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Development of a co-dominant SCAR marker linked to the *Ph-3* gene for *Phytophthora infestans* resistance in tomato (*Solanum lycopersicum*)

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Abstract Random amplified polymorphism DNA (RAPD) and bulk segregant analysis (BSA) approaches were used to characterize the molecular marker linked to the *Phytophthora infestans* resistance gene *Ph-3* in tomato. A total of 800 RAPD primers were screened. One RAPD marker UBC#602 was identified to be tightly linked to the *Ph-3* gene. The marker was successfully converted into a co-dominant sequence characterized amplified region (SCAR) marker. The SCAR marker SCU602 was used to analyze 96 F_2 progenies and fitted the expected 1:2:1 Mendelian segregation ratio. Forty one tomato inbred lines were screened using the SCAR marker in comparison with a reference marker linked to the *Ph-3* gene and both markers gave the same results. SCU602 was further validated for association to resistance and its potential in MAS in 72 tomato lines and cultivars. The marker identified three genotypes harbouring the resistance allele. This SCAR marker can be used in breeding programs for the selection of the *Ph-3* gene for *Phytophthora infestans* resistance.

Keywords Late blight · Resistance · Tomato · Phytophthora infestans · Marker · Marker-assisted selection

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Introduction

The oomycete *Phytopthora infestans*, causing late blight in tomato, can quickly devastate tomato and potato crops at any time during ontogeny. Late blight (LB) has been identified as a major disease of tomato and potato, and it can destroy the crops within several days of occurrence in the field. It is difficult to detect if levels of *P. infestans* are low in the field, and by the time the disease is detected it is often too late to save the crop through fungicide application (Fry and Goodwin 1997). In addition, most current *P. infestans* isolates are resistant to metalaxyl, the only available

fungicide to combat the disease (Gisi and Cohen 1996). However, fungicides are expensive, harmful to environment and humans. Thus, the use of at least partially resistant cultivars is the most promising method to control LB. Resistance to *P. infestans* can be classified into two categories: race-specific and race non-specific resistance. Race-specific resistance is typically controlled by a single gene and can be quickly broken down by emergence of races of the pathogen. On the contrary, race non-specific resistance is polygenic resistance or quantitative resistance, and usually governed by more than one gene and may be more durable (Foolad et al. 2008). The race non-specific resistance confers partial resistance to multiple isolates/races of the pathogen and makes it more difficult to use in breeding. Race-specific resistance genes have been identified in wild tomato species, in particular *S. pimpinellifolium*. In addition, race non-specific resistance has been reported in *S. pimpinellifolium* accession L3707 (PI365951) (Irzhansky and Cohen 2006). *Ph-1* and *Ph-2* genes conferring resistance to *P. infestans* race 0 and 1, respectively, were identified in *S. pimpinellifolium* and subsequently introduced into cultivated varieties (Foolad et al. 2008); however, the resistance was overcome by new *P. infestans* isolates. *Ph-3* gene was identified in S. *pimpinellifolium* accession L3708 (PI365957) (AVRDC 1994). This gene is a partially dominant gene and confers resistance to a wide range of *P. infestans* isolates that overcome *Ph-1* and *Ph-2* genes and was introduced into market and processing tomato lines (Kim and Mutschler 2005). The *Ph-3* gene was mapped to the long arm of chromosome 9 that closed to RFLP marker TG591A.

DNA markers are useful tool for plant breeding since the presence of genes can be detected at an early stage of plant development without phenotypic evaluations. RAPD technique is one of the most widely used applications because of identification of markers linked to traits of interest without the necessity of mapping the entire genome. Martin et al. (1991) have described an efficient method based on the RAPD technique to isolate DNA segments linked to certain traits. This approach was based on near-isogenic lines (NILs) and has been successful in identifying markers linked to disease resistance genes in tomato (*Lycopersicon* sp.) (Martin et al. 1991), lettuce (*Lactuca* sp.) (Paran et al. 1991), and common bean (*Phaseolus vulgaris*) (Adam-Blondon et al. 1994). Klein-Lankhorst et al. (1991) had identified chromosome specific RAPD markers in tomato by screening a *Lycopersicon esculentum* substitution line. Successes in identification of markers linked to resistance genes or objective traits using BSA and RAPD methods have been reported (Michelmore et al. 1991; Barua et al. 1993; Liu et al. 2011; Du et al. 2011; Li et al. 2011; Shobha and Thimmappaiah 2011; Bartoszewski et al. 2012). However, disadvantages of RAPD markers include dominance (Williams et al. 1990); hence the statistical information generated is less per marker in F_2 populations, difficulties with repeatability, and skewed segregation ratios (Thormann et al. 1994). To overcome these disadvantages, RAPD markers have been converted into highly specific SCAR markers (Paran and Michelmore 1993). Such SCAR markers have proven useful for marker-assisted selection and high-resolution mapping (Kaplan et al. 1996; Paran and Michelmore 1993).

Chunwongse et al. (2002) had used BSA and amplified fragment length polymorphic (AFLP) approaches to identify six AFLP markers associated with LB resistance in L3708. Of these, only marker L78 was tightly linked to the *Ph-3* gene. An attempt has been made to convert this marker into a SCAR marker (Park et al. 2010); however, the SCAR marker is a dominant marker and not easily used in MAS, because heterozygous plants cannot be distinguished from homozygous plants. Robbins et al. (2010) have developed two co-dominant CAPS markers for use in marker-assisted breeding for *Ph-3*. These markers were re-examined by Panthee and Foolad (2012), however, fragments in heterozygous lines were less intense and marker TG591 could not be verified. In this paper, we report the conversion of a RAPD marker linked to the *Ph-3* gene into a co-dominant and highly specific SCAR marker that would enable its use in marker-assisted selection.

Materials and methods

Plant materials and DNA extraction

Three tomato genotypes for each of *P. infestans* resistance and susceptibility were provided by Vegetable Research Division, National Institute of Horticultural & Herbal Science (NIHHS), Rural Development Administration (RDA), Korea, and Fruit and Vegetable Research Institute (FAVRI), Vietnam.

Resistant genotypes included CLN2037B, L3708, which were provided by NIHHS, and 08TP73-10-4 (an inbred line derived from the cross between TP76, highly susceptible to *P. infestans*, and CLN2037D, an inbred line carrying the *Ph-3* gene), which was provided by FAVRI. Susceptible lines were 08TP03-15-3-1 (received from FAVRI), TS33, and WVa700 (received from NIHHS). An F_2 population segregating for late blight resistance was generated from a cross between inbred lines 08TP03-15-3-1 and 08TP73-10-4. F_2 seeds were produced by allowing self-pollination of F_1 plants. The population consisting of 96 F_2 plants was developed. In addition, 41 tomato inbred lines and 72 tomato genotypes were provided by NIHHS. All genotypes belonged to *S. lycopersicum*.

Genomic DNA of the materials used in this study were extracted from leaves of young seedlings (three to four true leaves) using DNeasy Plant Kit (96-well format) from QIAGEN (Qiagen GmbH, Hilden, Germany). The DNA concentration was measured on a Nanovue spectrophotometer (GE Healthcare, U.K). The quality of the DNA was inspected using agarose gel electrophoresis and spectral absorbance (the A_{260}/A_{280} ratio).

Evaluation of resistance to *Phytophthora infestans*

The *P. infestans* isolate belonging to physiological race R0.1.3.5.6.10.11, a dominant race in Kangnung (Zhang and Kim 2007), provided by Kangnung University (Korea) was used for inoculation. The isolate was maintained on a V8 agar Petri plate and incubated at 18 °C. Inoculation was performed following the detached leaflet test as described by Legard et al. (1995) with slight modifications. Briefly, inoculum was prepared from 12-day old V8 agar Petri plate cultures grown in the dark at 20 °C. A sporangial suspension diluted in sterilized water to 10^5 sporangia/ml was incubated at 12 °C for 2 h to induce zoospore release. Before inoculation 1 day, three full sized tomato leaflets, taken at the 5–6 true leaf stage from the third or fourth leaf from the stem apex to synchronize leaf age and size, were collected and placed on the surface of 1.8 % agar Petri plates. The plates were kept at 20 °C for 18 h to keep the leaves wet. For each leaf, 30 μl of sporangia suspension was gently dropped in the centre. After inoculation, the leaflets were incubated for 48 h at 17 °C without light; thereafter, maintained at 18 °C for 6 days with saturated humidity under 12 h light (70 $\mu E\ M^{-2}S^{-1}$) period. Disease reaction was evaluated by counting sporangia produced under a microscope. Briefly, three inoculated leaves of each plan were placed in the Falcon tube that contained 3 ml of distilled water. The tube was shaken for 3–5 s by vortex to release sporangia from the leaves. Twenty microlitres of the aliquots containing sporangia was pipetted for counting. Plants were classified into three groups based on the number of sporangia counted, ≥ 1: resistant; 1.1 - 10: intermediate; and > 10: susceptible.

Bulk segregation analysis

An equal amount of DNA from 10 resistant F_2 plants and 10 susceptible F_2 plants were pooled into an R-pool and an S-pool, respectively, as described by Michelmore et al. (1991). These pools were used to screen random amplified polymorphic DNA (RAPD) primers, which showed polymorphism between parents, and resistant and susceptible reference inbred lines. Once DNA bands were found corresponding to the resistant parent and R-pool, or to the susceptible parent and S-pool, the bands were cloned and sequenced.

RAPD analysis

A total of 800 UBC (University of British Columbia) RAPD primers (synthesized by Bioneer, Korea) were pre-screened on the parents and reference resistant and susceptible inbred lines. The PCR reactions were performed in Eppendorf Mastercycler Gradient (USA). The 15 μl reaction volume included 2.5 mM $MgCl_2$ (Roche, Korea), 200 μM deoxyribonucleotide triphosphate mix (Roche, Korea), 10 X PCR buffer, 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Genet Bio, Korea), and 0.25 μM of random primer and 15–20 ng of genomic DNA. The amplification reactions

Table 1 List of plant materials used for preliminary evaluation

Accessions	Species	Characteristics[a]
TS33	*S. lycopersicum*	*Ph-1*
WVa 700	*S. pimpinellifolium*	*Ph-2*
CLN2037B	*S. lycopersicum*	*Ph-3*
L3708	*S. pimpinellifolium*	*Ph-3, Ph-4*

[a] Different genes conferring resistance to *P. infestans* present in tomato inbred lines

Fig. 1 Efficiency of different genotypes involving in *P. infestans* resistance

were carried out using the following thermal profile: 94 °C for 3 min (1 cycle); 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min (40 cycles); 72 °C for 7 min (1 cycle). Amplified products were incubated with a 1:10,000 dilution of the SYBR Green I nucleic acid gel stain (Invitrogen, USA) for 20 min and separated on a 1 % agarose gels using 0.5 X TBE buffer for three and half hours at 120 V and photographed under UV light. A 100 bp ladder was used as a molecular weight marker.

Conversion of RAPD marker into SCAR marker

Cloning and sequencing RAPD fragment

The RAPD fragment obtained from tomato line CLN2037B was excised from 1 % agarose gels and purified with a QIAquick gel extraction kit (Qiagen, Germany). The fragment was cloned using TOPO TA Cloning kit following the manufacturer's instructions (Invitrogen, USA). Plasmid DNA was extracted using Core-one plasmid mini-prep kit (Korea) and sent to the sequencing company CoreBio (Korea) for sequencing. The sequence was analyzed using the program BioEdit 7.0 (Hall 1999).

Primer design

Primers were designed according to the sequence obtained using the program Primer3 4.0 (Rozen and Skaletsky 2000). Oligonucleotide primers were synthesized by Bioneer Corp. (Korea).

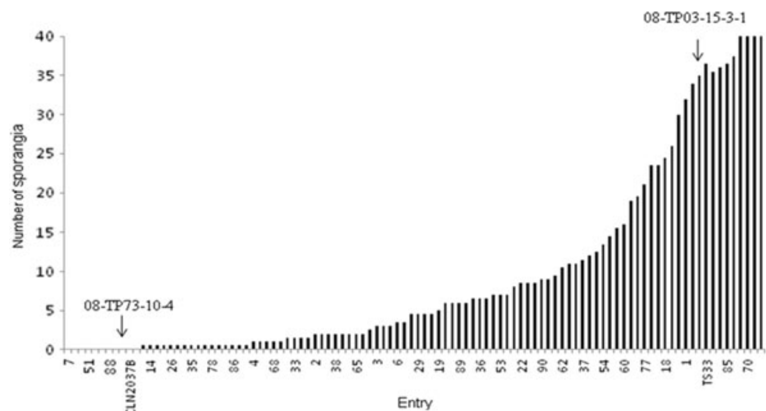

Fig. 2 Disease reactions of F_2 lines, parents and reference inbred lines

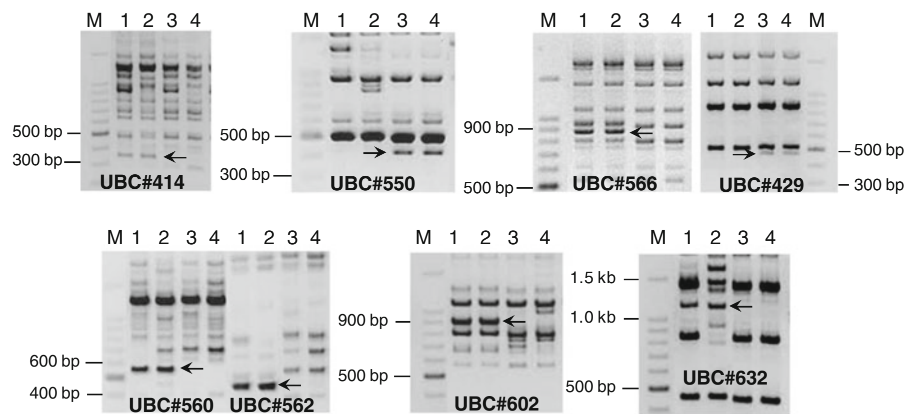

Fig. 3 Polymorphisms of selected polymorphic RAPD primers. Lanes M, 100 bp molecular ladder, *1*, resistant parent; *2*, R pool; *3*, susceptible parent; *4*, S pool. Polymorphic markers are indicated by *arrows*

SCAR analysis

Each PCR reaction was carried out in a total reaction volume of 25 μl containing 15–20 ng of genomic DNA, 200 μM deoxyribonucleotide triphosphate mix (Roche, Korea), 10 X PCR buffer, 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Roche, Korea), and 0.25 μM of each primer. PCR was performed on an Eppendorf Mastercycler Gradient (USA). The amplification profile consisted of an initial denaturation for 5 min at 94 °C followed by 35 cycles of PCR amplification under the following parameters: 20 s at 94 °C, 1 min at the annealing temperature of 55 °C, and 1 min of primer elongation at 72 °C. A final incubation at 72 °C for 10 min was programmed to allow completion of primer extension. Amplified products were visualized on an agarose gel as described previously.

Results

Resistance to *P. infestans* isolate in F_2 population

In order to confirm whether the *P. infestans* isolate used in this study overcame the *Ph-1* and *Ph-2* genes, a preliminary evaluation was conducted using four reference inbred lines carrying different genes for resistance against *P. infestans* as shown in Table 1. TS33 and WVa700 showed disease symptom but not CLN2037B and L3708 (Fig. 1). The result indicates that the *Ph-1* (present in TS33) and *Ph-2* (present in WVa700) genes were not overcome by the pathogen isolate used. CLN2037B and L3708 carrying the *Ph-3* gene displayed good resistance level to late blight. The resistant parent 08TP73-10-4 displayed the same level of resistance as CLN2037B and L3708, while the susceptible parent 08TP03-15-3-1 was as highly susceptible as TS33 and WVa700 (Fig. 2). Out of 96 F_2 plants, 29 were resistant, 40 were intermediate, and 27 were susceptible, fitting a monogenic segregation ratio.

Identification of RAPD markers linked to the *Ph-3* gene

A total of 800 RAPD primers were preliminary screened on the resistant and susceptible parents. Of these, 54 were detected to be polymorphic between the two parents, and reference resistant (CLN2037B and L37080) and susceptible (TS33 and WVa700) inbred lines. These primers were then screened on R- and S-pools and together with the parents, but only 12 primers showed polymorphism between R- and S- pools (Fig. 3). Ten of these (UBC#414, 460, 535, 560, 562, 566,602, 632, and 698) were associated in a coupling phase linkage with the *Ph-3* gene, amplifying the polymorphic fragments only in the resistant parent. The other two RAPD fragments (UBC#429 and 550)

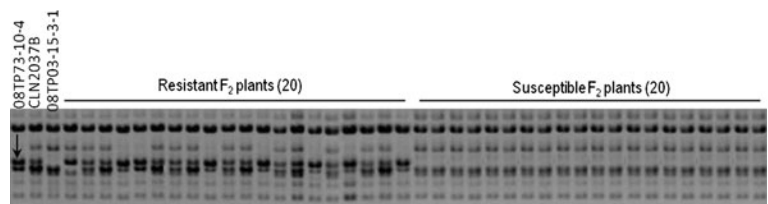

Fig. 4 Segregation pattern of the RAPD marker UBC#602 linked to the *Ph-3* gene. Polymorphic marker is indicated by *arrow*

Table 2 List of new primers designed from the sequence of the UBC#602 fragment

Marker	Forward primer (5′-3′)	Revert primer (3′-5′)	Annealing Temperature
SCU602F1R1	GTATTGTCCAAGGGTTTCAA	GAAATGATCAAAACCCTAGA	55
SCU602F2R2	AGCCTCATGGCTTGTATTTA	GAGACCAACCTCTATGA	55
SCU602F3R3	ACAAACTAAATGGCCAAGTG	ATGATAGCTCTTCTCGGGA	55

amplified polymorphic fragments only in the susceptible parent and thus were associated in repulsion phase linkage with the *Ph-3* gene (Fig. 3). These primers were then used to analyze the 20 individuals comprising the bulks to determine whether there was significant linkage to the resistance trait. However, only one marker UBC#602 (GCG AAG ACT A) revealed a 100 % linkage in the individual plants comprising the contrasting bulks (Fig. 4).

Development of SCAR marker

The UBC#602 fragment was successfully cloned and sequenced. A 983-base-pair sub-clone of fragment UBC#602 was identified and the terminal 10 bases of 5′-ends and 3′-end of the clones exactly matched the sequence of primer. In order to screen primers using high resolution melting (HRM) system when primers are not polymorphic using regular PCR, three SCAR primer pairs were designed covering the sequence: the first one amplifying from bases 36 → 473 (SCUF1R1), the second one from bases 234 → 723 (SCUF2R2), the third one from bases 495 → 900 (SCUF3R3). Primer sequences are listed in Table 2. However, only the third primer pair produced two polymorphic fragments in the parents (Fig. 5), indicating either a deletion or insertion mutation between the two alleles in the end of the sequence. The fragment amplified in the resistant parent was about 400 bp and in the susceptible parent was about 450 bp.

Marker-assisted selection

Forty one tomato inbred lines were genotyped using a reference marker linked to the *Ph-3* gene (provided by Park Pue Hee, personal communication) were used for comparison with the SCAR marker SCU602F3R3 (Table 3). All the inbred lines have the same genotyping results using two markers. In addition, 72 tomato genotypes from our germplasm were tested (Table S1). Of these, 3 tomato genotypes such as IT200624, K121155, and K133673 carry the resistant allele. Thus, the SCAR marker developed in this study makes possible to monitor the *Phytopthora infestans* resistance gene *Ph-3* commonly used in tomato breeding programs.

Discussion

In this study, the BSA method provided a rapid and simple alternative technique to identify one RAPD marker linked to the *Ph-3* gene. UBC#602, one of the 800 random primers screened, reproducibly produced polymorphism among parents and the two bulks. The RAPD marker UBC#602 was successfully converted into co-dominant SCAR marker SCU602F3R3. The marker amplified about 400 and 450-bp long bands in resistant and susceptible parents, respectively and was able to identify the heterozygote. The SCAR marker SCU602F3R3 was used to analyze 96 F_2 progenies, of which 29 were predicted to be homozygous resistant, 45 to be heterozygous, and 22

Fig. 5 Segregation pattern of the co-dominant SCAR marker SCU602F2R3 linked to the *Ph-3* gene

Table 3 List of tomato inbred lines used for genotyping

Entry	Genotype	
	SCU602F3R3	Reference marker[a]
	Inbred lines	
06-9-23	S	S
06-9-27	S	S
06-9-34	S	S
06-9-35	S	S
06-9-36	S	S
06-9-37	S	S
06-9-56	S	S
06-9-57	S	S
06-9-58	S	S
07-15x195-A	R	R
07-T-1076	R	R
06-9-62A	S	S
BS67x195-A	R	R
1036-31	R	R
1036-116	R	R
1033-3	R	R
1033-4	R	R
1033-5	R	R
1033-6	R	R
1033-7	R	R
1033-8	R	R
1033-9	R	R
1033-10	R	R
1033-11	R	R
1033-12	R	R
1033-14	R	R
1033-15	R	R
1033-16	R	R
1033-18	R	R
1033-20	R	R
1033-21	R	R
1033-22	R	R
1033-24	R	R
1033-25	R	R
1033-26	R	R
1033-28	R	R
1033-29	R	R
1033-30	R	R
1033-111	R	R
1033-112	R	R
1033-164	R	R
	Control checks	
CLN2037B	R	R
L3708	R	R
08TP73-10-4	R	R
TS33	S	S
WVa700	S	S
08TP03-15-3-1	S	S

[a] Park et al. 2010: *R* resistance; *S* susceptible

to be homozygous susceptible for the *Ph-3* gene. These numbers fitted the expected 1:2:1 segregation ratio at the 95 % level (χ^2=1.4). In addition, 41 tomato inbred lines were genotyped using the SCAR marker in comparison with a reference marker linked to the *Ph-3* gene (Park et al. 2010) and gave the same results. SCU602F3R3 identified three accessions among 72 additional tomato genotypes tested possessing the *Ph-3* gene. Recently, co-dominant CAPS markers linked to the *Ph-3* gene have been developed (Robbins et al. 2010). Panthee and Foolad (2012) have reexamined these markers and the result showed that marker TG591 could not be verified, and marker TC328 produced expected fragment sizes in resistant and susceptible parents, but taking into account that fragments in heterozygotes are always stained less intensely. Park et al. (2010) have converted successfully one of six AFLP markers linked to the *Ph-3* gene (Chunwongse et al. 2002) into a dominant SCAR marker. This marker shows promise in identifying the *Ph-3* gene; however, it cannot differentiate homozygous plants from the heterozygous plants. Thus, it is not applicable in MAS. The SCAR marker in this study possesses the characters of being PCR-based, simple, reliable, cost efficient, no restriction required, and amenable to large numbers of samples, which are the key requirements for large scale marker implementation of MAS in plant breeding (Eagles et al. 2001). In pyramiding strategies that are considered for development of durable disease resistance, identification of molecular markers for each desired resistance gene is required. SCA602F3R3 offers some potential advantages to tomato breeders. The marker can detect heterozygous plants, which allows strict backcrossing

that saves time and is efficient. Thus, the developed SCAR marker could be used in MAS of *Ph-3* in tomato breeding programs for LB resistance.

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