe, and *Rpi-sto1* and *Rpi-blb3* were still applicable in the Netherlands, but *R3a* was widely broken.

Recently, a similar experiment with *Pi* isolates from Argentina was described. The response of local isolates to ten different cultivars unexpectedly showed that cv. Russet Burbank possessed an *Rpi* gene, which was useful in this country (Andreu et al. 2010). However, in the United States, this *Rpi* gene is broken because of the general susceptibility of cv. Russet Burbank to *Pi* isolates from the USA (Staples 2004). The examples above show that isolates collected from specific geographical areas can predict the value of resistant varieties or help to select broad spectrum *R* genes for gene stacking. Consequently, this information could contribute to resistance breeding for specific geographical areas, where local field experiments have to be confirmed.

The triple *Rpi* gene construct showed a transformation efficiency of 14 and 59% at the fifth and ninth month after the transformation. These results showed that the transformation efficiency of this triple *Rpi* gene construct can be as high as that from transformations with single *Rpi* gene constructs, according to

in house transformation experience (Heeres et al. 2002), but with a certain time delay. It is important to be aware of this phenomenon for future transformation experiments with stacked genes in one vector.

The aim of this study was to achieve durable resistance to *Pi* by stacking broad-spectrum *Rpi* genes. This approach inherently causes difficulties to select late blight isolates for determining biological functionality of each individual *Rpi* gene within the same plant. In this study, biological functionality could be proven for *Rpi-vnt1.1* by using isolate Pic99189, but not for the other two *Rpi* genes simultaneously. Therefore, agro-infiltration with the three *Avr* effectors *IpiO1* (*Avrsto1*), *Avrvnt1* and *Avr2* (*Avrblb3*) was used to determine the biological functionality of each integrated gene. This not only provides a strategy to detect multiple functional genes in the same plant, but also confirmed the necessity of the screening and cloning of cognate *Avr* effectors of the *R* genes used. Twenty-eight transformants were investigated in detail in this study, among which, 23 harbored all three *Rpi* genes. They gave complete resistance in the detached leaf assay, as well as HR after agro-infiltration using corresponding *Avr* effectors. It was observed that there were no silencing effects among these 23 transformants. All the integrated *Rpi* genes functioned properly.

On the other hand, there were five out of 28 transformants only harboring the selection marker gene *nptII*. This phenomenon is not unique. In experiments with rice, transformation was performed with multiple transgenes, *VHb* (*Vitreoscilla* hemoglobin gene), *tzs* (*trans*-zeatin secretion gene) and *EPSP* (modified 5-enolpyruvylshikimate-3-phosphate synthase gene) in addition to the hygromycin-gene as a selection marker, located close to the T-DNA left border (Cao et al. 2005). Cao et al. observed that three out of 113 transformants contained only the *hyg* selection marker gene, but not any target gene (*VHb*, *tzs* or *EPSP*). Our results indicated, at a much higher frequency, that T-DNA transfer might also start in a way only resulting in the functional insertion of the *nptII* gene close to the left border of T-DNA, but not simultaneously for all three *Rpi* genes.

Another significant observation is the fact that a 100% match between disease test reaction and agro-infiltration induced hypersensitivity reaction was observed for *Rpi-vnt1.1*, in 23 triple *Rpi* gene transformants (Table 2). If a transformant produced resistance in the DLA, it also showed HR response to matching *Avr* effectors. Similarly, we found that *Rpi-blb3* and *Rpi-sto1* were biologically active from both disease test and agro-infiltration experiments. This shows that agro-infiltration could be potentially a substitute for DLA on testing multiple *Rpi* gene containing plants. This predictive potential will be tested in the future for more *R-Avr* gene combinations and for different varieties. In addition, a 100% match was observed between the presence of *Rpi* genes indicated by gene specific PCR results and their biological activities after disease test and effector agro-infiltration, respectively. This indicates that, for this construct, PCR on *Rpi* genes can be directly used as an indication not only for the presence but also for the biological functionality of all three integrated *Rpi* genes in cv. Desiree transformed with this construct.

This paper adopted the approach to stack broad spectrum *Rpi* genes by *Agrobacterium*-mediated transformation using one transformation vector. This one vector and one-step transformation strategy, in comparison with re-transformation or multiple plasmids transformation, has the advantage of creating an artificial cluster of *Rpi* genes at one locus of the plant genome (Hiei et al. 1997). The plants produced in this paper, harboring these triple *Rpi* genes at presumably one locus, can be efficiently used as a breeding parent

to transfer all three *Rpi* genes simultaneously into offspring plants.

Testing of durability of *Rpi* genes is an important phenomenon. The classical differential set *R1-R11* has been used for many years as trap plants in potato fields for *Pi* population studies in order to check the presence or appearance of new (complex) virulent isolates (Dr. G. Kessel, Plant Research International, Wageningen UR, The Netherlands). In this differential set, *R2*, which is highly homologous to *Rpi-blb3*, is already a representative of *Rpi-blb3*. This type of field study can now be extended to differential plants harboring *Rpi-sto1* and *Rpi-vnt1.1* and compared with triple *Rpi* gene transformants with *Rpi-sto1*, *Rpi-blb3* and *Rpi-vnt1.1*, in order to study and compare the potential differences in trapping candidate virulent isolates.

In conclusion, this paper showed an important way to come to more durable resistance by one-step stacking of three resistance genes using one vector in potato, showing an expected broadened resistance spectrum, without the problem of any silencing effects. The use of *Avr* genes matching the *Rpi* genes to be stacked in this approach is crucial.

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