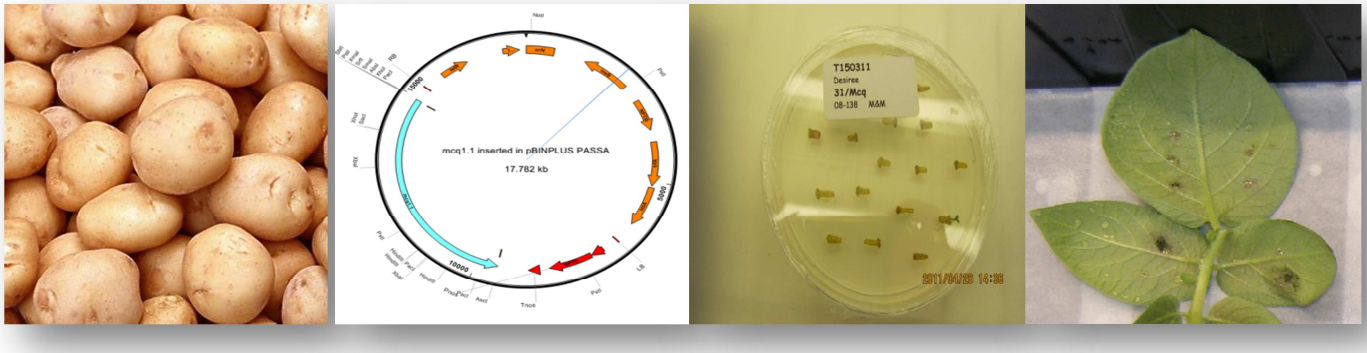
Improvement of late blight resistance on potato by transgenesis and cisgenesis



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Abstract

This project aimed at the improvement of late blight resistance in potato by means of transgenesis and cisgenesis. In transgenesis, *Rpi-mcq1* was successfully cloned into vector pBINPLUS. After single *R* gene transformation or by transformation of multiple different *R* genes, positive transformants were detected by PCR and functional assays in cisgenesis. Different transformation approaches were followed in this project in order to find out an efficient method for generating marker free plants. The results indicated that co-transformation of selection marker containing and marker free constructs is much more efficient in generating *R* gene positive plants than transformation of marker free constructs alone. However, several cisgenic plants were also selected from marker free transformation. Moreover, *in vitro* disease assay could be efficiently used as a pre-selection for positive *R*-gene containing regenerants.

Keywords: potato; marker free; (co-)transformation; *R*-gene; functionality

Thesis Outline

To improve the late blight resistance in potato by cisgenesis, three experiments were carried out.

1. To build up a Desiree transformants differential set, *Rpi-mcq1* gene was cloned from vector pCLD04541 with the amplification primers which were designed by removing some nucleotides to reduce the length of gene from the patent (Jones et al., 2010). It was transferred into the vector pBINPLUS.

2. In order to get sufficient numbers of cisgenic plants, marker free transformation with two *R* genes, *Rpi-sto1* and *Rpi-blb3*, was carried out in previous experiments. Also, co-transformation of two different binary plasmids, one with a selection marker and the other with the gene of interest, leads to integration at different genomic loci. Marker containing and marker free T-DNA will segregate in T1 progeny after which marker-free cisgenic plants can be selected (Chakravarty et al., 2007). Co-transformation of pBINPLUS: *Rpi-sto1: Rpi-blb3+Rpi-vnt1.1* and pBINPLUS: *nptII* was started in August 2010. Regenerants from both experiments were screened by PCR to select the *R* gene containing plants. In addition, insertion of vector backbone genes was analysed in order to fit the concept of cisgenesis.

3. Functional detection of *Rpi-sto1* and *Rpi-vnt1.1* genes in plants produced from different transformations was carried out in experiment 3. Agro-infiltration carried out with the *Avr* effectors was co-related with the PCR detection of relevant *R* genes. By using different *Pythophthora* isolates in detached leaf assays (DLA), the results were co-related with ATTA and PCR results for the relevant *R* genes. Finally, the efficiencies of both functional assays were compared.

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General introduction

Potato (*Solanum tuberosum*) is a perennial tuberous crop, which belongs to the *Solanum* family. Most varieties are tetraploid (2n=4x=48), whereas, there are also diploid, triploid, pentaploid and hexaploid tuber bearing *Solanum* species. As the third food crop in the world, potato increasingly gains importance in the daily life of consumers.

Late blight is the most destructive disease for potato cultivation worldwide, causing billion-dollar losses every year (Kamoun, 2001). The casual pathogen is *Phytophthora infestans*, an oomycete which also infects tomato (Judelson, 1997).

The control of late blight nowadays mostly focus on fungicide use, the use of healthy seed tubers and host resistance. However, the frequent use of fungicides increased the awareness of health and environmental risks. The high cost of fungicide application and world-wide pressure in minimizing use of chemical sprays also make host resistance a priority (Fry and Goodwin, 1997).

Most of the resistance obtained from wild species belong to 'vertical resistance' type. In this type, the resistance gene (*R* gene) or virulence gene in plants will interact with the avirulence gene in the pathogen, which is known as gene for gene model (Flor, 1971). This single *R*-gene based resistance has proved not to be durable (Malcolmson and Black, 1966). 'Horizontal resistance' refers to be polygenic or a quantitative trait loci (QTL) confers resistance to multiple races of a particular pathogen, which is provided by partial resistance genes in contrast to the vertical resistance by major *R* genes. Horizontal resistance, also called field resistance, is polygenically inherited, it is non-race specific and it is believed to provide more durability (Gebhardt, 1994; Tan et al., 2008).

If the valuable *R* gene sources were to be used one by one in new varieties, it will be very difficult to maintain resistance to the pathogen. Stacking of selected *R* genes in varieties is believed to provide enhanced durability of resistance. In traditional breeding, it is very difficult to combine *R* genes in one variety. Linkage drag of undesirable genes is another problem in traditional breeding, which needs a long time for selection. Stacking of *R* genes would prolong selection time. Genetic modification

could avoid linkage drag and directly realize a one-step gene transfer. This would save a lot of time and speed up the breeding for good cultivars. However, strict regulations on genetically modified organisms (GMO) constrain the application of this method. Recently, cisgenesis, which is the transformation of genes originating from the crop species itself or from crossable species, is becoming more acceptable. (Schouten et al., 2006; Jacobsen and Schouten, 2008). Cisgenesis does not introduce linked alien or bacterial genes. It could be a safe and quick approach for breeding of durable resistance to *Phytophthora infestans (P.infestans)* in potato.

1. Sub cloning of *Rpi-mcq1* into pBINPLUS for building a transgenic differential set in cv Desiree.

1.1 Introduction

So far, many *Rpi* genes have been cloned: *R1* (Ballvora et al., 2002) and *R3a* (Huang et al., 2005b) from *S. demissum*; *RB/Rpi-blb1* (Song et al., 2003; Van Der Vossen et al., 2003), *Rpi-blb2* (Vossen et al., 2005), *Rpi-bt1* (Oosumi et al., 2009) and *Rpi-blb3* (Lokossou et al., 2009) from *S.bulbocastanum*; *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al., 2008) and *Rpi-vnt1.1*, *Rp-vnti1.2* and *Rpi-vnt1.3* from *S.venturii* (Foster et al., 2009; Pel et al., 2009), *Rpi-mcq1* from *S. mochiquense* (Smilde et al., 2005). Additionally, Lokossou et.al (2009) have described one of the major late blight resistance gene clusters in which *Rpi-abpt*, *R2* and *R2–like* were identified. *Rpi-phu1* on chromosome 9 (Sliwka et al., 2006), R_{ber} on chromosome 10 (Ewing et al., 2000) and *R3b*, *R6*, *R7*, *R10*, *R11* on chromosome 11 (El-Kharbotly et al., 1994; El-Kharbotly et al., 1996) have also been identified.

Rpi-mcq1 was identified and genetically mapped on chromosome 9 (Smilde et al., 2005) and cloned to vector pCLD04541 in 2010 (Jones et al., 2010). Effector studies have shown that *Rpi-mcq1* recognized *Avr2* (Internal communication), which is also recognized by *Rpi-blb3* (Lokossou et al., 2009).

To meet the requirement of building a transgenic differential set in C.V. Desiree, *Rpi-mcq1* was supposed to be sub cloned to vector of pBINPLUS, which is commonly used in the Laboratory of Plant Breeding, Wageningen University and Research Centre. A smaller T-DNA part can be more easily inserted in the plant genome by *A.tum.* To make the target genes smaller in size, the genomic sequence was

downloaded from NCBI. Gene positions were predicted using Fgenesh. By using DNA star, primers were designed to amplify the complete *Rpi-mcq1* gene.

1.2 Materials and methods

Gene *Rpi-mcq1* in vector pCLD04541 in *E. coli* was kept in glycerol stocks at -80℃ and maintained by Marjon Arens. The vector pBINPLUS was given from Paek Yong-Gi.).

1.2.1 PCR amplification of Rpi-mcq1

Long range PCR with *Phusion* DNA polymerase: 50 µl reaction mixture was prepared containing 10 ng of DNA template, 2.5 µl of the forward primer (10 µM), 2.5 µl of the reverse primer (10µM), 1ul of dNTPs (10 mM), 10ul of 5**Phusion* HF buffer, 0.01 units of *Phusion* DNA polymerase (0.02 U/µl). A three -step protocol was used in the PCR program: 98℃ for 30s for initial denaturation; 98℃ for 10s, 58℃ for 30s, 72℃ for for 4 mins, during 30 cycles; 72℃ for 10mins. The primers for amplifying *Rpi-mcq1* were designed based on the sequence published and specific enzyme sites of *sbfI* and *AscI* were added by including some nucleotides in front to make restriction sites. *SbfI* and *AscI* were selected based on restriction maps of pBINPLUS-PASSA and the published sequence of *Rpi-mcq1*. No sites were present within the *Rpi-mcq1* gene, and the sites were adjacent to each other in the multiple cloning site of the vector. The sequence for this pair of primers was listed in Appendix 1.

4 µl of PCR reaction together with 6 µl of Milli Q water and 2 µl of 10* loading red were mixed and loaded on 0.8% agarose gel to check about the PCR results.

1.2.2 PCR product purification

PCR product was extracted by phenol-chloroform extraction in order to remove *Phusion*. Successively, an ethanol precipitation was performed in order to concentrate the DNA. The pellet was air dried and dissolved in 10 µl of MQ water. The DNA quality and quantity were checked on the gel.

1.2.3 Enzyme digestion and ligation

A 1 µg aliquot of DNA for *Rpi-mcq1* from chloroform extraction and ethanol purification was used in this step in a 50 µl reaction volume. 5 µl of *SbfI* (10U/µl, Fermentas, Netherlands) was added in the reaction mixture for overnight incubation at

37℃. It was inactivated by incubation at 80℃ for 20min. DNA of vector pBINPLUS passed the same procedure as that of *Rpi-mcq1* PCR product.

Sbf1 digested fragments were concentrated using ethanol precipitation. and were successively digested with *Asc*I., *SAP* enzyme was used after *AscI* in vector pBINPLUS for avoiding the self-ligation happening in the vector itself. It was inactivated at 65℃ for 20mins. After that, the purified fragments from ethanol precipitation were run on the gel to check the digestion efficiency and quantity of DNA amount.

A ligation mixture (20 µl) containing 100 ng of vector DNA, approximately 135 ng of digested DNA of *Rpi-mcq1,* 10*T4 DNA *Ligase* Buffer and 5 units of T4 DNA *ligase* was incubated at 16℃ for overnight. After ligation, the T4 DNA *ligase* was inactivated by incubation at 65℃ for 10min.

1.2.4 Desalting and Transformation of E. coli

Inactivated ligation mixture was desalted firstly. A small petri dish was poured with T10E1 buffer at the bottom to ½ the volume. A 0.025 µl VSWP filter (Millipore) was placed with the shiny side up on the surface right of the buffer. Ligation mixture was pipetted on the filter and lid was closed. For 3 hours at room temperature incubation, the ligation mixture was desalted. It was transferred into a new tube for electroporation. Competent MegaX DH10B cells (Invitrogen, Netherlands) was defrosted on ice firstly. 9 µl of the cells was pipetted into the pre-cooled tube containing of desalted ligation mixture approximately 1 µl. Gene PulserII (Bio-Rad) was set at 2.0 KV, 25 µ F and 200 MOhm. The electroporation was successful with a time constant between 4 and 5. 500 µl clean LB medium was added to the electroporation mixture and the cells were recovered by shaking at 225 rpm for 1 hour at 37℃.

1.2.5 Blue/white selection

Agar plates containing LB broth and Kanamycin were poured out before use in flow cabinet. 40 µl of IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 µl of Xgal (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside) were spread on top of the plate with a triangular spreader. After 30 min the plates were dried enough for using.

100 µl of the transformation culture was spread on the plate. The remaining 400 µl of culture was spread on another plate to make a comparison with the 100 µl spread after overnight culture in the oven at 37℃ with upside down.

1.2.6 Colony PCR

PCR reaction mixture with a volume of 25 µl was prepared before use. It contains 0.5 µl of the specific forward primer (10 µM), 0.5 µl of the specific reverse primer (10µM), 0.5 µl of dNTPs (10 Mm), 2.5 µl of 10*Dream *Taq* buffer, 0.25 units of Dream *Taq* DNA polymerase (5 U/µl) and 20.5 µl of sterile water. Primers were designed based on the sequences in the insertion and in the vector backbone. Forward primer was located at the start of *Rpi-mcq1* gene and reverse primer was located on the vector backbone near *sbfI* cutting side. They will give a product with size of 710 bp. The detailed information of the specific primers can be found in Appendix 2.

White colonies were picked up by sterile tip and transferred on a new agar plate with the clear label at bottom. After placing potential positive colonies on the plate with specific labels for each one, the tip was put in prepared PCR reaction mixture directly after and stranded for several seconds.

Totally, 16 colonies were picked up for each construct. Colony PCR was carried out with the following program: 95℃ for 30s for initial denaturation, 95℃ for 5mins, 58℃ for 30s, 72℃ for 1min, 72℃ for 10mins during 30 cycles.

1.2.7 Enzyme digestion for confirming the construct

Colonies that were positive in the colony PCR were grown and plasmids were isolated. Two different enzymes were used for digestion of the plasmid DNA isolated from *E. coli*. Enzyme selection was based on the restriction enzyme sites inside the expected construct. *HindIII* will cut the plasmid into 3 parts producing 3 bands in different positions in the gel. Also *BglII* digestion was carried out. Two bands were expected to be in the gel. A 10µl mixture with 1 µl of DNA (0.5-1 µg/µl), 1 µl of 10*Buffer Red (Fermentas, Netherlands), 0.5 µl of *HindIII or BglII* (10 U/µl) and 7.5 µl of nuclease-free water were mixed gently and incubated at 37℃ for 2 hours. Digestion results were checked from agar gel by mixing with 2 µl of 10*loading Red.

1.2.8 DNA sequence analysis

Two colonies 3-15 and 1-13 showed the right band in colony PCR and being confirmed by enzyme digestion in 1.2.7. They were cultured in liquid LB medium supplemented with Kanamycin at a concentration of 50 ng/ml for overnight at 37℃. In order to get enough DNA, a larger volume of culture was done by taking 30 ul of the pre-culture into 15 ml new culture with the Kanamycin in the same concentration. The DNA was isolated by following Midi prep protocol of QiaGen (Hilden, Germany). DNA concentration was measured from the gel together with the λ-DNA. Sequencing reaction was undertaken with the guide of Greenomics with the covered primers. Information about the sequence primers was listed in Appendix 3. The insert sequence of 3-15 turned out to contain a mutation leading to an amino acid change and was discarded. 1-13 was checked without mutations in the insert and used.

1.2.9 Transformation of Agrobacterium tumefaciens and colony PCR

The successfully built construct 1-13 was transformed to *A.tum* strain *AGL1+pVirG* by electroporation. Frozen tubes of competent cells were placed on ice for 5-10mins before using. The cuvettes were cleaned by ethanol and water with drying in flow cabinet and placed on ice before using. A mixture with 20 µl of *A.tum* and 2µl of plasmid DNA (with the amount at 100-200 ng) was pre-prepared in the flow cabinet. The electroporation machine Gene PulserII (Bio-Rad) was set at 25 in micro FP capacitance, 250 in capacitance extender and 200 in pulse controller. The voltage was set at 1.4. The cuvette went through electric at that set for a time constant between 4 and 5. 500 µl of clean LB medium was added and the transformation mixture was transferred into a 1.5 ml tube. For culturing, the bacterium was kept in an incubator at 30℃ with shaking. After 3 hours, the bacteria was plated out in the previously dried LB-agar plate supplemented with Carbenicillin at 100 ng/ml, Chloramphenicol at 75 µg/ml and Kanamycin at 50 ng/ml. The plates were kept upside down in the small oven at 30℃ for 2 days. Colony PCR was carried out on the picked up colonies from transformation of *A. tum* with the same method mentioned in 1.2.6.

1.2.10 Transformation of E.coli and Enzyme digestion for stability test

Positive colony from step 1.2.9 was maintained in glycerol stock and cultured at 30℃ by shaking. The plasmid DNA was isolated by using mini prep kit (Qiagen) and transformed into *E. coli* again. The method was the same as 1.2.4.

Colonies were picked up from transformation of *E. coli* and were done with colony PCR. Plasmid DNA was isolated from the cultured positive colonies. Enzyme digestion from *Hind III* was carried out on the isolated DNA, which aimed to see whether the construct was stable. The method used here was mentioned in 1.2.6 and 1.2.7 as well.

1.2.11 Gene functionality test from N.benthamiana complementation assay

The use of *N. benthamiana* complementation assay is an efficient tool for rapid functional screen of putative *R* genes. Infiltration of *Agrobacterium* strains carrying the candidate *Rpi-mcq1* and *Avr2* were performed in *N. benthamiana.* A detailed description of the *A. tum* strain preparation was described in Appendix 4.

1.3 Results

Rpi-mcq1 was amplified from vector pCLD04541 as the template with primers *Rpi-mcq1*-F1 and *Rpi-mcq1*-R1. A 5.4 kb fragment carrying the *Rpi-mcq1* promoter, open reading frame (ORF) and terminator was amplified as shown. When compared with the marker ladder in lane1, the fragment is higher than the band of 4000 bp. From the gel picture, the brightness of the bands also showed the DNA amount was enough for following experiments. There were no other bands except for the right size band, so the quality of the DNA was also sufficient to proceed with the following work (Figure1a). Purified PCR products which contained fragment of *Rpi-mcq1* with modified enzyme sites of *sbfI* and *AscI* were cut with these two enzymes. A bright band with the right size was seen between 4000 bp and 5000 bp. The cut out small fragments were several nucleotides length and are not visible from gel. There were also some weak bands appearing above and under the position of right size band (Figure 1b). Also the cloning vector, pBINPLUS, was digested with *sbfI, AscI* and treated with *SAP* (Figure 1c). The brightest band in lane 1 clearly showed the digested vector fragment was at the right position of 12.4 kb. One extra band was found below the right one. The quality reflected from the right size band showed the amount was enough for ligation in next step. PUC57 showed the expected size at around 2700 bp (Figure 1c lane 2).

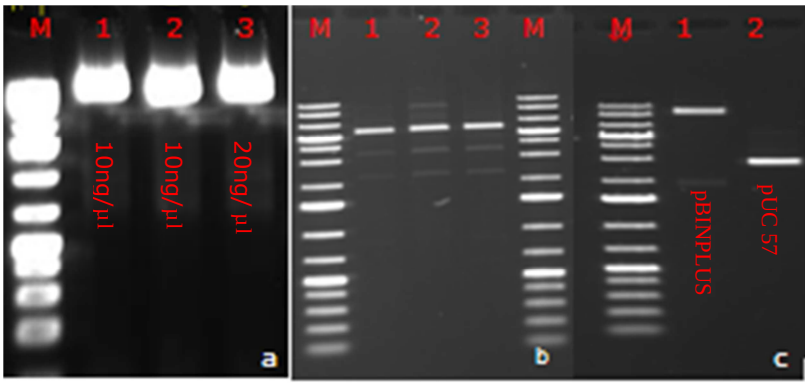


Figure 1 PCR amplification of *Rpi-mcq1*, PCR product purification and enzyme digestion of *Rpi-mcq1* vector of pBINPLUS

Picture a, the amplification of *Rpi-mcq1* started with different DNA concentrations in PCR reaction, which were indicated from the numbers in each lane. Picture b, PCR products were purified and digested by *sbfI & AscI*. Picture c, the digested vector pBINPLUS by *sbfI & AscI* was checked from gel, with pUC 57 as the control.

At last, two samples which were indicated from lane 1 and lane 2 were used in the ligation (Figure 1b). Colony PCR was carried out to screen for positive recombinants in ligation. 16 colonies in each construct were picked up from the plate and done with PCR. Two positive colonies, showing a product of 5.8 kb since the same primers were used as in the amplification, were identified: 3-15, 1-13 (Figure 2a). The result from picture b clearly confirmed that the recombinants were successfully made in 3-15 and 1-13 (Figure 2b).

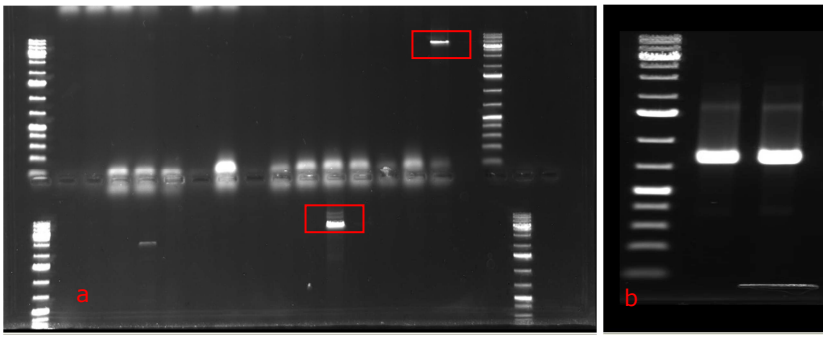


Figure 2 Colony PCR from *Rpi-mcq1* recombinant clones in *E.coli*.

Positive colony gave a band of approximately 5.8kb in picture a. There were 2 colonies selected (red box) and named as 3-15, 1-13 respectively. In picture b, PCR on the recovered bacterial culture was carried out by using primers *mcq1*-F1 and pBIN-R1, which gave a product of 710 bp.

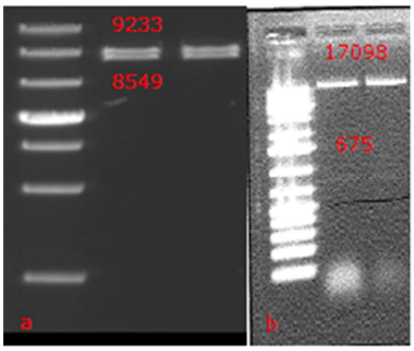


Figure 3 Confirmation of successful constructs in *E. coli* by enzyme digestion of *BglII* and *HindIII* respectively.

DNA isolated from 2 recombinants of 3-15 and 1-13 were digested by *BglII* in picture a. Picture b shows the digestion results from enzyme *HindIII* on the same recombinants as in picture a.

In order to confirm the integrity of the construct, *BglII* and *HindIII* digestion on the isolated DNA from positive colonies 3-15 and 1-13 were performed (Figure 3). There were two bands in the expected sizes from *BglII* digestion (Figure 3a). 3 parts were cut out from *HindIII* in pBINPLUS*::Rpi-mcq1*. As shown in Figure 3b, two bands were visible with the right positions at 17098 and 675 bp, the 9 bp fragment was not visible due to the small amount.

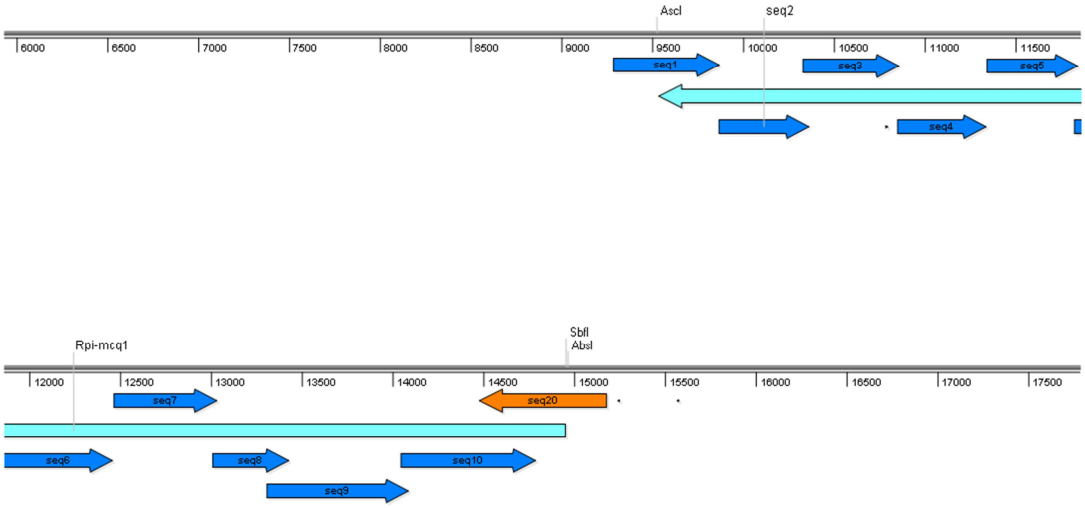


Figure 4 Sequencing map of *Rpi-mcq1*.

The light blue fragment means the amplified sequence of *Rpi-mcq1*. It starts from *sbfI* site and ends at *AscI* site, which were indicated from the arrow and the enzyme sites. The small

fragments with dark blue and orange colour were the single strand sequences from sequencing and aligned to the template DNA.

The inserts from clones 3-15 and 1-13 were sequenced in order to rule out PCR artefacts. Nine internal primers were designed and Results are summarized in figure 4. The whole sequence of *Rpi-mcq1* was covered. Sequence results analysis showed that one nucleotide was mutated in 3-15. And there were no mutations found in 1-13. Construct of *Rpi-mcq1* in pBINPLUS was successfully made in 1-13.

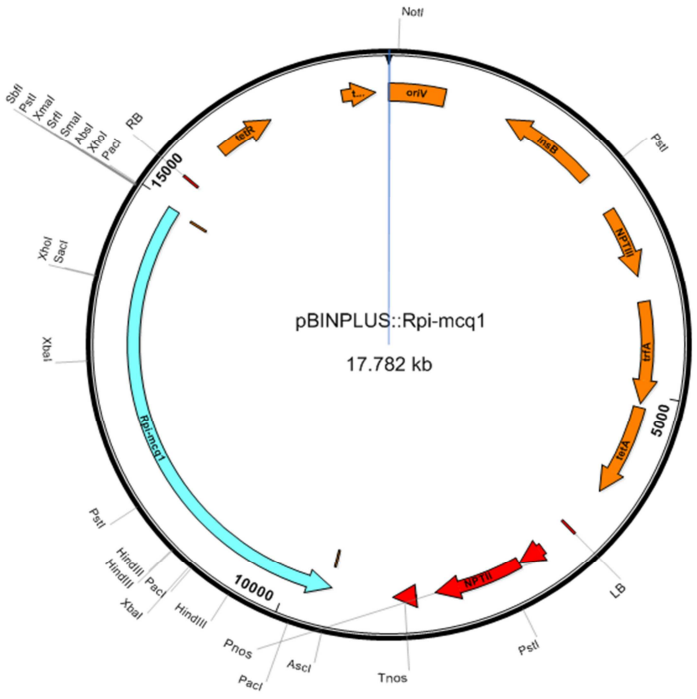
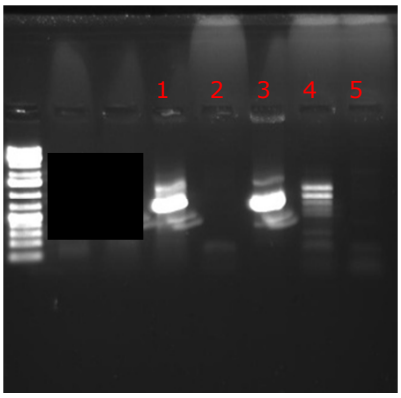


Figure 5 Map of pBINPLUS::*Rpi-mcq1* from Genestat. *Rpi-mcq1* was marked with the light blue arrow with the orientation from *sbfI* to *AscI*.

The backbone genes are also visible from this map with the orange and red arrows indicated. Some enzyme sites are also marked out from this map.

Transformation of construct 1-13 into *A.tum* was carried out successfully. Two colonies were picked up and cultured in liquid LB medium. They were named as 2(1) and 2(2) from 1-13. Colony PCR showed colony 2(1) positive in using the specific primers (Figure 6).



1. Colony 2(1)
2. Colony 2(2)
3. Positive control
4. Atlantic genomic DNA (negative control)
5. Water control

Figure 6 Colony PCR after transformation of *A. tum.*

Lane 1 and 2 are PCR results of colonies picked up from 1-13 transformation with primer pair of mcq1-F1 and pBIN- R1Positive control is the plasmid DNA isolated from 1-13 (Lane 3). And negative control here are Atlantic genomic DNA and milli Q water (Lane 4 and 5).

Stability of the constructs in Agrobacterium was tested. The picked up colonies from transformation of *E.coli* all showed the target band in PCR reaction with the specific primers (Figure 7). Enzyme digestion from *HindIII* was followed out. And the cut out bands also gave bands in the right positions at 17098 bp, 675 bp. The 9 bp fragment was not visible in this gel. The 10 colonies showed the consistent results and had confirmed the construct could be stably used in transformation.

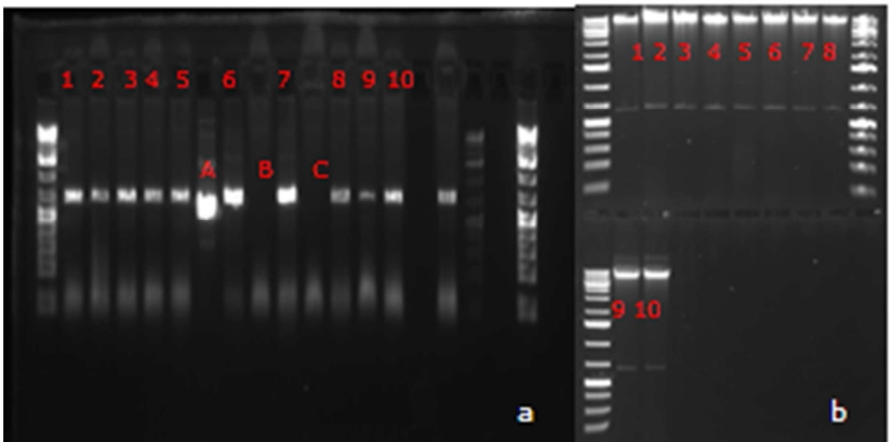
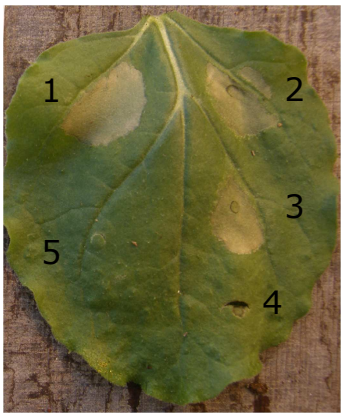


Figure 7 Colony PCR for transformation of *E. coli* and enzyme digestion on the selected out positive colonies.

Lane A was the positive control of plasmid DNA from 1-13. Lane B was from negative control of isolated DNA of vector pcld04541:: *Rpi-mcq1*. Lane C was the milli Q water as negative control. Number 1 to 10 means the ten colonies picked up from transformation of *E. coli*.



1. *Avr2+Rpi-blb3* OD600nm: 0.2

2. *Avr2+Rpi-mcq1* OD600nm: 2.0

3. *Avr2+Rpi-mcq1* OD600nm: 1.0

4. *Rpi-mcq1* OD600nm: 2.0

5. *Rpi-Blb3* OD600nm: 0.2

Figure 8 Complementation of *Rpi-mcq1* by transient expression in *Nicotiana benthamiana*.

Spot 1 is the positive control from co-infiltration of *Avr2* and *Rpi-blb3.* Spot 2 and 3 are the co-infiltration of recombinant 1-13 and *Avr2* at different OD values. Spot 4 and 5 are the negative controls from infiltration of recombinant and Rpi-blb3 respectively.

Functional assay of the recombinant was carried out by ATTA. Colony 2(1) which was the transformation of *A.tum* from 1-13, showed hypersensitive response when complementing with *Avr2* at both OD600nm of 1.0 and 2.0. Positive control in spot 1 worked well at an OD600nm of 0.2 with hypersensitive response while negative controls in 4 and 5 were showing no response as expected.

In summary, construct pBINPLUS:: *Rpi-mcq1* was successfully made in colony 1-13. This colony has been tested of being stable used in transformation. Further research would be carried out on this sub-cloned gene of *Rpi-mcq1*.

2. Different transformation methods in getting cisgenic plants

2.1 Co-transformation using marker containing and marker free vectors followed by sexual crosses

2.1.1 Introduction

Several methods were reported in making marker free transgenic plants, which include simultaneous transformation of two marker genes(co-transformation), the movement of a transgene segment within the genome (transposition), and recombination between two specific sequences that are not necessarily homologous (site-specific recombination) (Darbani et al., 2007). Co-transformation of two

independent T-DNAs, one with a selection marker and the other with the gene of interest, leads partly to integration at different loci which will segregate in the progeny, generating marker-free offspring plants (Chakravarty et al., 2007). Transposition can also be recognised as marker free transformation by designed vector with the movement of transgene (marker gene). Co-transformation as one way of getting cisgenic plants was carried out in this experiment. The efficiency of both two methods in generating cisgenic plants was compared to investigate an efficient way for improvement of late blight disease resistance in potatoes.

2.1.2 Materials and Methods

2.1.2.1 Materials

There were 200 explants cut out from the stems of cv. Atlantic and used for co-transformation of two independent vectors, one with the selection marker *nptII* and the other with the stacked *R*-genes of *Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*. The co-transformation was performed under four different ODs, which was marked as four groups.

Table 1 Overview of starting ODs and explant numbers in different groups of co-transformation of pBINPLUS::*nptII* and pBINPLUS:: *Rpi-sto1+Rpi-vnt1.1+ Rpi-blb3*.

Group	OD values from two vectors	Explant numbers	Regenerants numbers
A	0.8/0.8	200	2
B	1.0/0.5	200	30
C	1.6/0.8	200	6
D	1.6/0.5	200	24

The first number / was the OD value for pBINPLUS::*nptII,* and the second number refers to OD of pBINPLUS:: *Rpi-sto1+Rpi-vnt1.1+Rpi-blb3*.

2.1.2.2 Methods

Explant maintenance and plantlet preparation

Explants were maintained in the flat plate (diameter 10 cm) filled with LB medium supplied of 8 g of agar, 1ml of Zeatine (1 mg/l), 2ml of Cefotaxime (100 mg/l), 2ml of Vancomycine (100 mg/l) and 2ml of Kanamycin (50 mg/l) for per litter. By refreshing the medium every two weeks, the explants stayed in the circumstance without

contamination. Kanamycin allowed the regenerants being pre-selected by marker *nptII.*

Regenerants were cut out from the explants from the bottom by removing the callus. It is to avoid that new regenerants are growing from the old position again. With the help of a knife, the lower part of the regenerant was removed and the upper side of the whole plantlet was transplanted to plastic pots (diameter 15 cm) with MS20 supplied only with 2 ml of Cefotaxime (100 mg/l) and 2 ml of Kanamycin (50 mg/l) per litter. All of these were done in sterile flow cabinet.

The filled plastic pots with 8 plantlets were marked with the date of harvesting and moved to the climate room. After about 2 weeks, they were rooted and big enough for DNA isolation.

Leaf collection and DNA isolation

Two or three leaves from one plantlet were collected in a flow cabinet. The box with 96 micro tubes was placed on ice to keep cool. DNA isolation on the 96 filled tubes was carried out by following the protocol of Mini method from King-fisher machine. DNA was directly used for PCR reaction in next steps since there was no large difference for concentrations of the 96 samples by using King-fisher machine.

PCR screen for R-genes and backbone genes

PCR reaction was carried out on the plant genome DNA by designed specific primers. The primer information can be found in Appendix 6. In order to separate the target band of *Rpi-sto1* with the background band from cv Atlantic, the gradient PCR was carried out firstly. The samples in testing *Rpi-sto1* all followed the temperature being selected. Backbone gene detection from PCR was to fit the aim of being cisgenic or not. All the PCRs were done with a reaction volume of 25 µl, containing 3 µl of DNA template, 2.5 µl of 10*Dream *Taq* buffer, 0.5 µl of dNTP, 0.5 µl of forward primer, 0.5 µl of reverse primer and 0.5 µl of *Taq* enzyme. The running programs are different based on the product size and Tm of primers.

Agar gel was run to check the results with a 12 µl of mixture containing 4 µl of PCR products, 6 µl of Milli Q water and 2 µl of gel red.

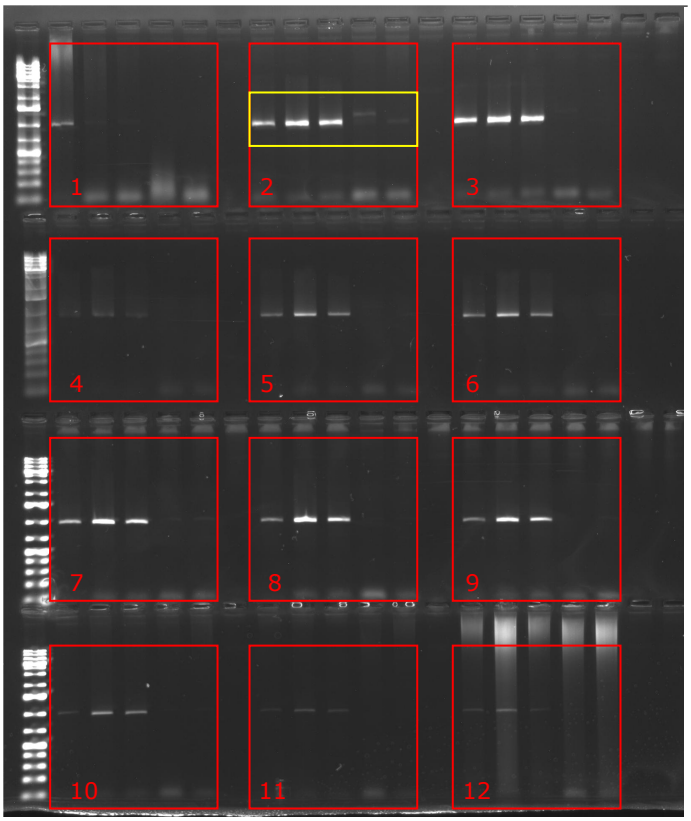
2.1.3 Results

Gradient PCR gel in Figure 9shows the separation of the specific fragment of *Rpi-sto1* and background bands in cv Atlantic. When annealing temperature was at 59.0℃, cv Atlantic showed one band higher than the target band of *Rpi-sto1* in yellow box in block 2. By following this temperature of the specific primer pairs, PCR results on all the samples showed that *Rpi-sto1* gene was present in all the regenerated, kanamycin resistant, plants. From the samples of transgenic cv Atlantic plants, DNA quantity can also be judged from the higher position background band from cv Atlantic.

PCR reaction for screening *Rpi-blb3* showed that 9 plants in 62 gave a product of 300bp compared with the positive control. Screening for *Rpi-vnt1.1* was also carried out on all the regenerants. There were 9 regenerants showing the target band at the right position of around 300 bp. These 9 plants corresponded with the positive plants selected for positive *Rpi-blb3* (Table 2).

In transformation, the proportion for *nptIII* insertion by PCR screening could indicate the whole backbone insertion in the plant genome at a level of 98% (Internal communication). While in co-transformation, it is less informative since there are two different T-DNAs inserted in the plant genome.

In order to get cisgenic plants, other vector backbone genes were also tested beside *nptIII*. From left border till right border, there were totally 7 genes being selected. *VirG* gene as a signal for evaluation of *A.tum* contamination was also included. Table 2 summarized results for *R* genes, backbone genes and *virG* by sorting regenerants in harvesting periods.



From block 1 to 12, there is a Tm gradient specifically with 58.8℃, 59.0℃, 59.4℃, 60.1℃, 60.8℃, 61.5℃, 62.3℃, 63.0℃, 63.7℃, 64.4℃, 64.8℃ and 65℃. In each block, there is a loading order from left to right of 55-3:11, 117-3:10, 12-2:7, Atlantic and Milli Q water. The first three samples are the positive *Rpi-sto1* containing plants which were detected previously. Cv Atlantic and Milli Q were both set as the negative control.

Figure 9 Gel electrophoresis results of gradient PCR for separating *Rpi-sto1* gene in plants containing this gene from cv Atlantic background genes.

Proportions of plants containing *R* genes, backbone genes and *virG* respectively in different groups were also calculated in Table 3. It turned out that a low frequency of plants was contaminated with *Agrobacterium*.

In group A, the starting ODs for the two constructs were both at 0.8. There were two regenerants harvested totally in this group. In group B, the starting OD in marker free construct with stacking *R* genes was 1.0. The other one for the *nptII* containing construct was 0.53 with OD value. 30 regenerants were harvested from explants in this group. Group C and group D were different with the *nptII* containing construct at OD of 0.8 and 0.53 respectively. The starting construct of the other one with *R*-genes was the same at OD of 1.6 (Table 3). The number of kanamycin resistant plants was much higher in group D.

For *R* genes screening by PCR to select cisgenic plants, group D gave a much higher proportion in number of plants containing the triple *R* genes. Group B was in the second place in efficiency of getting the target plants. There were no triple *R* genes containing plants in group A and C. For the backbone genes detection, group D

showed the lower insertion proportion in most of the genes except for *traJ* when compared with group B. For contamination comparison from *virG*, group D also showed lower proportion than B.

To get plants which were positive in stacking *R* genes and also free in backbone genes, the triple *R* genes containing plants are shown in Table 4. All 9 plants were *nptIII* free but only 4 of them were cisgenic, without any backbone. They were H62-co1-4-5, H62-co2-4-3, H62-co2-4-4 and H62-co2-4-8. From results of *virG* testing in these 4 plants, they were also showing no contamination with *A. tum.* The first 6 plants from this table were carried out with trial PCR detection for estimating direct linkage between the inserted *Rpi-sto1* and *nptII* in the genome.

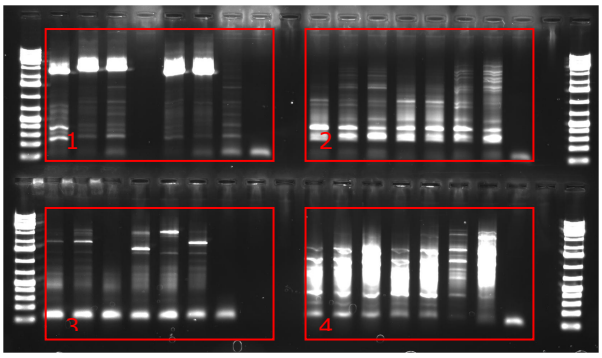


Figure 10 PCR Trial on plants containing triple R-genes and resistant to Kanamycin with different primer pairs in measuring the distance between *Rpi-sto1* and *nptII*.

In each block, a different primer pair was used. The loading order in each block is the same from left to right: H62-co2-4-3, H62-co2-4-8, H62-co2-4-4, H62-co1-4-2, H62-co1-4-3, H62-co1-4-5, cv Atlantic and Milli Q water. Block 1, 2, 3 and 4 were results from primer pairs corresponding with the primers in Appendix 7 and Figure 10.

In block 1 of figure 10, there were consistent bands in the six samples except for H62-co1-4-2. The size for this band was around 2800bp. While in block 2, 3 and 4, bands in different samples were not comparable. Primer pair 1 in construct pBINPLUS PASSA*::Rpi-sto1+Rpi-vnt1.1+Rpi-blb3* showed that the amplification from this primer pair would give a product size of 2770bp. In conclusion, *Rpi-sto1* and *nptII* were connected together in the genome of plants being detected positive in containing *R*-gene(s), which are predicted to be difficult for separation by sexual crossing in future.

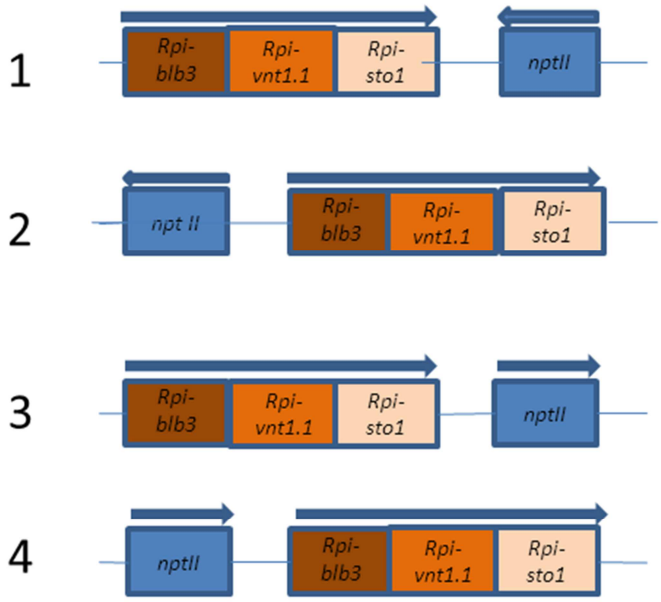


Figure 11 Four different insertion ways in determining four pairs of primers.

In 1 and 2, the triple gene construct and *nptII* construct were supposed to be inserted in their original order in the map Appendix 8. There is another way around in 3 and 4, which described that *nptII* construct was inserted in the opposite orientation compared with 1 and 2. The arrows on top of the genes mean the orientation for insertion.

In conclusion, there are totally 9 regenerants showing positive in containing the triple *R*-genes while 4 of them are backbone free. As mentioned before, these four regenerants are predicted not to be successful after sexual crossing in separating the triple *R*-gene construct from *nptII* because of the obserbed linkage between both constructs.

2.1.4 Discussion

This experiment demonstrated that group D, with a high OD for the triple *R*-genes construct and a low OD for the construct with selection marker *nptII*, was efficient in getting the triple *R*-genes containing plants compared with the proportions in the other groups. The Agrobacterium-mediated co-transformation frequency for various species was reported to be 100% for linked genes in a unique T-DNA or for linked T-DNAs in a single binary plasmid, and ranged from 30% to 85% for independent T-DNAs in separate binary plasmids (Block and Debrouwer, 1991; Komari et al., 1996; Daley et al., 1998; Vidal et al., 2003). In this experiment, the co-transformation

efficiency was 100% for *Rpi-sto1* and 33% in group D for the simultaneous presence of all 3 linked *R*-genes. Delivery of two DNAs from different concentrations were compared, and group D was significantly higher in efficiency of co-transformation in generating triple *R*-genes contanining plants. PCR analysis of the backbone genes and *A.tum* contamination also indicated that group D performed well with lower proportion for most of the genes tested. Therefore, transformants free from selection marker and harbouring stacked *R* genes had higher chance of being from Group D.

The long term goal in this experiment is to get cisgenic plants after sexual crossing. The two T-DNAs had to be inserted in different part of the genome in order to generate the plants being available to be crossed in future. Molecular analysis of the segregation of marker genes are usually done in the progeny by selfing or backcrossing with a non-transformed plant. Successful segregation of marker genes was reported as drug resistance and GUS expression behaving as two independent Mendelian loci in progeny (Komari et al., 1996). Here, we proposed a pre-selection method for the molecular analysis by PCR reaction with specific primer pairs. In this experiment, samples being choosen from backbone genes free plants indicated that the marker gene probable was linked with the R genes(s) from the consistant band from block 1 (Figure 10). Therefore, these plants would not be used for the future crossing to discard the marker gene. However, cross experiments have to be made to confirm this conclusion. Transformants showing all positive for *Rpi-sto1* gene but not for all 3 *R*-genes appeared to be a strange phenomenon in this experiment. More research is needed to confirm and explain this observation.

Table 2 Summary of 4 groups of plants after co-transformation by PCR analysis for presence of *R*-genes, backbone genes and *virG*.

			Positive No. in *R*-gene test			Positive No. in backbone-gene test (from RB to LB)							
Groups	Harvesting time	No. of regenerants	*Rpi-sto1*	*Rpi-blb3*	*Rpi-vnt1.1*	*tetR*	*traJ*	*oriV*	*nptIII*	*trfA*	*tetA*	*ori3+5*	*virG*
A	1	1	1	0	0	0	1	0	1	1	1	0	0
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	1	1	0	0	0	1	0	1	1	1	0	0
B	1	8	8	0	0	2	4	3	3	4	6	0	0
	2	2	2	1	1	2	1	2	3	2	3	1	1
	3	20	20	0	1	14	0	9	13	16	17	1	0
C	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	5	5	0	0	0	0	0	2	1	1	0	0
	3	1	1	0	0	0	0	0	1	0	1	0	0
D	1	8	8	3	3	3	5	3	1	5	3	0	0
	2	8	8	4	4	0	1	0	1	1	0	0	0
	3	8	8	1	2	5	5	4	4	6	6	1	0

Table 3 Proportion of transgenic plants containing *R*-genes, backbone genes and *virG* respectively in different groups.

Groups	No. of regenerants	*Rpi-sto1*	*Rpi-blb3*	*Rpi-vnt1.1*	*tetR*	*traJ*	*oriV*	*nptIII*	*trfA*	*tetA*	*ori3+5*	*virG*
A (0.8/0.8)	2	100%	0%	0%	0%	100%	0%	100%	100%	100%	0%	0%
B(1.0/0.5)	30	100%	3%	3%	60%	17%	47%	63%	73%	87%	7%	3%
C(1.6/0.8)	6	100%	0%	0%	0%	0%	0%	50%	17%	33%	0%	0%
D(1.6/0.5)	24	100%	33%	33%	33%	46%	29%	25%	50%	38%	4%	0%

In brackets from A, B, C and D, it is referred to the concentrations for the starting constructs. For example, in group A, 0.8/0.8 means the co-transformation from marker free with R genes containing construct and nptII containing construct started with the ODs at 0.8 and 0.8 respectively.

Table 4 Analysis of triple *R*-genes containing plants from co-transformation under PCR screening for backbone genes and potential linkage between *Rpi-sto1* and *nptII*

	R-gene test			Backbone-gene test(from RB to LB)								
Plants codes	*Rpi-sto1*	*Rpi-blb3*	*Rpi-vnt1.1*	*tetR*	*traJ*	*oriV*	*nptIII*	*trfA*	*tetA*	*ori3+5*	*virG*	*Rpi-sto1+nptII*
H62-co1-4-5	+	+	+	-	-	-	-	-	-	-	-	+
H62-co2-4-3	+	+	+	-	-	-	-	-	-	-	-	+
H62-co2-4-4	+	+	+	-	-	-	-	-	-	-	-	+
H62-co2-4-8	+	+	+	-	-	-	-	-	-	-	-	+
H62-co1-4-2	+	+	+	-	+	-	-	+	-	-	+	-
H62-co1-4-3	+	+	+	+	+	+	-	+	+	-	+	+
H62-co3-2-15	+	+	+	-	+	-	-	+	+	-	-	~
H62-co3-4-1	+	+	+	+	+	-	-	+	-	-	+	~
H62-co3-4-7	+	+	+	-	-	-	-	-	+	-	-	~

2.2 Marker Free multiple R genes transformants in one vector.

2.2.1 Introduction

To distinguish rare transformation events against large amount of non-transformed cells is a major challenge in the generation of transgenic plants. Selectable marker genes (SMGs) have been widely used in enabling plant transformation because the transformed cells are resistant to the appropriate antibiotic or herbicide. However, the SMGs are known to inhibit growth and regeneration of transformed cells thereby decreases transformation frequency. In addition, SMGs integrated into the plant genome, arises concerns about widespread occurrence of such transgenes in novel ecosystems (Zuo et al., 2002).

For selection of marker free transgenic plants, PCR screening is mostly used. To apply this strategy on thousands of regenerants is still a tough thing. Thereby a fast and accurate pre-selection from *in vitro* disease test of potentially transformed plants is an alternative. Based on gene to gene theory, *in vitro* plantlets went through the inoculation of appropriate *P.infestans* isolates. Plants which contain the relevant *R*-gene would be resistant to the isolates tested.

Potato is very amenable to tissue culture. In an *in vitro* environment, humidity and temperature were manipulated for *P.infestans* to germinate and develop on potato plantlets (Huang et al., 2005a).Thus, the *in vitro* disease assay as an pre-selection for the potential resistant transgenic plants is valuable in discarding the susceptible plants. This method narrowed down the PCR screening numbers of plants, which is also economic friendly.

P.infestans isolate 90128 harbours avirulence to *Rpi-blb3 and also to Rpi-sto1*. Therefore, it could be used for a pre-selection of functional T-DNA insertion.

2.2.2 Materials and Methods

Materials

Double gene construct pBINPLUS:: *Rpi-sto1+Rpi-blb3* with removed marker gene of *nptII* was used in transformation of c.v. Atlantic. Regenerants were harvested and maintained from this transformation.

P. infestans isolate 90128 was used in this study. The information is listed in Table 5.

Table 5 Characteristics of *P. infestans* isolate 90128

isolates	origin	Mating type	Virulence profile	Year isolated
90128	Geldrop, Netherlands	A2	1.3.4.7.8.10.11	1990

Methods

Explants maintenance and plantlets preparation

For stem explants maintenance, it is the same as 2.1.2.2 except for the supplied antibiotics in the plate. Here, 1 ml of Zeatine (1mg/l), 2 ml of Cefotaxime (100mg/l), and 2 ml of Vancomycine (100mg/l) were added in the LB medium together with 8g of agar. Regenerants were planted in plastic jars. After 2 weeks for rooting, the plantlets were ready to be multiplied. Fresh shoots or single nodes were cut and transplanted to plastic pots (diameter 15 cm) with MS30. 8 cuttings from 4 plantlets were included in one jar. After one or two weeks of rooting, the plantlets were ready to go with *in vitro* disease test.

P. infestans isolates, maintenance, and inoculum preparation

P. infestans isolate 90128 was used in this study. A fresh sample of sporangiospores was placed on rye agar medium supplemented with 20g sucrose (Caten and Jinks, 1968). After two weeks of growing, the plate will be covered with mycelium. Sterile water was added to the sporulating mycelium. The sporangiospore suspension was pipetted into a new tube and incubated at 4℃ for 3h. The concentration was adjusted to $5*10^4$ zoospores ml^{-1} for inoculation (Bradshaw et al., 2006).

Inoculation

Fully developed leaves of *in vitro* plants were used in the inoculation by pipetting 10ul droplets on the abaxial side (1 spot per leaf). Inoculum preparation and inoculation were performed in the flow cabinet to avoid being contaminated. Finished jars with the inoculated plantlets were removed to a chamber which held a 24-hour relative humidity of 100%, a 16-hour light period, a stable temperature at 15℃. Each regenerant was 2 times present and both were inoculated. After 2 weeks, plantlets were scored with *R-R* and *R-S.*

Resistance score

Inoculated plantlets were scored at 21 days post inoculation (dpi). Compatible interactions were scored in two classes, which include susceptible (spreading lesion with massive sporulation) and less susceptible (spreading lesion with no or little sporulation). They were both referred to S. Incompatible interactions were scored as R (no symptom or localized HR-like necrosis) (Huang et al., 2005a). The two repeats of plantlets for one genotype were finally scored with *R-R* or *R-S*.

R-R and R-S plantlets multiplication and DNA isolation

Selected, potentially resistant, plantlets were multiplied from the stock. After 1 or 2 weeks, leaves were big enough for harvesting and DNA was isolated from the same method of 2.1.2.

PCR screening on R-R and R-S plantlets

Positive plants with *R*-gene(s) were tested with the backbone gene from vector pBINPLUS and also *A.tum* contamination by using signal gene *virG*. Method was the same as 2.1.2.

2.2.3 Results

From May 2010 till November 2010, 3405 plants were harvested from marker free transformation. 3101 out of 3405 plants were tested with the *in vitro* disease essay. In Table 6, the numbers in each period were listed. After *in vitro* assay, R-R and R-S plant were calculated based on the resistance evaluation. The results were available separately from column R-R and R-S in Table 6. PCR screening was carried out on the pre-selected plants of R-R and R-S. The column “PCR positive”, showed the number of plants potentially harbouring *R*-gene(s). Between brackets, two numbers are visible. They are the positive plants showing

Table 6 Plantlets undertaken of *in vitro* disease test and PCR results comparison

Harvesting period	Total plants No.	Plants undertaken of in vitro disease test	R-R	R-S	PCR positive	Efficiency in R-R	Efficiency in R-S
2010/05*	9	9	1	8	0	0	0
2010/06*	311	311	8	42	4(3,1)	0.375	0.024
2010/07*	1273	1273	18	84	8(8,0)	0.444	0
2010/08	780	780	26	71	7(6,1)	0.231	0.014
2010/09	608	608	29	67	3(2,1)	0.069	0.015
2010/10	120	120	1	4	1(1,0)	1	0
2010/11[1]	304	0	0	0	1	-	-
Total	3405	3101	83	276	24(20, 3,1)[2]	0.241	0.011

*Harvested by the other persons.

[1] Not done with *in vitro* assay.

[2] 20, total number of positive PCR plants from *R-R*; 3, total number of positive plants from *R-S*; 1, not from anyone of *R-R* or *R-S*.

- No results

R-R and R-S reaction respectively. Efficiency in both PCR screening on R-R and R-S were calculated and available in the last two columns as percentage.

In November 2010, 304 plants were directly screened from PCR, showing 1 plant positive with *R*-gene specific primers.

There were 20 plants from 83 *R-R* plants showing positive in PCR screening, representing a percentage of 24.1%. While in the selection of PCR positive plants from *R-S* plants, the percentage was only 1.1%. For the efficiency in each individual period, the highest frequency of PCR positive plants coming out was after 2-3 months (transformation started in April 2011), which is indicated in Figure 11.

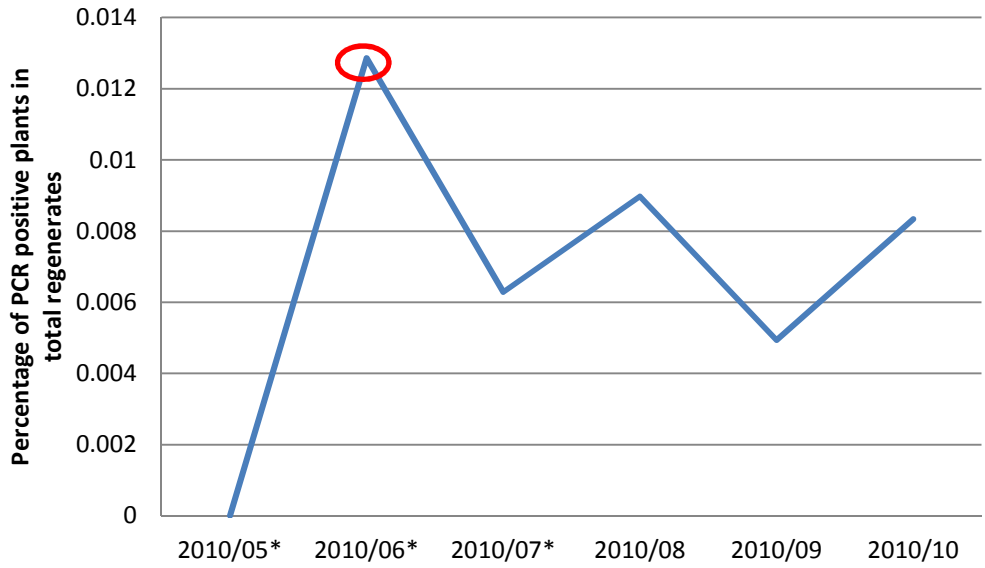


Figure 12 Graphical map of percentage of PCR positive plants among total regenerants obtained from each period.

In Figure 12, there was a peak in Jun. 2010. One month after setting up of the experiment in April 2011, regenerants started to be harvested. In the third months after the transformation, regenerants gave a highest percentage in harbouring *R*-gene.
11 new screened plants harbouring *R*-gene(s) were also tested by backbone detection from PCR. Results were summarized in Table 7. Seven plants out of 11 were R gene(s) harbouring and also backbone genes free, which were 2nd-8-7, 685-2, 621-2, 617-3, 617-6, 645-7, 648-2 and 654-5. It can also be viewed from Table 6 the seven cisgenic regenerants were positive in PCR screening for *Rpi-sto1* and *Rpi-blb3* but

negative for *Rpi-vnt1.1*. Of the positive R-genes containing regenerants, there were 7 regenerants showing backbone free.

Table 7 Plants from marker free transformation undertaken of PCR detection

	R-gene test				Backbone-gene test(from RB to LB)						
Plant code	*Rpi-sto1*	*Rpi-vnt1.1*	*Rpi-blb3*	*tetR*	*traJ*	*oriV*	*NPTIII*	*trfA*	*tetA*	*ori3+5*	*virG*
2nd-9-8	+	-	+	-	-	+	+	+	+	+	+
2nd-8-7	+	-	+	-	-	-	-	-	-	-	-
685-2	+	-	+	-	-	-	-	-	-	-	-
621-2	+	-	+	-	-	-	-	-	-	-	-
617-3	+	-	-	-	-	-	-	-	-	-	-
617-6	+	-	-	-	-	-	-	-	-	-	-
645-7	-	-	+	-	-	-	-	-	-	-	-
648-2	-	-	+	-	-	-	-	-	-	-	-
654-5	+	-	-	-	-	-	-	-	+	-	-
704-4	+	-	+	-	+	+	+	+	+	-	-
2nd-5-2	+	-	-	+	-	-	-	+	-	-	-

2.2.4 Discussion

In vitro assay can be used as a pre-selection for potential marker free transformants.

In this experiment, we carried out *in vitro* assay to select potentially marker free transformants from large amounts of regenerants. Result indicated that efficiency of getting PCR positive plants from *in vitro* assay was comparable with the efficiency of directly from PCR detection, which was approximately the same at 1% (Internal communication). *In vitro* assay could be an alternative method of large scale analysis from PCR. This method was described before as perfect correspondence between *in vitro* assay and the DLA for studying the gene for gene interaction between potato and *P.infestans* (Huang et al., 2005a). The advantages for *in vitro* assay as a pre-selection method in reducing the PCR numbers and increasing work efficiency were obvious. In addition, *in vitro* assay can also be predictive in gene for gene interaction which could increase the credibility of PCR positive plants in containing the *R* genes. On the other hand, *in vitro* disease assay could miss some PCR positive plants showing resistance in DLA or in the field..

R-R plants from in vitro assay were more predictive than R-S plants when compared to PCR results.

In order to determine the proportion of PCR positive plants in *R-R* and *R-S*, the efficiency in each was calculated and compared. The results demonstrated that most of the PCR positive plants werecoming from *R-R*, which could give reference for future work to focus on *R-R* plants when carrying out *in vitro* assay.

3. Functional characterization of detected *Rpi-sto1* and/or *Rpi-vnt1.1* gene(s) in MF plants produced from (co-) transformation.

3.1 Introduction

Phytophtora infestans infects potato by invagination of a feeding structure (haustoria) into the host cell plasma membrane, establishing an intimate contact with this host, and delivering effector molecules (virulence effectors) into the plant cell. The plant reacts to the invasion by recognizing the avirulence effectors and activating the innate immunity via complex pathways that arrest proliferation of the pathogen, thus conferring disease resistance (McHale et al., 2006).

So far, five matching pairs of *PiAVR* and potato *R*-genes have been identified, *PiAVR3a/3a*(Armstrong et al., 2005), *AVR4/R4*(van Poppel et al., 2008), *AVR-blb1/Rpi-blb1*(Vleeshouwers et al., 2008), *AVR-blb2/Rpi-blb2*(Oh et al., 2009) and *AVRvnt1/Rpi-vnt1.1*(Pel, 2010).

In effector screening of *IpiO*, the two variants of *IpiO1* and *IpiO2* triggered a cell-death response in *S. bulbocastabum* plants carrying the late blight *R* gene *Rpi-blb1*(alternatively named *RB*)(Song et al., 2003). *Rpi-sto1* as homologue of *Rpi-blb1* also showed cell-death response to *IpiO1* and *IpiO2*, which provides functional evidence for the *R-Avr* interaction (Champouret et al., 2009).

In this experiment, transformation and co-transformation were undertaken on cv Atlantic. In order to get efficient numbers of cisgenic plants, plantlets were screened from PCR detection for *R* genes. Positive ones were selected out followed by the backbone gene detection. Two different functional assays were performed. *Agro*bacterium Transient Transformation Assays (ATTA) is to deliver *Avr* effectors to the plant cell followed by checking hypersensitivity reaction on the leaf. It is a powerful tool for the analysis of gene function in plants. Threshold of concentrations in *Avr* effectors were recorded in order to compare the sensibility of transgenic plants in resistance response to *Avr* effectors. A second method is the detached leaf assay (DLA), which is used to mimic *Pi* infection in the field (Huang S W, et al., 2005). Different isolates of *Phytophthora infestans* were used on the same plant. Results were compared to see the response of the same plant to different *P. infestans* isolates.

3.2 Materials and methods

3.2.1 Materials

Marker free transformants of cv Atlantic, which are from single transformation events of pBINAW2c:: *Rpi-vnt1.1*, pBINAW2e:: *Rpi-vnt*1.1+*Rpi-sto*1 and co-transformation of pBINAW2c:: *Rpi-vnt1.1* & pBINAW2e:: *Rpi-sto1*. Also the same genes in another vector were co-transformed to cv Atlantic: pBINAW2LB4:: *Rpi-vnt1.1*&pBINAW2LB4:: *Rpi-sto1*.

Effectors

Several effectors were used in ATTA experiments. Table 8 indicated the information of the effectors.

Table 8 Overview of effectors used in the study

Effector name	Vector	*A.tum* strain
Ipio1	pK7WG2	*AGL1*
Avrvnt1	pMDC32	*AGL1*
Rpi-sto1	pBINPLUS	*AGL1*
Rpi-vnt1.1	pBINPLUS	*AGL1*

Isolates

4 isolates were used in DLA test. The characteristics were described in the next table including the origin and mating type, race and year isolated (Table 9).

Table 9 Characteristics of *P. infestans* isolates used in this study.

Isolates	origin	Mating type	Virulence profile	Year isolated
EC1	Ecuador	A1	1.3.4.7.10.11	1998
USA618	Toluca Valley, Mexico	A2	1.2.3.6.7.10.11	Not known
DHD11	North Korea	A1	1.2.3.4.6.7.10.11	2007
Pic99189	Metepec, Mexico	A2	1.2.5.7.10.11	1999

3.2.2 Methods

Plantlet and leaflet preparation

Fresh shoots or single nodes were cut and transplanted on Murashige and Skoog (MS) supplemented with 30g of sucrose per litre (MS30) in plastic pots (diameter 15cm). 8 cuttings from 4 plantlets were included in one pot. After two weeks for rooting, the *in vitro* plantlets were transferred into trays (with 5cm*5cm*5cm in width, length and height) for pre-exercise. They were transferred again to big pots with a straight wooden stick for inducing the plantlets growing vertically. The plants were then grown in the greenhouse. Depending on the potato genotype, it took 2 to 4 weeks from transplanting to inoculation.

Leaves for detached leaf assay were collected before ATTA. Primary leaves from the third to the sixth counting from the bottom of plants were excised at the stem. Their petioles were cut firstly to be sharp and then inserted into 5 cm*5cm green flower foam which was marinated in water for 15min. The leaves in the foam were placed with adaxial face down on sterile water-soaked filter paper in the covered tray. Together with the label fixed with a wooden stick on the foam, two leaves from the same transgenic plant were used.

Avr effectors and R-gene preparation

Method was described in Appendix 4. Positive controls were mixed with *Rpi-sto1* and *Avr* effector of *Ipio1*. Another positive control as mixture of *Rpi-vnt1.1* and *Avrvnt1* was also undertaken to adjust all the samples into the same background.

Infiltration

Three leaves from lower side of the plant were infiltrated with MMA culture at an OD gradient of 0.2, 0.05 and 0.0125 using a 1ml syringe. Leaves were infiltrated from the abaxial side with the same amount of cultures. After finishing one concentration or one effector, the gloves and syringe need to be refreshed to avoid cross contamination.

Resistance score

At 6 days post infiltration, leaves were scored with a number from 0 to 2. Infiltration spots which show hypersensitive response (HR) compacted will be recorded as 2.0. Spots with several lesions around the infiltration place but on which the total area is less than the area of HR will be recorded from 0.5 to 1.5. “NR” means no response.

A full table was filled in with the positive and negative controls. Threshold for HR happening in each plant was written down based on the data of ATTA. If there were 4 out of 8 data giving a number more than 1.5 in this plant at an OD of 0.2 in the *Avr* effectors, the plant will be recorded as a threshold of 0.2. It was the same assessment method in other ODs.

P.infestans isolates, maintenance, and inoculum preparation

A fresh sample of sporangiospores was placed on rye agar medium supplemented with $20gl^{-1}$ sucrose (Caten and Jinks, 1968). After two weeks of growing, the plate will be covered with mycelium. Sterile water was added to the sporulating mycelium. The sporangiospore suspension was pipetted into a new tube and incubated at 4 ℃ for 3h. The concentration was adjusted to $5*10^4$ zoospores ml^{-1} for inoculation (Bradshaw et al., 2006).

Inoculation

Each leaf was inoculated with eight droplets (10 µl each) of sporangiospore solution on the abaxial side. After 6 to 8 days, the leaves were evaluated for the development of *P.infestans* disease symptoms.

Resistance score

Leaflet which shows 8 spots (6 in the two sides of the main leaf, 2 in the detached two leafs separately) resistant to *P. infestans* was recorded with Resistant (R). Leaflet gives 6 spots of HR response, 2 spots in the detached leafs of susceptible response was marked with RQ; SQ was also found because of the 4 spots out of 8 were scored as susceptible to *P. infestans;* S means the 8 spots in leaflets were all susceptible (Figure 13).

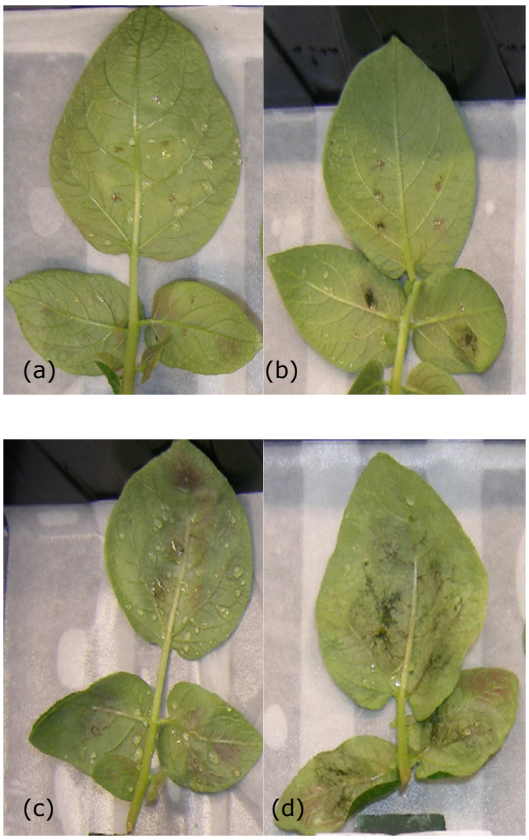


Figure 13 Resistance score for *P. infestans* from detached leaf assay

Leaflet in (a) shows 8 spots (6 in the two sides of the main leaf, 2 in the detached two leafs separately) show resistant to *P. infestans*; in (b), the leaflet gives 6 spores of HR response, 2 spots in the detached leafs of susceptible response; (c) was marked as SQ because of the 4 spots out of 8 were scored as susceptible to *P. infestans*; (d) was the picture for S, with all 8 spots of susceptible response.

3.3 Results

90 plants from different transformations including double genes single transformation and co-transformation with a single *R*-gene in each construct were tested with PCR reaction to distinguish the presence of *R* genes. ATTA and DLA results were scored on these plants and the correlations between *R*-gene presences, isolates detection and effector response were analysed. Detail information can be found in Appendix 9. Backbone gene *nptIII* was tested on the positive *R*-gene plants to evaluate the backbone insertion proportion. Plants were separated from the *nptIII* plus (+) and minus (-) as seen from Table 10. Results showed that in *nptIII* free plants, there were 4 plants containing double *R* genes. These 4 plants were from 2 transformations of pBINAW2e:: *Rpi-vnt1.1+Rpi-sto1* and pBINAW2LB4:: *Rpi-vnt1.1*&pBINAW2LB4:: *Rpi-sto1*. While in the plants only containing single *R*-gene, 16 and 23 were the numbers from the 2 transformations respectively (Table 10).

In *nptIII* containing plants, the same sorting was carried out based on the *R*-genes *stol1* and *vnt1.1*. Data showed that 16 plants contain both genes inside and 18 plants were only containing *vnt1.1* and 12 plants only *stol1*. Cultivar Atlantic was included in this experiment as a negative control.

Table 10 Plants from transformants harbouring *Rpi-sto1* or *Rpi-vnt1.1* were sorted by presence or absence of the *nptIII* gene**.**

nptIII	Plants numbers containing the gene(s)			
	Rpi-sto1 & *Rpi-vnt1.1*	*Rpi-sto1*	*Rpi-vnt1.1*	Total
-	4[1]	16	23[2]	43
+	17[3]	18	12[4]	47

- *nptIII* free

+ With *nptIII*

[1] One plant came from transformation of pBINAW2e:: *vnt1.1+sto1*.

[2] Three plants of 23 were transformed of pBINAW2c:: *vnt1.1*.

[3] One plant came from transformation of pBINAW2e:: *vnt1.1+sto1*.

[4] There were 3 plants from transformation of pBINAW2c:: *vnt1.1*.

Other plants are from co-transformation of pBINAW2LB4:: *vnt1.1*&pBINAW2LB4:: *sto1*.

Table 11 described the correlation between genes and functional assay from both ATTA and DLA. The correlation was calculated from the percentage of hypersensitive response numbers out of the total plants numbers for individual gene detection. In plants tested with *Rpi-sto1* inserted in the genome, isolate EC1 recognised 85% of the plants, which means there were 85% plants being scored as R in DLA. Isolate USA618 contains both gene *Avr* effectors. *Rpi-sto1* containing transformants were tested for this isolate as well and72% from DLA results matched with resistance. DHD11 was also reported harbouring both *Avr* effectors. From the table shown, 42% of DHD11test results fit the PCR gene screening. *Rpi-sto1* as homologue of *Rpi-blb1* would happen cell-death response to *IpiO1* as well. A proportion of 32% in total was shown to respond in ATTA test. When isolate Pic99189 was used for the DLA functional assay for *Rpi-vnt1.1*, there were 80% plants corresponded with PCR results. While in both genes bearing plants, the number was 100%. EC1 recognised 86% of *Rpi-sto1* gene in both genes containing plants. Isolate USA618 and DHD11 had high percentage of 94% and 100% respectively for both genes positive for the PCR. ATTA testing by using *Avrvnt1* for *Rpi-vnt1.1* detection showed 34% plants responding.

In ATTA test for both genes containing plants, positive control of *Rpi-vnt1.1* and *Avr vnt1* were mixed and co-infiltrated to the leafs. For scoring of the positive control in double gene containing plants, there was no HR found in contrast to the plants only harbouring single gene in the genome. For using of *Ipio1* and *Avrvnt1* in *A. tum* for infiltration, the leaves did not give response either.

Table 11 Response between genes and functional assay from both ATTA and DLA.

R-gene test	Different isolates used in DLA test				Two different *Avrs* in ATTA test	
	Pic99189	EC1	USA618	DHD11	*Ipio1*	*Avrvnt1*
Rpi-sto1	0	85%	72%	42%	32%	ND
Rpi-vnt1.1	80%	ND	ND	80%	ND	34%
Rpi-sto1+Rpi-vnt1.1	100%	86%	94%	100%	-	-

- There is no response on the leaves; ND means not done.

Table 12 Relation of PCR screen for individual R genes and functional assay of ATTA and DLA with isolate DHD11.

			Plants numbers correspond to ATTA and DLA		
NptIII	Genes for screening	Plants positive in PCR screen	R/R	S/S	Q
+	*Rpi-sto1*	18	7	7	4
	Rpi-vnt1.1	12	1	2	9
	Rpi-sto1+Rpi-vnt1.1	17	-	-	-
-	*Rpi-sto1*	16	4	6	6
	Rpi-vnt1.1	23	11	4	8
	Rpi-sto1+Rpi-vnt1.1	4	-	-	-

R/R refers to plants undertaken of both functional assays of ATTA and DLA with the result of both resistant; S/S was targeted with plant in getting susceptible of both assays; Q means questionable plants, the results from both assays were not corresponded.

In *sto1* functionality test from ATTA of the single gene containing *nptIII* free plants, 4 out of 16 plants gave hypersensitive response in two replicate plants at the level of 0.2 of *IpiO1*. Results from DLA test by using isolate DHD11 in these 4 plants showed R. In DHD11 test susceptible plants which were recognised as S in column DHD11, 6 plants had no response with *Ipio1* in both repeats. Plants KB06-29 and KB04-18 were triggered as S in DLA while plant 1 in KB06-29 and one leaf from KB04-18 showed hypersensitive response. Plant KB06-64 had the same situation with KB06-29. There

were 2 other plants which were recognised by R in DLA test but only one leaf had HR in KB06-17. In KB06-55, two repeats showed HR and No Response (NR) respectively. Plant KB06-10 was scored as Q in both repeats of ATTA and MS in DLA test (Appendix 8).

From *Rpi-vnt1.1* functionality test in the *nptIII* free PCR positive plants, there were 15 plants out of 23 in DLA showing the corresponding results with ATTA test (Table 11). In the R plants of these 15 plants, the ATTA results were showing both two repeats have response at level of 0.2. In S plants of DLA, NR was found in each plant. In plants of KB06-16, 27, 44, 46 and 65, they were marked as R in DLA while for ATTA test only one plant gave HR. In plant of KB06-46 and KB06-65, the HR happened at level of 0.05. Plant KB04-5 was tested susceptible in DLA while the ATTA results showed NR in one plant but one leaf of the other plant gave HR. There were also other two plants of KB03-12 and KB04-6 not matching in both tests, which was resistance in DLA but no response in ATTA (Appendix 8).

In *nptIII* containing plants, 18 plants were tested with only *sto1* inside. For these 18 plants, DLA and ATTA results were scored and compared as well. Results of 14 out of 18 plants in both DLA and ATTA were corresponding with each other (Table 11). six out of 7 plants which were marked as R in DLA, gave a resistance level at 0.05. The other one showed resistance at 0.2. Plants with S in DLA in the 14 plants gave a uniform HR in each plant. There were other 4 plants sorted in different groups. KB04-10, KB06-4 and KB06-54 were tested susceptible in DLA but at least one plant showed resistance in ATTA. KB06-41 was given R in DLA but there were no response in both plants in ATTA test (Appendix 8).

NptIII plants containing *Rpi-vnt1.1* were tested for ATTA and DLA. The results from 3 plants in DLA combined with the results from ATTA, which includes 2 susceptible plants and 1 resistant plant. The other 9 plants were questionable in comparing the results from both sides (Table 12).

For the plants containing both *R* genes from PCR screen, in ATTA test the positive control as *Rpi-vnt1.1* mixed with *Avrvnt1* did not show HR as expected. The plants did not happen with hypersensitive response either. So the results in ATTA were not comparable with DLA test on the same plants containing both R-genes. As shown in table 10 and table 11, the relevant lanes were marked with '-' showing not

comparable and no response respectively. In *stol1* containing plants for both *nptIII* free and *nptIII* containing, the co-infiltration of *Rpi-sto1* and *ipiol1* at 0.2 as a positive control described at least one plant showed HR in all samples. Co-infiltration of *Rpi-vnt1.1* and *Avrvnt1* at 0.2 was carried out as the positive control in only *Rpi-vnt1.1* containing plants. The HR was also found in at least one plant of two repeats in all plants containing *Rpi-vnt1.1*. *AGL1* as a negative control in all the plants for ATTA testing gave no response on leafs (Appendix 8).

Cultivar Atlantic was co-infiltrated with *Rpi-blb1* and *IpiO1*, *Rpi-vnt1.1* and *Avrvnt1* as the control for all the transgenic plants with the same background of cv Atlantic. But there was no response from both *R-gene* positive controls. Infiltration of *Rpi-vnt1.1*, *IpiO1* and *AGL1* as the negative controls worked as no response. Cv Atlantic is susceptible when it was set as a negative control by inoculating with DHD11.

3.4 Discussion

DLA is better for detecting the functionality of R genes than ATTA for Atlantic transformants.

In this study, both ATTA and DLA were developed as the routine methods for monitoring major *R* genes in potato and their corresponding *Avr* genes in *P.infestans.* Correspondence between ATTA and DLA concluded that DLA has more recognition than ATTA on monitoring the same *R*-gene(s). Effector research shows that there are two models between effector and effector target, which are one effector-one target and one effector-multiple targets (Hogenhout et al., 2009). Referring to *Ipio1*, Vleeshouwers et al reported that *Rpi-blb1* and *Rpi-sto1* recognise the same *P.infestans Ipio1* specifically. Isolates used in DLA test occur as small gene families consisting of different virulence profiles. Race-nonspecific *R* genes are more aggressive when challenged with *P.infestans* isolates. Evidence was obtained from *Rpi-sto1* gene complementary characterization detection. Specific complementary detection from effector in ATTA has a lower percentage in matching with the *R*-gene test from PCR compared with DLA test by EC1, USA618 and DHD11. Zhu et al. described that agro-infiltration could be potentially a substitute for DLA on testing multiple *Rpi* gene containing plants(Zhu et al.), which was not corresponding with this experiment.

Transformants with stacked R genes are more resistant in DLA test in late blight.

DLA essay for two *R* -genes harbouring plants has higher match with PCR than with plants only containing one *R*-gene (Table 4). On the other hand, the higher number of match means that transformants with stacked *R* genes are more frequently resistant to late blight. In rice, after stacking of bacterial blight (BB) resistance genes, a wider spectrum and a higher level of resistance to the BB pathogen was observed than for the parental lines which contain the single *R*-gene(Huang et al., 1997). This result was also confirming that stacking R genes in potato plants could increase the durability of late blight resistance, which afforded evidence to research on increasing resistance to late blight by stacking *R* genes (Jacobsen, 2007).

ATTA was not predictive in cv Atlantic harbouring two transformed R genes from co-transformation.

As the experiment setting, Agro-infiltration was also applied to plants harbouring two transformed *R* genes detected by PCR. No hypersensitive response was found on leafs of cv Atlanta plants bearing two *R* genes from co-transformation (Table 4 and Table 5), in contrast with plants from transformation of stacked genes with showing HR complimentary with each gene. Zhu et al. (2010) tested the triple *R* genes transformants from transformation of stacked *R* genes vector pBINPLUS by ATTA, which indicated that the 23 transformants functionally expressed all three *Rpi* genes with using the specific effectors (Zhu et al.). It is not clear how to explain this discrepancy. This phenomenon need to be further analysed.

Conclusions

1. *Rpi-mcq1* was successfully cloned to pBINPLUS and first potential transformants have been obtained.

2. In co-transformation, group D (3:1 ratio of *R*-gene containing construct and *nptII* containing construct) is expected in generating higher frequency of MF cisgenic triple *R* genes harbouring plants. However, kanamycin resistance seems to be linked frequently with *R*-gene(s), which will, after sexual crossing with a susceptible plant, not bring the segregation needed for the selection of cisgenic plants.

3. *In vitro* assay can be used as a pre-selection method and *R*-*R* plants are highly predictive for positive PCR results; transformation efficiency is highest in the third month post transformation.

4. Double *R* genes containing cv Atlantic plants did not show *Avr* reaction. The reason for that has to be investigated in more detail.

5. Co-transformation is efficient in getting high frequency resistant plants with stacked R-genes.

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Appendix

1. Information of primers used for amplification of gene Rpi-mcq1 from vector pCLD 04541.

Primer name	Sequence(5' to 3')	Tm	Created sticky end
Rpi-mcq1-F1	tgaccctgcaggGTGCTGAGATTACCATTGTTGTC	62℃	*SbfI*
Rpi-mcq1-R1	tgacggcgcgccTGCATTTGTCCTTTCACCTTC	62℃	*AscI*

2. Overview of the specific primers for colony PCR in construct of pBINPLUS:: *Rpi-mcq1*.

Primer name	Sequence	Tm	Product
mcq1 F1	TGTGGGAGTCCTATTACACCACGAA	58℃	710bp
pBIN R1	CTCGAACGACGTCACCGCCC	60℃	

3. Overview of primers used for sequencing of *Rpi-mcq1*

Primer name	Sequence	Sequence results by alignment
Seq.1	TTTACCTTCCCTCCCTCAATCG	9279-9871
Seq.2	TCAAATATCGAACCGCTAAGGACC	9860-10365
Seq.3	TCCCTACACGTTACTTCAGAC	10322-10860
Seq.4	GAGGCAATGAGTACAAGTTTGG	10843-11340
Seq.5	CTCGGTGTGAAAGAAGTTGC	11335-11845
Seq.6	AGTTTGGTGAAGCATTGGTC	11817-12460

Seq.7	ACACGATCAATGTCCACAACC	12459-13035
Seq.8	AGAAGGAAGGAACAACACATGC	13003-13431
Seq.9	GGCATTCGATTCTTCGGTTC	13301-14090
Seq.10	CAAGGTACAACACCTCTAAGCAG	14039-14790
seq.20	TGCCGTAAAGCACTAAATCG	15180-14470

4. A.tum strain preparation

Two days before infiltration, *A.tumefaciens* containing *Avr* effectors and different R-genes in vectors were grown over night at 30℃. in LB medium with appropriate antibiotics and right concentrations(appendix) respectively. After 16h growth, the OD_1 was measured and 50ml of YEB medium was inoculated with x µl of LB culture and grown overnight at 30℃ in order to get an OD_2 of 1.0 the next day($x=z/OD_1$ with $z=80000/2^{(\text{delta time}/2)}$). The following day, 45ml of YEB culture was centrifuged for 10mins at 3600rpm. The pellet was re-suspended with y ml of MMA containing 1ml/L of acetosyringone. $Y=22*OD_2$ enabled the standardization of the different cultures at an OD_3 of 2.0. Every re-suspended pellet was incubated for an hour at room temperature.

5. Overview of the primers in PCR reaction of plants in screening for cisgenic ones.

Label of primer pairs	Sequence (in 5'→3' order)	Annealing temperature	Length of products	Remarks
nptII	TCGGCTATGACTGGGCACAACAGA AAGAAGGCGATAGAAGGCGATGCG	55.5°C	722bp	T-DNA
Rpi-sto1	ACCAAGGCCACAAGATTCTC CCTGCGGTTCGGTTAATACA	65°C	890bp	T-DNA

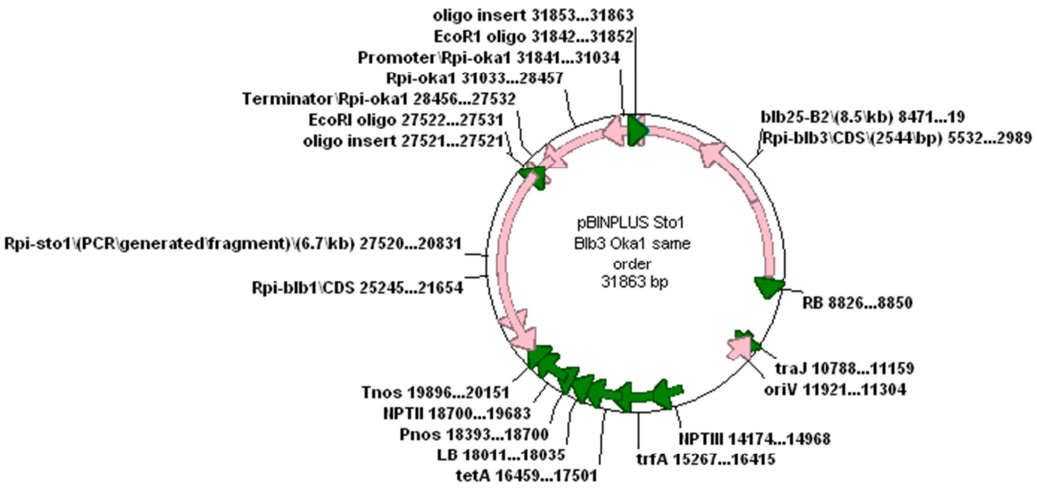
Rpi-vnt1.1	CCTTCCTCATCCTCACATTTAG CTCATCTAATAGATCCTCCAC	60°C	302bp	T-DNA
Rpi-blb3	AGCTTTTTGAGTGTGTAATTGG GTAACTACGGACTCGAGGG	63.5°C	305bp	T-DNA
traJ	ACGAAGAGCGATTGAGGAAA CAAGCTCGTCCTGCTTCTCT	62.5°C	260bp	Vector Backbone
oriV	ATAAGTGCCCTGCGGTATTG GCAGCCCTGGTTAAAAACAA	60.8°C	246bp	Vector Backbone
insB	GCGCTATCTCTGCTCTCACT AACGGCCTCACCCCAAAAA	62.7°C	1872bp	Vector Backbone
nptIII	GAAAGCTGCCTGTTCCAAAG GAAAGAGCCTGATGCACTCC	60.8°C	162bp	Vector Backbone
trfA	CGTCAACAAGGACGTGAAGA CCTGGCAAAGCTCGTAGAAC	61.5°C	146bp	Vector Backbone
tetA	CTGCTAGGTAGCCCGATACG CCGAGAACATTGGTTCCTGT	61.4°C	296bp	Vector Backbone
tetR	GGGGGAGGGGATGTTGTCTA AGGGGTATGTTGGGTTTCAC	60°C	843bp	Vector Backbone
oriv3+5	TGCGGCGAGCGGTATCAG CTTCTTGATGGAGCGCATGGG	63°C	1045bp	Vector Backbone

6. Overview of primers for measuring the distance between *Rpi-sto1* and *nptII*.

Pair Number	Name	Sequence(5'-3')	Tm
1	TL1 F	GCAGCACCTAACAACCCTCACAAG	61℃
	TL2 R	TCTCCTGTCATCTCACCTTGCTCC	61℃
2	TL3 F	AAGTCACTGTTGCAGCTCG	56℃
	TL4 R	GTTCCTGCCATTGTGATTGC	56℃
3	TL5 F	CAGGAGCAAGGTGAGATGACAGG	60℃
	TL6 R	TCAGCGTACTCTACCTGTTGTGG	59℃
4	TL7 F	GTAATAGCTTTGTGGCTGCATC	56℃
	TL8 R	AGTCTTCTTGAGCAGTTCTTCC	56℃

F forward; R reverse

7. Map of construct of pBINPLUS:: *Rpi-sto1&Rpi-blb3&Rpi-vnt1.1*



(Source: Suxian Zhu)

8. Detailed information in functional characterization of detected *Rpi-sto1* and/or *Rpi-vnt1.1* gene(s) in MF plants produced from (co-) transformation

	Sto1	*Vnt1.1*	*nptIII*	*virG*	Pic99189	EC1	USA618	DHD11	ATTA				Positive control			
									Ipio1		*Avrvnt1*					
						Paek			plant1	plant2	plant1	plant2	plant1	plant2	plant1	plant2
H43-KB05-4	+	+	-	-	R	ER		R	Q	NR	0.2	0.2	Q	Q		
H48-KB06-14	+	+	-	-	R	ER		R	NR	NR	NR	NR	NR	NR		
H48-KB06-21	+	+	-	-	R	ER		R	NR	NR	NR	NR	NR	NR		
H48-KB06-51	+	+	-	-	R	R		R	NR	Q	NR	0.2	NR	0.2		
KB04-11	+	-	-	-	S	MS		S	NR	NR					NR	NR
KB04-15	+	-	-	-	S	R		R	0.2	0.2					0.2	0.2
KB04-18	+	-	-	-	S	R		S	Q	NR					0.2	0.2
KB04-3	+	-	-	-	S	ER		S	NR	NR					NR	NR
H48-KB06-10	+	-	-	-	S	MR		MS	Q	Q					0.2	0.2
H48-KB06-17	+	-	-	+	S	MR		R	NR	Q					0.2	0.2
H48-KB06-23	+	-	-	-	S	R		S	NR	NR					NR	NR
H48-KB06-29	+	-	-	-	S	R		S/MS	0.2	NR					0.2	NR
H48-KB06-34	+	-	-	-	S	ER		R	0.2	Q					0.2	0.2
H48-KB06-38	+	-	-	-	S	ER		R	0.2	Q					0.2	0.2
H48-KB06-52	+	-	-	-	S	MR		S	NR	NR					NR	0.2
H48-KB06-55	+	-	-	-	S	R		R	0.2	NR					NR	0.2
H48-KB06-57	+	-	-	-	S	MR		S	NR	NR					NR	NR
H48-KB06-6	+	-	-	-	S	R		R	0.2	0.2					0.2	0.2
H48-KB06-64	+	-	-	-	S	MR		S	0.2	NR					0.2	NR
H48-KB06-68	+	-	-	-	S	MR		MS	NR	NR					NR	NR
H15-KB03-12	-	+	-	-	ER/R	S		R			NR	NR	NR	NR		
H15-KB03-4	-	+	-	-	R	S		R			0.2	0.2				
H15-KB03-7	-	+	-	-	R	S		R			0.2	0.2				
KB04-19	-	+	-	-	R	S		R			0.2	0.2	0.2	0.2		
KB04-5	-	+	-	-	S	MS		S			Q	NR	0.2	NR		
KB04-6	-	+	-	+	R	MS		R			NR	NR	Q	Q		
H48-KB06-11	-	+	-	-	S	MS		S			NR	NR	NR	NR		
H48-KB06-16	-	+	-	-	R	S		R			0.2	NR	NR	NR		
H48-KB06-18	-	+	-	-	R	S		R			0.2	0.2	NR	NR		
H48-KB06-19	-	+	-	-	S	S		S			NR	NR	NR	NR		
H48-KB06-2	-	+	-	-	R	S		R			0.2	0.2	0.2	0.2		
H48-KB06-20	-	+	-	-	S	S		S			NR	NR	NR	NR		
H48-KB06-25	-	+	-	-	R	S		R			0.2	0.2	0.2	NR		

H48-KB06-27	-	+	-	-	R	S		R			0.2	NR	NR	NR		
H48-KB06-28	-	+	-	-	R	S		R			0.2	0.2	0.2	0.2		
H48-KB06-33	-	+	-	-	R	S		R			0.2	0.2	0.2	Q		
H48-KB06-42	-	+	-	-	R	S		R			0.2	Q	NR	NR		
H48-KB06-43	-	+	-	-	R	S		R			0.2	Q	0.2	0.2		
H48-KB06-44	-	+	-	-	R	S		R			0.2	NR	NR	NR		
H48-KB06-46	-	+	-	-	R	S		R			0.05	NR	0.2	Q		
H48-KB06-53	-	+	-	-	S	S		S			NR	NR	NR	NR		
H48-KB06-65	-	+	-	-	R	S		R			0.05	NR	NR	NR		
H48-KB06-8	-	+	-	-	R	S		R			0.2	0.2	Q	Q		
KB04-14	+	+	+		R	R	S	R	-	-	-	-	NR	NR		
KB04-17	+	+	+		R	MS	S	R	-	-	-	-	NR	NR		
KB04-22	+	+	+	-	ER/R	ER	R	R					NR	NR		
H43-KB05-5	+	+	+		R	MR	R	R	-	-	-	-	NR	NR		
H48-KB06-12	+	+	+		MR	R	R	R	-	-	-	-	NR	NR		
H48-KB06-13	+	+	+		R	ER	R	R	-	-	-	-	NR	NR		
H48-KB06-15	+	+	+		R	ER	R	R	-	-	-	-	NR	NR		
H48-KB06-24	+	+	+		R	ER	R	R	-	-	-	-	NR	NR		
H48-KB06-26	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-3	+	+	+		R	S	R	R	-	-	-	-	NR	NR		
H48-KB06-35	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-37	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-45	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-47	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-5	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
H48-KB06-56	+	+	+		R	ER	R	R	-	-	-	-	NR	NR		
H48-KB06-7	+	+	+		R	R	R	R	-	-	-	-	NR	NR		
KB04-10	+	-	+		S	MS	Q	S	0.2	0.05					0.2	0.2
KB04-13	+	-	+		S	R	R	R	0.2	0.2					0.2	0.2
H48-KB06-32	+	-	+		S	MR	R	S	NR	NR					NR	0.2

H48-KB06-39	+	-	+		S	R	R	R	NR	NR					NR	NR
H48-KB06-4	+	-	+		S	R	R	S	0.2	0.2					0.2	0.2
H48-KB06-40	+	-	+		S	R	R	R	0.05	0.05					0.2	0.2
H48-KB06-41	+	-	+		S	ER	R	R	NR	NR					0.2	0.2
H48-KB06-49	+	-	+		S	R	R	S	NR	NR					Q	Q
H48-KB06-50	+	-	+		S	S	S	S	-	-					NR	NR
H48-KB06-54	+	-	+		S	R	S	R	0.05	0.2					0.2	0.2
H48-KB06-58	+	-	+		S	R	R	S	NR	0.2					0.2	NR
H48-KB06-59	+	-	+		S	R	R	R	0.05	0.05					0.2	0.2
H48-KB06-60	+	-	+		S	R	R	R	0.05	0.0125					0.2	0.2
H48-KB06-61	+	-	+		S	S	S	S	NR	NR					NR	NR
H48-KB06-62	+	-	+		S	R	R	R	0.05	0.2					0.2	0.2
H48-KB06-66	+	-	+		S	R	R	R	0.05	0.05					0.2	0.2
H48-KB06-67	+	-	+		S	MS	R	S/MS	NR	NR					0.2	0.2
H48-KB06-9	+	-	+		S	MR	S	S	NR	NR					0.2	NR
H15-KB03-11	-	+	+		R	MR		R			NR	NR	NR	NR		
H15-KB03-2	-	+	+		MR	S		R/MR			NR	NR	0.2	0.2		
H15-KB03-5	-	+	+		R	S		S/MS			0.05	NR	0.2	NR		
KB04-16	-	+	+		S	S		S			NR	NR	NR	NR		
KB04-20	-	+	+		S	MR		S			NR	NR	Q	Q		
KB04-21	-	+	+		R/MR	S		R			0.2	NR	0.2	NR		
KB04-9	-	+	+		R	S		R			NR	NR	NR	NR		
H48-KB06-22	-	+	+		R	S		R			0.2	NR	0.2	0.2		
H48-KB06-30	-	+	+		R	S		R			0.2	0.2	0.2	0.2		
H48-KB06-31	-	+	+		R	S		R/MR			NR	NR	0.2	0.2		
H48-KB06-36	-	+	+		R	S		R/MR			NR	NR	NR	NR		
H48-KB06-63	-	+	+		R	S		R			NR	NR	NR	NR		
Atlantic					S	S	S	S								

H43: pBINAW2e:: *vnt1.1&sto1*; H48: pBINAW2LB4: *vnt1.1*&pBINAW2LB4-*sto1*; H15: pBINAW2c: *vnt1.1*; NR: no response

R: resistance; S: susceptible; MR/MS: middle resistant or susceptible