

Molecular Markers Associated with *Ph-3* Gene Conferring Late Blight Resistance in Tomato

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Abstract

Late blight (LB), caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of tomato. Three major genes *Ph-1*, *Ph-2* and *Ph-3* conferring resistance to LB have been identified and mapped to the chromosomes 7, 10 and 9, respectively. However, PCR-based molecular markers associated with these genes are limited. Molecular markers are extremely useful in the screening and selection of tomato lines for the development of LB resistant genotypes. The objective of this study was to identify molecular markers associated with *Ph-3* gene conferring LB resistance in tomato. Four co-dominant markers were found to be associated with *Ph-3*, all of which were sequence characterized amplified region (SCAR) type. Breeding lines and cultivars were inoculated with a field isolate of *Phytophthora infestans* to collect phenotypic data on disease resistance. Genotypic data from molecular markers associated with *Ph-3* were in close agreement with the phenotypic data for the lines tested. With the verification of genotypic data from novel molecular markers in known genotypes supported by phenotypic data, the novel molecular markers may be useful in screening tomato populations aiming to develop LB resistant genotypes or cloning the LB resistant genes.

Keywords

Late Blight, Molecular Breeding, *Phytophthora infestans*, Resistance Breeding, Tomato

1. Introduction

Late blight (LB), caused by the oomycete *Phytophthora infestans* (Montagne, Bary), is one of the most poten-

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tially devastating diseases of tomato in areas with high humidity and cool temperatures and can cause 100% crop loss in unprotected tomato fields or greenhouses. Because of its devastating economic impact, this disease has been the subject of intensified pathological and genetic research since the occurrence of the Irish potato famine in the 1840s. Genetic resistance to LB in tomato has been of interest for many years, and three major resistance genes have been identified in the red-fruited tomato wild species *S. pimpinellifolium*, including *Ph-1*, *Ph-2* and *Ph-3*, which have been mapped to tomato chromosomes 7, 10, and 9, respectively. *Ph-1* is a single dominant gene providing resistance to race T-0, but it was rapidly overcome by new races of the pathogen. *Ph-1* was mapped to the distal end of chromosome 7 using morphological markers [1]. However, no molecular marker associated with this resistance gene has been reported. Currently, *P. infestans* race T-1 predominates, rendering the resistance conferred by *Ph-1* ineffective. The resistance conditioned by *Ph-2*, a single incomplete-dominant gene mapped to the lower end of the long arm of tomato chromosome 10 [2], provides partial resistance to several isolates of race T-1 [1] [3]. *Ph-2* slows, but does not stop the disease progress [2]. Furthermore, *Ph-2* often fails in the presence of more aggressive isolates [4] [5]. *Ph-2* has been mapped to an 8.4 cM interval on the long arm of chromosome 10 between RFLP markers CP105 and TG233 [2]. A much stronger resistance gene, *Ph-3*, was discovered in *S. pimpinellifolium* accessions L3707 and L3708 (a.k.a. LA 1269 or PI365957) at the Asian Vegetable Research and Development Center (AVRDC) in Taiwan [4]. Currently this gene is much more useful than *Ph-1* and *Ph-2* and confers incompletely dominant resistance to a wide range of *P. infestans* isolates of tomato, including those that overcome *Ph-1* and *Ph-2* [6]. *Ph-3* has been mapped to the long arm of chromosome 9 near RFLP marker TG591a [6]. Recently, this gene was fine-mapped in the 0.5 cM genomic region of long arm of chromosome 9 in between Indel_3 and P55 molecular markers [7]. Utilizing the fine-map information, and considering the significance of the gene in late blight resistance, it was further characterized by cloning a 24 kb region [8]. Recently, co-dominant sequence characterized amplified region (SCAR) [9] and cleaved amplified polymorphic sequence (CAPS) markers have been developed for use in marker-assisted breeding for *Ph-3* (M. Mutschler, *pers. comm.*) [10]. Identification of these markers associated with *Ph-3* has been extremely useful to transfer the *Ph-3* gene into the desirable genetic background by marker-assisted selection (MAS). However, it has been suggested that the full resistance conferred by L3707 and L3708 (the original sources of *Ph-3*) is conferred by more than just one *Ph-3* locus, and that *Ph-3* alone in either homozygous or heterozygous conditions would not be highly commercially desirable as it does not confer strong resistance against aggressive isolates such as US-7 and US-17 [11] [12]. The presence of yet undetermined additional hypostatic gene(s) present in L3708 is necessary to provide full resistance. Further, it has been determined that there are new *P. infestans* isolates which have overcome the *Ph-3* resistance [6]. However, it appears that a combination of *Ph-2* and *Ph-3* confers strong resistance to most such isolates. Recently, several tomato-breeding programs around the world, including the North Carolina State University, Pennsylvania State University, Cornell University (M. Mutschler, *pers. comm.*), and AVRDC—The World Vegetable Center (P. Hanson, *pers. comm.*), have succeeded in transferring LB resistance genes to fresh-market and/or processing tomato breeding lines or hybrid cultivars using a combination of phenotypic screening and MAS. For example, most recently, several fresh-market tomato breeding lines (e.g., NC1 CELBR (*Ph-2* + *Ph-3*) and NC2 CELBR (*Ph-2* + *Ph-3*) and hybrid cultivars Plum Regal (*Ph-3*), Mountain Magic (*Ph-2* + *Ph-3*) and Mountain Merit (*Ph-2* + *Ph-3*)) have been released by the North Carolina State Tomato Breeding Program, USA [13] [14]. Also, more breeding lines and cultivars are in the pipeline from these and other tomato breeding programs. However, the availability of more useful PCR-based markers for *Ph-2* and *Ph-3* will make the selection and breeding for LB resistance in tomato more expedient. The objective of this study was to identify molecular markers associated with *Ph-3* gene conferring LB resistance in tomato.

2. Materials and Methods

Tomato lines used in this study are described in **Figure 1**. The control lines used were NC 1CELBR (Resistant homozygous), NC 123S (Susceptible homozygous), and Mountain Merit F1 hybrid (NC 1CELBR × NC 123S) (Heterozygous) for *Ph-2* and *Ph-3*. Lines NC 25P (Resistant homozygous), NC 30P (Susceptible homozygous) and hybrid Plum Regal (NC 30P × NC 25P) (Heterozygous) for *Ph-3* were also included. Additional lines with known status for *Ph-2* and *Ph-3* included were 63EB(2002)-1C, NC 870(2002)-1B and the F1 hybrid, NC03220, from the cross between these two lines. The original source of the *Ph-3* gene used in developing NC 1CELBR (L3707) and the source of the *Ph-2* gene for NC 1CELBR (Richter's Wild Tomato) were also included. Other

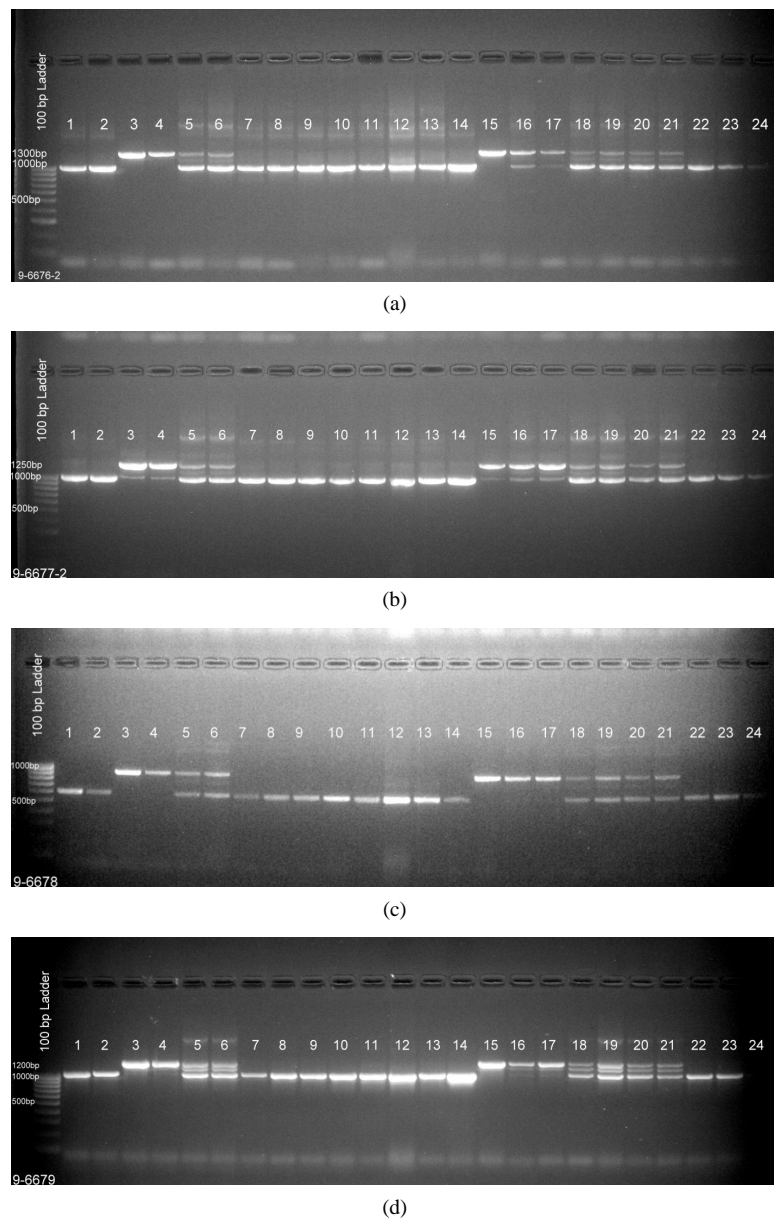


Figure 1. Molecular markers associated with *Ph-3* mediated late blight resistance in tomato. In the gel, the sample order is: 1: NC1CELBR-R, 2: NC25P-R, 3: NC123S-S, 4: NC30P-S, 5: Mtn. Merit-H, 6: Plum Regal-H, 7: 08135(x)-1W, 8: 08135(x)-6W, 9: 145L-1(2008), 10: 62L-1(2008), 11: 08135(x)-11W, 12: 860-1A(2008), 13: 860-1B(2008), 14: LA3707, 15: Micro Tom, 16: 68L-1(2008), 17: 146L-1(2008), 18: 22L-2W(2008), 19: 22L-1W(2008), 20: 69L-1(2008), 21: 70L-1(2008), 22: 870(2002)-1B, 23: 63EB(2002)-1C, and 24: NC03220. (a) NCLB-9-6676 SCAR-type molecular marker associated with *Ph-3* gene conferring late blight resistance in tomato. Resistant genotypes NC 1CELBR and NC 25P produced 1000 bp PCR product whereas susceptible genotypes NC 30P and NC 123S produced 1300 bp PCR product. Both fragments were present in heterozygous genotypes Plum Regal and Mountain Merit. (b) NCLB-9-6677 SCAR-type molecular marker associated with *Ph-3* gene conferring late blight resistance in tomato. Resistant genotypes NC 1CELBR and NC 25P produced 1000 bp PCR product and susceptible genotypes NC 30P and NC 123S produced 1250 bp PCR product. Both fragments were present in heterozygous genotypes Plum Regal and Mountain Merit. (c) NCLB-9-6678, SCAR-type molecular marker associated with *Ph-3* gene conferring late blight resistance in tomato. Resistant genotype NC 1CELBR and NC 25P produced 600 bp PCR product whereas susceptible genotypes NC 30P and NC 123S produced 900 bp PCR product. Both fragments were present in heterozygous genotypes Plum Regal and Mountain Merit. (d) NCLB-9-6679 SCAR-type molecular marker associated with *Ph-3* gene conferring late blight resistance in tomato. Resistant genotype NC 1CELBR and NC 25P produced 1000 bp PCR product whereas susceptible genotypes NC 30P and NC 123S produced 1200 bp PCR product. Both fragments were present in heterozygous genotypes Plum Regal and Mountain Merit.

lines with known status for *Ph-2* and *Ph-3* included 96LB, an early generation backcross line derived from L3707 and 139LB(2001)3W-8 and 139LB(2001)3W-64, both early backcross generation lines derived from “Richter’s Wild Tomato”. Other lines tested were resistant selections made from field trials where late blight was present but that had not been identified for their status of the *Ph-2* and *Ph-3* genes. Three grape tomato lines (08135 selfed lines from F4 generation) that had been selected for superior horticultural traits and had limited testing for late blight resistance were included. The lines were planted with two replications per line and three plants per replication in 24-cell plastic trays. Plants were maintained in a controlled condition with 25/15°C day and night temperatures, 70% - 90% relative humidity, and 12/12 hour photoperiods in the greenhouse. Field isolate of *Phytophthora infestans* was collected from naturally infested susceptible tomato genotypes from the field at Mountain Horticultural Crops Research & Extension Center (MHCREC) in the summer of 2012 and inoculum was maintained on NC 123S (susceptible genotype) leaves under controlled conditions. In the greenhouse, seven-week-old plants were inoculated with the field isolate of *Phytophthora infestans* at the rate of 10,000/mL sporangia. Individual plants were hand misted twice at 30 minute intervals to ensure that all plants received uniform inoculum pressure. Plants were evaluated after one week using a score from 0 - 5 (where, 0 was no lesions, and 5 was for plant parts completely covered with lesions or dead).

The main basis for designing primers in this study is the mapping information available for *Ph-2* and *Ph-3*. These two genes are mapped to chromosome 10 and 9, respectively. The latest information on functional genomics resources from these two chromosomes was utilized, which was given as follows: NC-LB-10-6395 (Solyc10g085460.1-CC-NBS-LRR, resistance protein); NC-LB-9-6676-2 (Solyc09g092280.1.1-CC-NBS-LRR, resistance protein); NC-LB-9-6677-2 (Solyc09g092290.1.1-CC-NBS-LRR, resistance protein); NC-LB-9-6678 (Solyc09g092300.2.1-CC-NBS, resistance protein fragment); NC-LB-9-6679 (Solyc09g092310.1.1-CC-NBS-LRR, resistance protein) (The downstream end of the integration depending on SNP data). Associated genome sequence was downloaded from Sol Genomics Network (http://solgenomics.net/organism/Solanum_lycopersicum/genome). Resources were also utilized from Solanaceae Coordinated Agriculture Project (<http://solcap.msu.edu/>). Twenty-five primer pairs were designed on the basis of this sequence using Primer 3.0 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) with default settings. A list of informative primers associated with *Ph-3* is reported in **Table 1**.

Genomic DNA was extracted from 2-week-old plants of each line using a standard CTAB protocol [15]. DNA concentration was measured using a Nanodrop-1000 (Nano Drop Products, Wilmington, DE) and adjusted to a final concentration of 20 ng/μL. All markers examined in this study were PCR-based, including cleaved amplified polymorphic sequences (CAPS) and sequence characterized amplified regions (SCAR). All PCR primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). PCR master-mix was used from Phenix Research Products (Candler, NC) and QIAGEN (Valencia, CA). PCR was conducted in 25-μL volume consisting of 12.5 μL master mix (1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 U *Taq* polymerase and 10× PCR buffer), 2 μL DNA (20 ng/μL), 1.5 μL (12.5 μM) primers (total of forward and reverse), and 7.5 μL of nuclease free water. DNA amplifications were performed in a thermal cycler (Eppendorf, New York) using the following cycling condition: one cycle of 92°C for 3 min, followed by 35 cycles of 92°C for 0.30 min, 52°C for 1 min, and 72°C for 0.30 min; and final extension at 72°C for 8 min, followed by holding at 4°C. Following PCR amplification, PCR products were either directly separated on 1.5% Agarose gel and DNA bands visualized with 0.5 μg/ml ethidium bromide staining or first digested with a restriction enzyme (*Hae III*) and then separated on Agarose gel (for CAPS markers). DNA fragment sizes were determined using a 100-bp DNA ladder (Phenix Research Products, Candler, NC). For all markers, PCR amplification and gel electrophoresis were carried out at least twice to confirm the results.

3. Results and Discussion

Four sequence characterized amplified region (SCAR) molecular markers were found to be associated with *Ph-3* gene. The novel co-dominant SCAR marker NCLB-9-6676 associated with *Ph-3* gene produced 1000 bp and 1300 bp PCR product from resistant and susceptible genotypes, respectively (**Figure 1(a)**). Similarly, NCLB-9-6677 was also found to produce the PCR product of 1000 and 1250 bp from resistant and susceptible lines, respectively (**Figure 1(b)**).

Yet another co-dominant SCAR molecular marker NCLB-9-6678 was also capable to discriminate resistant

Table 1. List of forward and reverse primers for informative sequence characterized amplified region (SCAR) markers associated with *Ph-3* gene conferring late blight resistance in tomato.

Marker	Position	Forward sequence	Reverse sequence	Resistant	Susceptible
NC-LB-9-6676	66,762,879	ACAGAAAAGTGCACGAAGTT	ATTTGAATGTTCTGGATTGC	1000	1300
NC-LB-9-6677	66,773,785	AAAGCCAATACCTTAGCACA	ATTCAACGGTCAAAAACCTTG	1000	1250
NC-LB-9-6678	66,784,000	CCTTAATGCAATAGGCAAAT	ATTTGAATGTTCTGGATTGG	600	900
NC-LB-9-6679	66,792,551	TCGGCTTATAGAAAAGCAAC	CGGAGAACAGTTTTGAACTC	1000	1200

and susceptible genotypes of tomato by producing 600 and 900 bp DNA fragments (**Figure 1(c)**). Hybrid genotypes Plum Regal and Mountain Merit had distinct double bands. Similarly, NCLB-9-6679 produced 1000 and 1200 bp PCR product from resistant and susceptible genotypes, respectively (**Figure 1(d)**). Chromosomal location of these markers associated with *Ph-3* gene is shown in **Figure 2**. Informative primers associated with *Ph-3* gene, and expected DNA fragments are given in **Table 1**.

Results were consistent for all lines for the four new SCAR markers associated with *Ph-3* gene. Important point to note was that genotypic data from new molecular markers on established genotypes (such as NC1-CELBR, NC123S, Mountain Merit, NC25P, NC30P and Plum Regal) with known gene status was in perfect agreement making the finding robust.

In the phenotypic ratings for late blight incidence, NC 1CELBR and 08135(x)-1W, both of which were R for *Ph-2* and *Ph-3* genotypic data, and Richter's Wild Tomato, which was R for *Ph-2* but S for *Ph-3* according to the genotypic data, were completely free of late blight. Two other lines, 860-1A(2008) and NC161L [16] both of which were R for both *Ph-2* and *Ph-3* according to the genotypic data, had a significantly higher ratings for late blight incidence than the above three lines. The hybrid "Mountain Merit" (NC 1CELBR xNC123S), which was H for both *Ph-2* and *Ph-3*, had a higher disease rating than the resistance parent NC 1CELBR (data not shown). Also, the hybrid "Plum Regal", which was heterozygous for the *Ph-3* gene alone, had a higher disease rating than its resistant parent, NC 25P.

We identified four co-dominant molecular markers associated with *Ph-3* gene. These molecular markers (NCLB-9-6676, NCLB-9-6677, NCLB-9-6678, and NCLB-9-6679) are novel and are expected to be useful for tomato breeding program in marker-assisted selection. Because *Phytophthora infestans* spores can travel a long distance by wind in a relatively short time, have a relatively short incubation period, and produce millions of spores before symptoms are visible, and screening is complicated [17]. Therefore, development of reliable co-dominant molecular markers is extremely important. Findings from this study are likely to pave the way towards fulfilling this necessity.

We adapted the approach of genome-based molecular marker development utilizing the currently available tomato genome (http://solgenomics.net/organism/Solanum_lycopersicum/genome). On the basis of this information, novel molecular markers NCLB-9-6676, NCLB-9-6677, NCLB-9-6678, and NCLB-9-6679 associated with *Ph-3* are located on 66,762,879, 66,773,785, 66,784,000, and 66,792,551 bp, respectively. With respect to previously identified RFLP molecular marker, TG591 associated with *Ph-3*, distance between TG591 and these molecular markers associated with *Ph-3* are within 8 to 83 kbp distance (<1 cM) in this study (<http://solgenomics.net/marker/SGN-M88/details>), which is within the genome sequence of *Ph-3* gene (**Figure 2**). This is potentially within the fine-map and cloned distance of the *Ph-3* gene [7] [8]. Furthermore, we utilized the annotation information including CC-NBS-LRR to select the genomic sequence to design the primers (see Materials and Methods), which was the protein found in characterizing the cloned *Ph-3* gene [8]. A strong association between genotypic and phenotypic data obtained from greenhouse experiments using field isolate was found in the present experiment. The combination of *Ph-2* and *Ph-3* confers resistance to late blight better than with a single gene, which is also observed in the present study.

Since molecular markers were found strongly associated with *Ph-3* gene conferring resistance to late blight, these molecular markers may facilitate the combination of *Ph-2* and *Ph-3* genes in tomato breeding lines even without field inoculation. However, testing of lines for late blight resistance in field, greenhouse, and growth chamber inoculation trials will likely be needed in addition to marker testing to achieve a high level of resistance. Some lines homozygous for the *Ph-2* and *Ph-3* genes separately or combined were highly resistant whereas other lines indicated to be homozygous for the resistance genes by marker results had a lower level of resistance. This

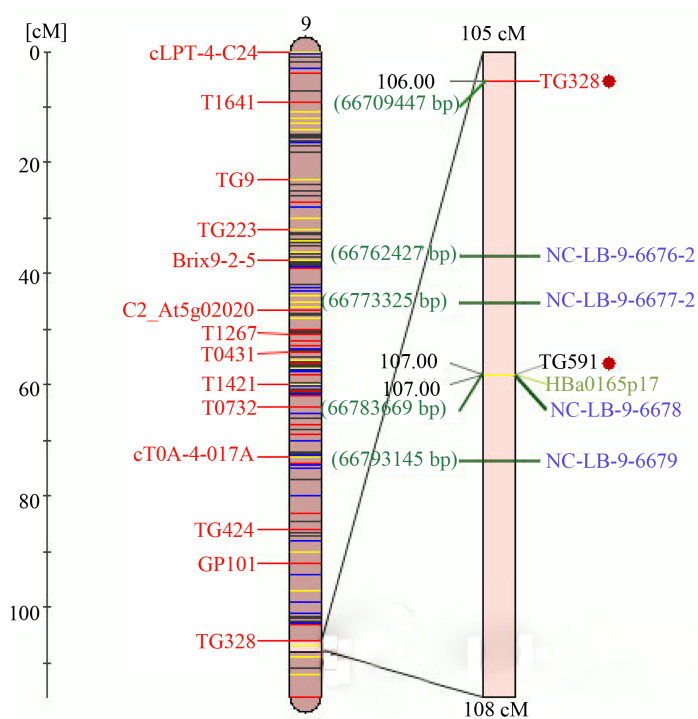


Figure 2. Chromosome 9 showing the positions of molecular markers identified in the present study with respect to *Ph-3* conferring resistance to late blight in tomato.

indicates the presence of additional modifying genes for late blight resistance or resistance gene analog (RGA) in addition to the *Ph-2* and *Ph-3* major genes. “Richter’s Wild Tomato” which had a high level of late blight resistance in field trials was chosen as a source of the *Ph-2* gene. However, the lower resistance level in the 63EB(2002)-1C line derived from backcrossing the *Ph-2* gene from “Richter’s Wild Tomato” indicates that there may have been modifying genes for late blight resistance that are lost in the backcross process. Earlier backcross generation lines (139LB(2001)3W-64 and 139LB(2001)3W-8) had a higher level of late blight resistance than the 63EB(2002)-1C line (data not shown).

Ph-2 and *Ph-3* genes have been combined by conventional methods in a large-fruited tomato [18]. However, these two genes are yet to be combined in plum and grape tomatoes, which are commercially important classes of tomatoes. With the availability of these markers, the process of combining these genes in plum and grape tomatoes may be expedited. The 08135(x) selections tested in this study are grape tomato lines. The highly resistant reaction of the 08135(x)-1W selection compared to the other 08135 selection, together with the marker results indicating it to be homozygous resistant for *Ph-2* and *Ph-3* combined, makes this line desirable for further selection and breeding for late blight resistance in grape tomatoes. Because of the incompletely dominant control of late blight resistance with both the *Ph-2* and *Ph-3* genes, it would be desirable to have the genes in both parents used to make commercial F1 hybrids to enhance the resistance level of the hybrids. The use of molecular markers to identify the *Ph-3* resistance genes and their status in lines will greatly facilitate the process of developing diverse lines with the resistance genes for use as parents in hybrids.

4. Conclusion

We have developed novel PCR-based molecular markers associated with *Ph-3* gene. With the verification of molecular data using phenotypic data generated from field isolate, it is expected that these markers will be useful to expedite the process of introgression of *Ph-3* gene into desirable genetic background. However, it is always desirable to verify the findings in populations derived from wider genetic backgrounds. SCAR markers associated with *Ph-3* are expected to be more useful since they are found more consistent in the present experiment.

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