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Extreme resistance to late blight disease by transferring 3 *R* genes from wild relatives into African farmer-preferred potato varieties

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Three late blight resistance (*R*) genes from wild potato species were transferred as a stack into the farmer-preferred varieties 'Tigoni' and 'Shangi'. Transgenic events were tested by detached-leaf assays (DLA) and whole-plant assays (WPA) with isolates of *Phytophthora infestans* using 20,000 sporangia / mL inoculum. For the first DLA, 9 out of 13 'Tigoni' and 10 out of 12 'Shangi' transgenic events had scores below 3% of leaf affected area. 17 of the 19 transgenic events with low scores were subjected to a second DLA using two different *P. infestans* isolates. 8 of them exhibited extreme LB resistance while the remaining 9 transgenic events showed hypersensitive response (HR). 6 transgenic events (2 'Tigoni' and 4 'Shangi') with extreme resistance by DLA were subjected to WPA and they all exhibited extreme resistance to LB. Hence, this study demonstrates that a simple DLA predicts high level of resistance to late blight. *R* gene expression analysis in 18 transgenic events showed different transgenic events exhibiting different levels of expression in the three genes. However, that pathogen induction and / or high *R* gene expression are necessary for extreme resistance when transgenic events bear a stack of three *R* genes was not observed.

Key words: Detached-leaf assay, late blight, *Phytophthora infestans*, resistance.

INTRODUCTION

Agriculture is the mainstay of African nations where 48% of the total African population and almost 70% in East Africa rely on agriculture for their livelihood (NEPAD, 2013). Therefore, the African Union (AU) chose to make agriculture one of the pillars of the New Partnership for African Development (NEPAD). It is geared towards contribution to development goals such as poverty eradication through improved crop production, job creation and boosting Africa trade and investments. Potato (*Solanum tuberosum* L.), being the world's fourth

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largest crop after maize, wheat and rice is universally significant (IYP, 2008). The crop is cultivated in tropical and subtropical regions of the world and can grow from sea level up to over 4,000 m above sea level (Wurster, 1977). Production and consumption have increased significantly in recent years mainly through expansion of cultivated areas (Devaux et al., 2014).

Processing of potatoes into food products has become increasingly important in African urban areas. Therefore, potato is a significant crop in Africa and its year-round production a common feature in many African countries which gives it a great potential for providing food security. At present, developing countries produce more potatoes than developed countries. In Sub-Saharan Africa, India and China, production continues to rise with the total production being close to 250 million tons up from 35 million tons in 1960 (Haverkort and Struik, 2015). In Kenya, potato is the second most important food and cash crop mainly grown by small-scale farmers (Laititi, 2014; Muthoni et al., 2013). It provides nutritional benefits such as vitamin C and provides employment for more than 2.5 million people (Kaguongo et al., 2013). Varieties available in Kenya include 'Shangi', 'Tigoni', 'Asante', 'Ambition', 'Rudolf', 'Kenya Mavuno' among others. The first two are some of the most preferred varieties in Kenya, due to their, taste, versatile use, short dormancy period and fast maturity of approximately three months (NPCK, 2015).

Late blight (LB) caused by the oomycete *Phytophthora infestans*, is the most devastating disease of potato in the world. It is a major biotic constraint to production and has been observed by farmers as a problem that causes heavy economic losses that can be as high as 90% if the disease strikes early in the growing season (Ssengooba and Hakiza, 1999). *P. infestans* is an economically important pathogen that was responsible for the Irish potato famine in 1840 which led to the death of 1 million people and the migration of 1.5 million others (Schumann and D'Arcy, 2005). Loss of potatoes due to LB in the world has been estimated at 5.2 billion euros per annum (Haverkort et al., 2009). Susceptible potato varieties require fungicide application as the only option to control the disease. However, the cost of fungicide, machinery and labor are unaffordable to resource poor farmers. Furthermore, fungicide resistance is on the rise and the population structure of *P. infestans* in East Africa is changing (Njoroge et al., 2016).

LB infection can be delayed by the use of certified seeds and through crop rotation (Hannukkala et al., 2007); but the most cost-effective way to control any plant disease is by host resistance since this offers prevention from the disease. Thus, conventional breeding has long been pursued for the development of host resistance. However, it takes several decades to obtain resistant cultivars from the first cross with resistant wild species and these varieties still suffer from linkage drag of undesirable alleles from wild relatives (Haverkort et al., 2009). Resistant cultivars often use single *R* gene-based resistance which is rapidly overcome by virulent *P. infestans* strains that have a compatible interaction with the resistant plants (Vleeshouwers et al., 2000).

Several *R* genes against *P. infestans* originating from a wide variety of wild species have been isolated in the last decade such as *RB* (same as *Rpi-blb1*) and *Rpi-blb2* genes from *Solanum bulbocastanum*, a Mexican wild species, and *Rpi-vnt1.1* from *S. venturii*, an Argentinean wild species. The latter has three similar allelic variants; *Rpi-vnt1.1, Rpi-vnt1.2* and *Rpi-vnt1.3*. These *R* genes confer extreme resistance to *P. infestans* but have not been extensively used in potato breeding due to the time lag to produce acceptable varieties and the short-lasting resistance mediated by single *R* genes (Vleeshouwers et al., 2011). Consequently, no virulent strains of *P. infestans* have been selected which make these genes likely to confer a longer lasting resistance than those previously introduced into potato varieties such as those from *S. demissum*.

Numerous *R* genes have been cloned and can be stacked in existing potato varieties which would result in new LB resistant varieties, in a much shorter time with longer lasting resistance and no linkage drag. This strategy has been explored and achieved extreme resistance to late blight disease (Zhu et al., 2012; Ghislain et al., 2018). Interestingly, Haesaert et al*.,* (2015) showed that by stacking several *R* genes (*Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3*), complete resistance to *P. infestans* was achieved in the field whereas 'Desiree' transgenic events transformed with single *R* genes only achieved partial resistance. Though their study covered only 2 to 3 years, plants with stacked *R* genes appeared more stable than the single *R* gene plants which got infected with virulent isolates during their field experiments.

Each of the three *R* genes, belonging to the nucleotide-binding site (NBS) and leucine rich repeats (LRR) domains, expresses proteins that recognize *P. infestans* AVR-BLB1, AVR-BLB2 and AVR-VNT1 effectors, activating the extreme resistance known as effector-triggered immunity (ETI; Oh et al., 2009). The ETI is characterized by a hypersensitive response (HR) and associated disease-resistance responses (Oh et al., 2014). The 2A_1 clonal lineage of *P. infestans* is the dominant genotype in Kenya that has completely displaced the old US-1 lineage on potato (Njoroge et al., 2016). 2A_1 is also rapidly spreading in the other east African countries and was also the only clonal lineage used for bioassays in this study. This *P. infestans* lineage expresses all the three corresponding AVR-BLB1, AVR-BLB2 and AVR-VNT1 effectors in the field and therefore will not be able to overcome quickly these 3*R* transgenic varieties (Ghislain et al., 2018).

In this study, 3 *R* genes was introduced as a stack made of genes from wild relatives of *S. tuberosum* (*RB* and *Rpi-blb2* from *S. bulbocastanum* and *Rpi-vnt1.1* from

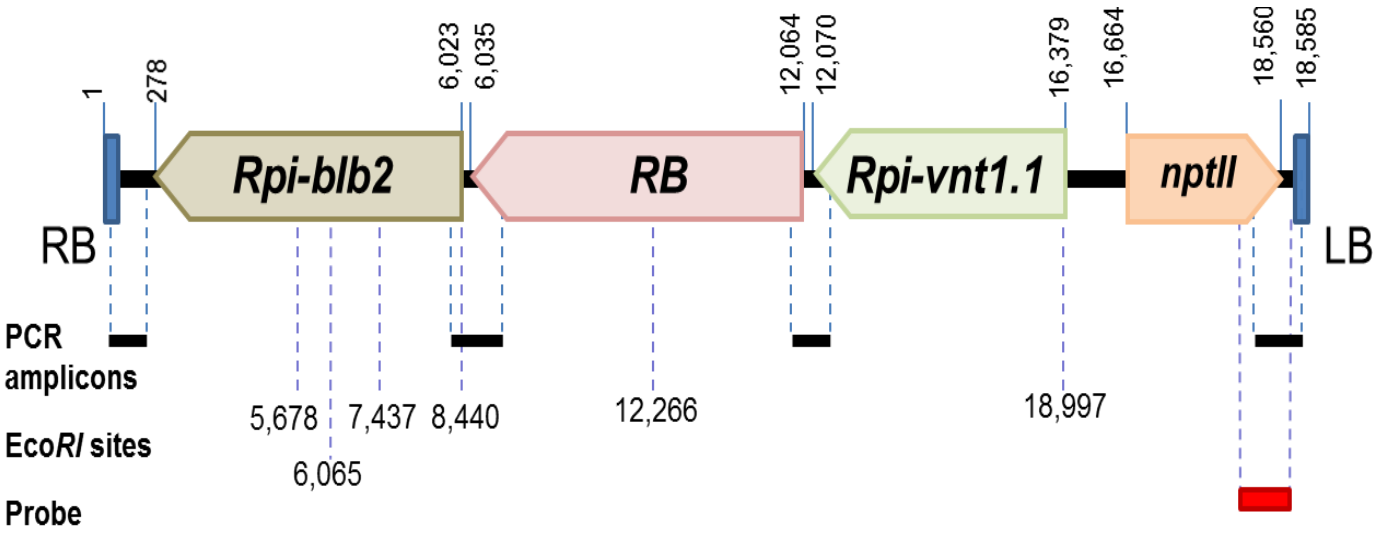

Figure 1. T-DNA carrying the 3 *R* genes (*Rpi-blb2*, *RB*, and *Rpi-vnt1.1*) and the selectable marker gene (*nptII*) used for transformation of potato. The T-DNA is a 18,585 bp long insert of the potato binary vector pCIP99 (24,819bp). PCR amplicons used to determine completeness of the inserted T-DNA are indicated by black lines. Positions of the *Eco*RI sites are indicated by dotted lines and numbers written below. The *nptII* probe which has been used for Southern blotting is indicated in red.

S. venturii) into African farmer-preferred potato varieties 'Tigoni' and 'Shangi'. Detached-leaf assay versus whole-plant leaf assay was tested to predict the level of resistance to late blight. Finally, the gene expression of each of the three *R* genes in transgenic events, from both varieties was analyzed.

MATERIALS AND METHODS

Construction of the 3*R* potato gene construct

The pCIP99 gene construct is described elsewhere (Ghislain et al., 2018). Briefly, the backbone vector is the pCAMBIA2300 plant transformation binary vector (vector provided by CAMBIA Australia) in which from the left border to the right border, the *nptII* gene in transcription orientation was inserted toward the left border and the three *R* genes in transcription orientation towards the right border (*Rpi-vnt1.1*, *RB* and then *Rpi-blb2*). The full size of the T-DNA is 18,585 bp whereas the resulting plant transformation vector pCIP99 is 24,819 bp long (Figure 1). The vector pCIP99 was then transferred by electroporation into the *Agrobacterium tumefaciens* hypervirulent strain EHA105 (Hood et al., 1993).

Agrobacterium-mediated transformation of 'Tigoni' and 'Shangi' varieties

Internodal explants from 4-week-old plantlets were used for transformation using previously published protocol with minor modifications (Cuellar et al., 2006). The *Agrobacterium tumefaciens* EHA105 strain bearing pCIP99 was incubated for 15 h at 28°C in LB semi-solid medium containing 100 mg/L kanamycin. A single colony was taken with a scalpel and then used to cut internodal explants of potato plantlets of the 'Tigoni' and 'Shangi' varieties. The infected internodes were placed on co-culture medium and subsequently transferred to regeneration medium (Cuellar et al., 2006) containing 50 mg/L kanamycin and 200 mg/L carbenicillin. To avoid false kanamycin resistant regenerants, calli were induced from leaf segments of the putative transgenic plantlets and cultured for 4 weeks on a highly selective medium (4.3 g/L salt Murashige and Skoog, 20 g/L sucrose, 20 g/L mannitol, 0.5 g/L 2-(*N*-Morpholino) ethanesulfonic acid, 0.5 g/L polyvinylpyrrolidone, 0.2 g/L glutamine, 0.04 g/L adenine sulfate, 1 ml/L of vitamins GAP, 1 mg/L naphthalene acetic acid, 0.1 mg/L 6-Bencyl-aminopurine, 200 mg/L kanamycin, and 2 g/L Gelrite, pH 5.6) under *in vitro* propagation conditions. Plantlets that did not form calli were deemed false kanamycin resistant regenerants and discarded.

Molecular characterization by PCR

Genomic DNA was extracted from putative transformed plants using a phenol-chloroform extraction method (Gawel and Jarret, 1991). To confirm the presence of the T-DNA in putative 'Tigoni' and 'Shangi' events, primers overlapping the region between *RB* - *Rpi-blb2* and *RB* - *Rpi-vnt1.1,* and primers overlapping the left and right border regions flanking the T-DNA insert, were designed and used for PCR amplification (Table 1). Conventional PCR was done in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) under the following conditions: 94˚C for 5 min followed by 40 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 1 min then 72˚C for 10 min and 10˚C hold.

Copy number analysis by Southern blot

Genomic DNA was extracted using a modified protocol of Gawel and Jarret (1991). 40 µg of the DNA was digested using *Eco*RI enzyme (20,000 U/ml *Eco*RI-HF™ NEB#R3101S) with 14 µL CutSmart Buffer 10X (NEB #B7204S) and 80 µL nuclease-free water to a final reaction volume of 200 µL. The DNA was digested overnight at 37°C and then separated in 0.8% agarose for 4 h at 55 V/20 cm. DNA was transferred to a positively charged nylon membrane (Roche) overnight by capillary method. Non-transgenic genomic DNA restricted by EcoRI was used as negative control and a transgenic event (Vic.1) by Ghislain et al. (2018) previously

Table 1. Primer sequences for molecular characterization of transgenic events.

Target region	Primer name	Primer sequence	Tm (°C)	Expected size (bp)
RB-Rpi-Vnt1.1 Overlap	RB-Vnt1F	AAAAAGAAGATTTCATGCGC	55	363
	RB-Vnt1R	CATTTTGGTTCATGAGTTCA	55	
RB-Rpi-Blb2 Overlap	RB-blb2F	GCTGCGTTAATTATTTACAT	55	587
	RB-blb2R	GTTGGTTGATTACTTGAACT	55	
nptII gene	35SNPTIIF	TCCTGTCATCTCACCTTGCTCC	60	400
	35SNPTIIR	ACGATTCCGAAGCCCAAACC	60	
Left Border	GSPc_LB	TCTCCATAATAATGTGTGAGTAGTTCCC	67	250
	LB_R	CAGCTTAGTTGCCGTTCTTCCG	67	
Right Border	GSPb_RB	ACCGATCGCCCTTCCCAACAGTTG	67	250
	RB_R	CACTATAGCAGCGGAGGGGTTG	67	
RB gene transcript	qRT-RB-F	CACGAGTGCCCTTTTCTGAC	60	132
	qRT-RB-R	ACAATTGAATTTTTAGACTT	60	
Rpi-blb2 gene transcript	qRT-Rpi-blb2-F	TTCAAAACCCCAAATAAGTTTCAAC	60	214
	qRT-Rpi-blb2-R	CCATGCTTGCTGTACTTTGCA	60	
Rpi-vnt1.1 gene transcript	qRT-Rpi-vnt1.1-F	GGTAAGGTATTGGCTCTG	60	81
	qRT-Rpi-vnt1.1-R	CTTCTCAGCAATCCACATA	60	
β-*tubulin* gene transcript	β-tubulin F	ATGTTCAGGCGCAAGGCTT	60	104
	β-tubulin R	TCTGCAACCGGGTCATTCAT	60	

proven to be single copy was used as a positive control. A Digoxigenin (Roche) labelled probe was made according to the manufacturer's instructions.

P. infestans isolate collection and purification

P. infestans isolates used in this study were collected from farmers' fields in major potato growing regions in Kenya including Kiambu (Limuru and Muguga), Molo (Elburgon, Turi and Kapsita) and Meru (Timau, and Kibirichia). Single lesion leaflets from different farms were collected during the potato-growing season of June to July 2016. The leaflets were taken to the laboratory at BecA/ILRI hub in Kenya for pathogen isolation. The diseased leaflets were washed under running tap water, tapped dry on paper towels and then incubated overnight at 18˚C in humid chambers (Petri dishes with moist paper) to induce sporulation of *P. infestans.* To obtain axenic cultures of the pathogen, fresh potato tubers of the susceptible variety Asante were cleaned under running tap water and disinfected in 10% bleach (commercially referred to as Jik; Reckitt Benckiser®) for ten minutes. A sterilized pocket knife was used to cut potato tuber slices of ~1 cm thickness in a laminar flow hood. Approximately 10 mm of the sporulating leaflets were cut and placed beneath the potato tuber slices in clean petri dishes. The petri dishes were sealed with Parafilm and kept at 18°C until mycelia grew through the tuber slices. Mycelia were then transferred to modified V8 agar media made as follows. 120 g of ripened tomatoes, 200 g of fresh carrots and 4 g of clean and young potato leaves were cut to small pieces and blended in 1 L distilled water. The mixture was sieved to obtain the filtrate that was measured to 200 mL in a glass measuring cylinder. The 200-mL filtrate was then mixed with 15 g of technical agar (Oxoid Agar No. 3™ LP0013), 3 g of calcium carbonate (Uni-Chem Calcium Carbonate powder) and 50 mg of β-sitosterol (Sigma). The mixture was topped up to 1 L and autoclaved at 121˚C for 15 min. After cooling, the media was dispensed into petri dishes and *P. infestans* isolates were maintained in culture. The cultures were observed under a microscope at a total magnification of 40X (4X * 10X) to confirm that the isolates were *P. infestans* based on morphological descriptors. This was done by observing the presence of the characteristic lemon shaped sporangium that is found in *P. infestans* and the aseptate tubular hyphae that are found in oomycetes. All the isolates selected for detached-leaf assay had the *P. infestans* characteristics.

Inoculum preparation

Inoculum was made by adding 5 mL of distilled water to the culture plates and gently scraping off mycelia using sterile blades. The mycelial fragments were then transferred to clean Falcon® tubes containing 15 mL distilled water, vortexed for about 5 min and then sieved through four layers of cheesecloth. The resulting sporangia suspension was quantified using a haemocytometer and afterwards standardized to a concentration of 20,000 sporangia/mL. The sporangia suspensions were incubated at 4˚C for 3 to 4 h to induce the release of zoospores. Presence of viable zoospores was confirmed under the microscope before the inoculations were performed.

Detached-leaf assays

Detached-leaf assays were done using the method described by Knapova and Gisi (2002). Leaf materials of uniform size from 'Tigoni' and 'Shangi' transgenic events and non-transgenic varieties were obtained from glasshouse grown plants at 45 days after planting. These were cleaned with distilled water and placed abaxial side up in 9 cm petri dishes containing moistened paper towels. Three isolates from Kiambu, Molo and Meru labelled, isolate 1 (Latitude 1˚10'52.08949" S; Longitude 36˚38'39.49569" E Elevation 2,142 m), isolate 2 (S 00'15'22.3", E 035'42'58.1" Elevation 2,533 m) and isolate 3 (S 00'05'11.3", E 037'35'43.6" Elevation 1,904 m) respectively were selected for the initial detached-leaf assay (DLA). For each isolate, five Petri dishes containing a single leaflet per potato event were used. Four leaflets

per event were inoculated at the midrib with 50 µL droplet of the 20,000 sporangia/mL suspension, while the fifth leaflet was inoculated with 50 µL droplet of sterile distilled water as a check leaflet. After inoculation, each set of five Petri dishes per isolate for each event was placed in transparent plastic bags and sealed to maintain humidity. The Petri dishes were then incubated at 18˚C for 7 days with a 12 h photoperiod and 80% relative humidity. The leaf affected area (LAA) was assessed visually on the 7th day and was defined as the necrotic plus green area covered with sporangia. The size of the affected area was measured using a Vernier digital caliper. This experiment was repeated once for events that exhibited low or no infections. The repetition was done to verify that indeed the resistant events from the first assay would hold their resistance when inoculated with different isolates. The second assay was done using isolates from Kiambu and Meru labelled isolate 4 (Latitude 1˚10'52.08949" S; Longitude 36˚38'39.49569" E Elevation 2,142 m) and 5 (S 00'05'11.3", E 037'35'43.6" Elevation 1,904 m) respectively. The length and width of the LAA of individual leaflets was measured in millimeters and used to calculate the average percentage of the leaf affected area using the formula (π*a*b) which calculates the area of an ellipsoid. π is the mathematical constant '*pi*', 'a' is the radius of the length and 'b' is the radius of the width. Analysis of variance (ANOVA) was performed to measure the variation in resistance of the potato events to *P. infestans* isolates. The resistance scale of 0 to 9 (0 being the most resistant and 9 being the most susceptible) of Yuen and Forbes, 2009, was used as follows:

*Standard scale= (Severity of test plant/Severity of control plant) *9*

The average of the two experiments was used to determine the resistance scale values since the experiment was repeated once.

Whole-plant assays

These assays were done according to the method adapted from Sharma et al*.,* (2013). Transgenic events were grown from first generation tuber seeds in the glasshouse with three replicates for each event and a non-transgenic control for each. At six weeks, the plants were moved to a controlled environment (CONVIRON, Argus controls) chamber prior to inoculation. The inoculum was made and quantified as previously described but consisted of mixed sporangia of five isolates from Muguga and Meru at a final concentration of 20,000 sporangia/mL. Inoculations were performed by spraying the test plants with the sporangial suspensions using a hand-held sprayer until all the leaves were wet. The plants were covered with a transparent plastic bag until 24 h after inoculation to maintain high humidity and enhance the infection process. The Conviron chamber was maintained at a relative humidity of between 80 and 100% with a 12 h photoperiod and temperatures of 18°C for 10 days. Plants were misted once daily with sterile distilled water and were monitored every day until the experiment was terminated. The severity of infection was recorded as a percentage of foliage area that was affected 10 days post inoculation (dpi). The same resistance scale used for the DLA was used to assign resistant score values in this WPA.

R gene expression

Transcript level of the *Rpi-vnt1.1*, *RB*, and *Rpi-blb2* genes in the transgenic events was done from infected plants by RTqPCR. The analysis was done at two-time points (1 day before inoculation referred to 'Day 0' and 3 days after inoculation referred to 'Day 3'). RNA was extracted using the RNeasy plant minikit (Qiagen) from the infected leaves which had been frozen with liquid nitrogen and stored at -80°C. Three repetitions of samples and RNA extractions (biological replicates) per treatment were done. The extracted RNA concentration was estimated by spectrophotometry using a NanoDrop Micro Photometer (Thermo Scientific). cDNA was synthesized using the *AccuPower*® CycleScript RT kit (Bioneer) following the manufacturer´s protocol. To assess the relative expression values of each of the 3*R* genes, qPCR reactions were set up using Luna® Universal Probe qPCR Master Mix with gene specific primer sets (Table 1) and qRT-Rpi-vnt1.1-F and qRT-Rpi-vnt1.1-R for *Rpi-vnt1.1* gene (Roman et al., 2017), qRT-RB-F and qRT-RB-R for *RB* gene (Kramer et al., 2009), qRT-Rpi-blb2- and qRT-Rpi-blb2-R for *Rpi-blb2* gene (EPA 2006). The RTqPCR was normalized to β-tubulin housekeeping gene with primer β-tubulin F and β-tubulin R (Kramer et al., 2009) (Table 1). All reactions were prepared in a total volume of 10 µl using Luna® Universal qPCR Master Mix (New England Biolabs) as follows: 5 µl of Luna® qPCR master mix, 0.5 µl of each primer (2.5 µM), 1 µL of cDNA and 3 µl of nuclease free water. The RTqPCR was done as follows; initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C or 15 s and 60°C for 1 min. The melting curve was run at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s. Data was analyzed using DataAssist^{TM} Software (Applied Biosystems).

RESULTS

Agrobacterium-mediated genetic transformation

3,000 internodes from both 'Tigoni' and 'Shangi' varieties were *agro*-infected. 282 putative transformed shoots was regenerated for 'Tigoni', of which 76 were confirmed to be highly resistant to kanamycin which represents a regeneration efficiency of 9.4%. In the case of 'Shangi', we regenerated 328 putative transformed shoots, of which 108 were confirmed to be highly resistant to kanamycin representing a regeneration efficiency of 10.9%. PCR was performed on 76 'Tigoni' and 108 'Shangi' putative events. 32 'Tigoni'' and 31 'Shangi' events were confirmed to be positive for the *nptII* gene which represents a transformation efficiency of 1.1% for 'Tigoni' and 1% for 'Shangi'. This transformation efficiency was lower than that reported by Orbegozo et al., (2016) of approximately 6% and Roman et al., (2017) at 7.5% for the Desiree variety transformed with *Rpi-blb2* and *Rpi-vnt1.1* single gene construct. However, the efficiency was comparable to that reported by Ghislain et al. (2018) at 1.5% for Victoria variety transformed with the same 3*R*-gene construct.

Molecular characterization of transgenic plants

PCR with overlapping primers to detect all the 3 *R* genes resulted in 22 'Tigoni' and 13 'Shangi' positive events. These transgenic events were further screened for the absence of vector backbone sequences by PCR. 13 'Tigoni' and 12 'Shangi' events did not have vector backbone sequences. Southern blot analysis was done on these 25 transgenic events to determine the transgene copy numbers using a 35S-*nptII* gene specific probe. 5 'Tigoni' and 5 'Shangi' (40%) events had single copies of the T-DNA while the rest had multiple copies (Figure 2).

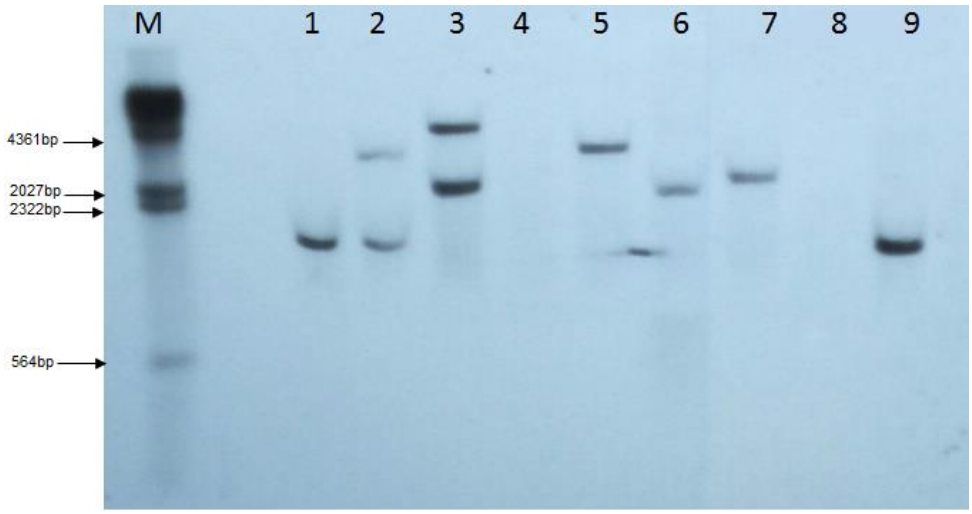

Figure 2. Southern blot analysis of DNA restricted by *Eco*RI using *nptII* gene as a probe from transgenic events from the variety 'Shangi' (sample 1 to 5) and 'Tigoni' (sample 6 to 8), non-transgenic 'Tigoni' variety (sample 8) and Vic.1 (sample 9) transgenic event containing a single copy as positive control. 'M' is the DIG labeled molecular size marker.

Table 2. ANOVA analysis at a *P*-value of 0.05 comparing the variation of individual transgenic events to isolates 1, 2 and 3.

ANOVA: Single factor						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between events[a]	42144.38	23	1832.36	21.75	3.87071E-18	1.76
Within events[b]	4044.33	48	84.26			
Total	46188.71	71				

[a]Differences amongst events as affected by different isolates 1,2 and 3; [b]Differences between events as affected by individual isolates; *P* values less than the alpha value (α=0.05) indicate significant variation while values greater than the alpha value do not.

This result was comparable to the result obtained by Ghislain et al. (2018) where 50% of the events had single T-DNA copies.

Detached-leaf assay to rapidly identify the late blight resistant transgenic events

The 13 'Tigoni' and 12 'Shangi' transgenic events that were positive for the 3*R*-gene stack were evaluated for resistance to *P. infestans* together with their non-transgenic equivalent varieties. Three *P. infestans* isolates were used for the first DLA and two isolates for the second DLA all being from the dominant 2A_1 clonal lineage in Kenya as reported by Njoroge et al., (2016). ANOVA results in Table 2 show how individual transgenic events exhibited resistance to inoculation by *P. infestans* isolates 1, 2 and 3 in the first assay. The output of the results showed highly significant differences ($P \leq 0.05$) amongst the events in the first DLA. Out of 25 events, 19 (76%) exhibited resistance ranging from 0 to 3% of the average leaf affected area by LB at 7 days after inoculation. This number of resistant events was comparable to what was reported by Ghislain et al. (2018) where 75% of transgenic events transformed with this *3R* gene construct were highly resistant to *P. infestans*. The non-transgenic 'Tigoni' and 'Shangi' varieties were completely susceptible. Transgenic events that showed susceptibility with a resistance scale values of 6-9 (Tig.8, Tig.16, Tig.141, Tig.225, and Sha.6) had multiple lesions with heavy sporulation. These events with susceptibility to late blight based on the first DLA results were not included in the second DLA. Results of the first and second assay are represented in Figure 3 where a few events exhibited susceptibility to LB in the first DLA.

The average of the resistance values in the first and second assay was used to calculate resistance values of the events. Of the 25 events tested, 19 exhibited high resistance (Table 3). The remaining five events were susceptible with visible lesions and sporulation. As expected, the two non-transgenic controls were highly

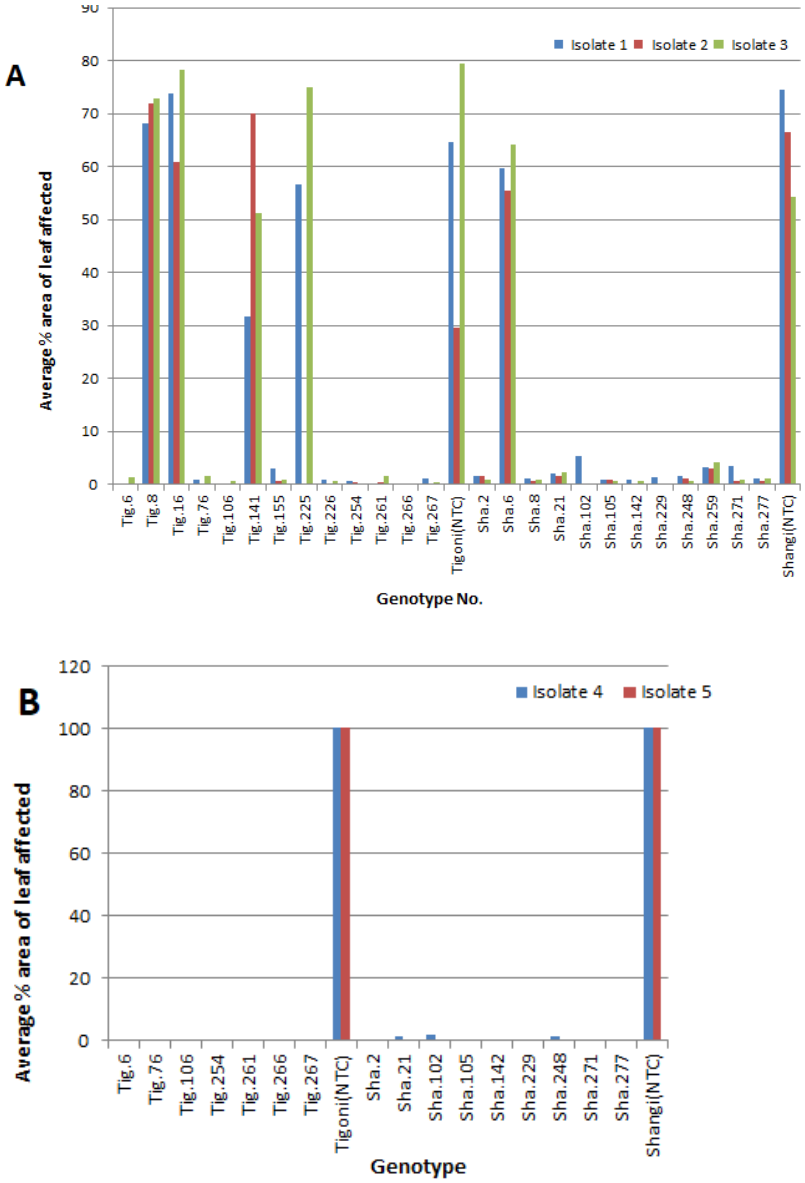

Figure 3. Detached-leaf assay of the high and low lesion transgenic events tested in the first (A) and second (B) assay showing the average percent of leaf affected area by *Phytophthora infestans* isolates 1, 2 and 3 (first DLA) and 4 and 5 (second DLA) for each genotype of 'Tigoni' (Tig), 'Shangi' (Sha) transgenic events and non-transformed controls (NTC). The average percent of leaf affected area was calculated based on the area of the lesions.

susceptible and high sporulation was evident.

Whole-plant assay on DLA late blight resistant transgenic events

Results of the whole-plant assay were comparable to those of the DLA. Six transgenic events (Tig.155, Tig.267, Sha.259, Sha.2, Sha.105, and Sha.248) randomly chosen from the 20 DLA-based resistant transgenic events, were tested in a single WPA and all exhibited complete resistance to LB with no infection (Figure 4). The non-transgenic 'Tigoni' and 'Shangi' varieties developed lesions at 3 days post infection (dpi) and at the end of the assay (10 dpi) the plant foliage had well over 80% infection. These results were like those observed by Ghislain et al. (2018) where transgenic events were all resistant to LB in WPA. This observation extended to the field where transgenic events that exhibited resistance in WPA maintained their resistance and non-transgenic varieties got LB (Ghislain et al., 2018). The percentage of damaged area varied much less among repetitions for

Table 3. Resistance scores of 'Tigoni' and 'Shangi' transgenic events infected by *Phytophthora infestans* isolates in detached-leaf assays on a resistance scale of (0-9); 0 being highly resistant and 9 being highly susceptible.

Genotype[a]	Average % of leaf affected area[b]	Standard scale values[c]	Genotype[a]	Average % of leaf affected area[b]	Standard scale values[c]
	TIGONI			**SHANGI**	
Tig.6	0.29	0.04	Sha.2	0.79	0.09
Tig.8	70.96	8.54	Sha.6	59.73	6.80
Tig.16	70.96	8.54	Sha.8	1.46	0.17
Tig.76	0.48	0.06	Sha.21	1.52	0.17
Tig.106	0.11	0.01	Sha.102	1.94	0.22
Tig.141	51.03	6.14	Sha.105	0.51	0.06
Tig.155	1.45	0.17	Sha.142	0.38	0.04
Tig.225	65.84	7.92	Sha.229	0.39	0.04
Tig.226	0.57	0.07	Sha.248	0.99	0.11
Tig.254	0.28	0.03	Sha.259	3.51	0.40
Tig.261	0.38	0.05	Sha.271	1.14	0.13
Tig.266	0.07	0.01	Sha.277	0.63	0.07
Tig.267	0.30	0.04	Shangi **(NTC)**	79.08	9.00
Tigoni (**NTC**)	74.77	9.00			

[a]Potato genotypes inoculated with *P. infestans;* [b]Average leaf affected area that formed lesion after the 1st and 2nd detached-leaf assays; [c]Values indicating the level of susceptibility/resistance of transgenic potato genotypes to *P. infestans;* Tig - Tigoni, Sha – Shangi and NTC - non-transgenic control.

the WPA than for the DLA and between replications for the non-transgenic varieties. Therefore, the WPA as a more reliable screening method for late blight resistance was considered.

R gene expression of transgenic events

Expression study of *Rpi-vnt1.1*, *RB* and *Rpi-blb2* genes was done by RT-qPCR on 17 transgenic events (9 for 'Tigoni' and 8 for 'Shangi') that were available for RNA extraction and had been tested as highly resistant by detached-leaf assays. β-Tubulin housekeeping gene demonstrated stability in expression and was therefore used for relative comparison to the target genes expression. Different transgenic events showed different patterns of expression at day 0 and day 3. The *Rpi-vnt1.1* gene showed a lower basal expression level compared to β-tubulin at day 0 in 15 out of 18 transgenic events and was always the lowest of the 3 *R* genes. The *RB* gene showed a higher basal level of expression in 13 out of 18 transgenic events compared to β-tubulin and presented for all of them a higher expression 3 days after infection. The *Rpi-blb2* gene showed a higher basal level of expression compared to β-tubulin at day 0 in all transgenic events and was overall the *R* gene with slightly higher expression but with the lowest induced expression than the other 2 *R* genes. Twelve transgenic events showed an increase in expression after inoculation for all the three *R* genes. Five events (Tig.266, Sha.102, Sha.142 and Sha.229) indicated a lower *R* gene expression in comparison to the reference gene expression for at least one of the three *R* genes after inoculation. There was insignificant change in expression levels of the *RB* gene for two transgenic events (Tig.76 and Sha.229). Different levels of expression of the three *R* genes were observed in different transgenic events. In some cases, expression levels were enhanced after inoculation and in other instances expression levels were diminished in one or all the three *R* genes after inoculation (Figure 5).

DISCUSSION

In this study, the ability of a 3*R*-gene stack containing *Rpi-vnt1.1*, *RB*, and *Rpi-blb2* genes was assessed to provide extreme resistance against *P. infestans* in farmer-preferred potato varieties 'Tigoni' and 'Shangi'. These varieties are popular in Kenya but are also grown in neighboring east African countries. The researchers decided to use the 3*R*-gene stack that had been shown to confer extreme resistance to *P. infestans* on the transgenic events from another farmer preferred variety 'Victoria' in Uganda as described by Ghislain et al. (2018). The resistance of 3*R* transgenic events of 'Tigoni' and 'Shangi' was characterized by a total absence of symptoms of LB after detached-leaf and whole-plant assays indicating a successful defense response. It was observed that the detached-leaf assay predicts very well the resistance in the whole-plant assay. In addition, the study demonstrated that the resistant transgenic events

Figure 4. Damaged Shangi non-transgenic control (NTC) and two healthy Shangi transgenic plants (3R) 10 days-post-inoculation with *P. infestans.*

display an increase in expression of at least one of the three *R* genes.

In the *Agrobacterium*-mediated genetic transformation, a transformation efficiency of 1.1% was obtained for 'Tigoni' and 1% for 'Shangi' which was lower compared to those reported previously at 3.4, 2.8, 6 and 7.5% for the variety Desiree (Ghislain et al., 2018; Ahmad et al., 2012; Orbegozo et al., 2016; Roman et al., 2017). However, this was similar to that reported for the variety 'Victoria' at 1.5% (Ghislain et al., 2018). This observation confirms that transformation efficiency is genotype dependent and lower for larger T-DNA (Heeres et al., 2002). A regeneration efficiency of 9.4% was obtained for 'Tigoni' and 10.9% for 'Shangi' which is within the range of those reported previously by Orbegozo et al. (2016) at 9.5% and Roman et al., (2017) at 13% for the 'Desiree' variety transformed with *Rpi-blb2* and *Rpi-vnt1.1* single gene constructs. This does not seem to indicate a significant difference in the regeneration efficiency of these two varieties.

The DLA technique was used for the first time in Kenya to evaluate resistance of potato to *P. infestans* infection in addition to WPA that had previously been used by Ghislain et al. (2018). It is demonstrated herein that detached-leaf bioassays can be used to quickly determine the level of LB resistance in transgenic potato events. For the first DLA, all the events that showed resistance were equally resistant to all three isolates. The resistant transgenic events were tested for the second time using 2 isolates and they similarly showed equal resistance. DLA facilitates handling of many plant genotypes and allows individual genotypes to be challenged with several pathogen isolates. Noteworthy, the first and second assays gave slightly different results in that, the transgenic events exhibited a higher resistance in the second assay. Eight of the transgenic events had no infection (0% of leaf affected area) in the second assay while they exhibited a low but visible % of leaf affected area in the first assay. This difference could be attributed to the decrease in pathogen aggressiveness during the lapse of time between the first and the second assay. While the first assay was performed when the isolates were freshly isolated from the field, the second assay was performed one month after the first DLA raising the possibility that the isolates had reduced aggressiveness. Aggressiveness of isolates is usually reduced when an isolate is frequently cultured in the laboratory, additionally, different isolates differ in aggressiveness, but the differences tend to be smaller in genetically similar populations (Cooke et al., 2006). A slight difference was observed between the WPA and the DLA where the events that had exhibited hypersensitive response in DLA, exhibited extreme resistance in the WPA. This could be attributed to the different physiological states of the plants. It is expected that all the transgenic events that exhibited resistance in the WPA will exhibit extreme resistance in the field since Sharma et al., (2013) and Ghislain et al. (2018) observed that transgenic events that exhibited partial resistance in the glasshouse were completely resistant in the field. This was anticipated due to a lower natural inoculum pressure in the field compared to that used for DLA or WPA at 20,000 sporangia/ml. Additionally, field conditions cannot be controlled where sub-optimal conditions

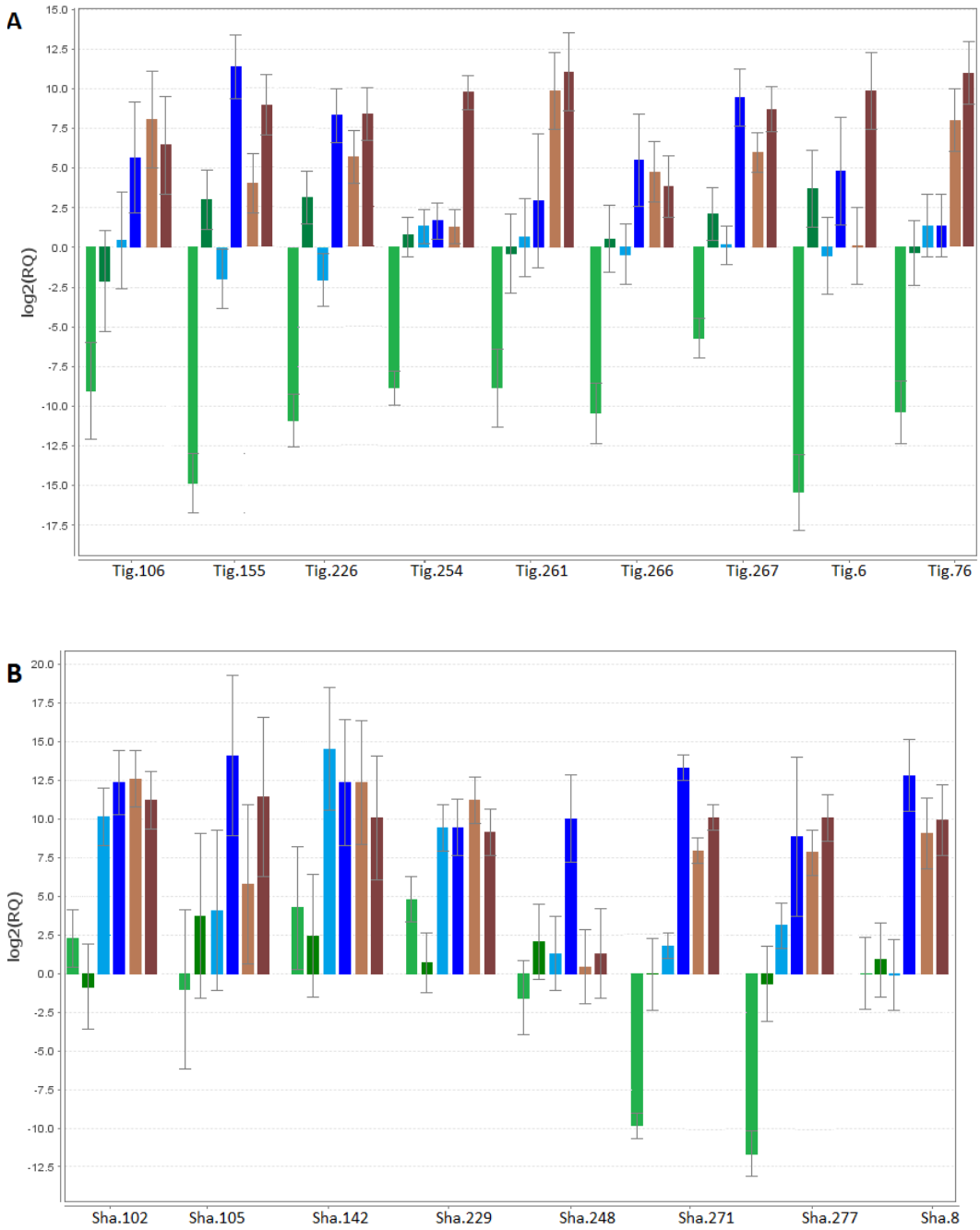

Figure 5. Relative quantification (RQ) plot of expression profiles of each of the *R* genes before *P. infestans* inoculation at day 0 and day 3 after inoculation for highly resistant transgenic events from 'Tigoni' and 'Shangi'. Figure A contains 'Tigoni' transgenic events and B has 'Shangi' transgenic events. Green bars represent *Rpi-vnt1.1* gene, blue bars represent *RB* gene and brown bars represent *Rpi-blb2* gene. Bars of the lighter shade represent day 0 and the darker shade represent day 3.

lead to a lower infection potential. It is therefore evident from this work that DLA is a reliable method to rapidly identify transgenic events that have extreme resistance to *P. infestans*. This reduces the amount of time and resources needed to evaluate resistance of transgenic events.

R gene expression was shown to be induced after inoculation with *P. infestans,* slowly decreasing over the subsequent three days (Kramer et al., 2009; Vleeshouwers et al., 2011; Orbegozo et al., 2016; Roman et al., 2017, Ghislain et al., 2018). In this study, there was significant variation in the expression levels of the three *R*

genes before and after inoculation. Ghislain et al. (2018) reported that there were insignificant differences of gene expression at different time points for the unique highly resistant transgenic event studied. Here, *R* gene expression was assayed on 17 highly resistant transgenic events. Results showed enhanced expression after inoculation in two third of the transgenic events while it reduces slightly in others. This observation appears to be consistent with that observed by Millet et al., (2009) in nwhich *RB* gene transcript abundance varied in different transgenic lines which was attributed to the location in which the *R* gene was introduced.

Five transgenic events that expressed a lower expression than that of the reference gene for at least one of their *R* genes still exhibited late blight resistance after inoculation. Millet et al., (2009, 2015) showed that disease resistance was affected by plant age and cultivar; even though *R* gene expression varied in young plants, they exhibited higher resistance in comparison to older plants and vice versa. In future analysis, screening of the transgenic events at more than two time points could provide more information on expression of the three *R* genes. Additionally, it would be important to do more experiments on *R* gene expression at different physiological stages of transgenic events. The pathogen induction of *R* gene expression behavior has been observed in many other plant species where expression of *R* genes has been enhanced after pathogen challenge (Kramer et al., 2009; Alice and Joy, 2016). However, due to the differences observed in the expression of the *R* genes before and after inoculation, the results do not support that an induction or high level of expression are necessary to obtain highly resistant transgenic events when using a 3*R*-gene stack.

In conclusion, it was demonstrated here that the three *R* genes (*Rpi-vnt1.1, RB* and *Rpi-blb2*) from the wild species *S. bulbocastanum* and *S. venturii* can confer extreme, possibly stable and durable, resistance to LB in the potato varieties 'Tigoni' and 'Shangi' cultivated in east Africa. Out of 13 'Tigoni' and 12 'Shangi' transgenic events, at least 9 transgenic events from each variety that are highly resistant were identified. These events have single T-DNA copy insertions and do not have vector backbone sequence inserted which is also desirable for releasing these transgenic events as varieties. Field evaluations under natural infection by *P. infestans* are needed to confirm the extreme resistance to the local *Pi* population since host-pathogen interactions is always influenced by environmental factors. These extremely late blight resistant transgenic events have the potential to be eventually commercialized for food security and be used in breeding programs to develop other late blight resistance varieties. However, it is yet to be demonstrated that at least one of them has conserved the integrity of all three *R* genes and has not interrupted an allele of an essential gene of the potato. A LB resistant variety will be important to smallholder farmers because farmers will realize increased yields and save on the costs of fungicides while reducing exposure of fungicides, thereby generating more revenue and improving their livelihoods. Genetic control of late blight in potatoes not only provides a strategy to improve food security but also boost economic development in Sub-Saharan Africa where agriculture employs more than half of the total manpower and provides a livelihood for numerous small-scale producers (IMF, 2012). However, social acceptance of biotech or genetically engineered crops in Africa will need to improve, especially in countries where transgenic crops have not yet been introduced and where the regulatory environment is still under development (Chambers et al., 2014).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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