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# DEVELOPMENT OF A MOLECULAR MARKER FOR THE RESISTANCE GENE *R11* OF POTATO TO LATE BLIGHT

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## ABSTRACT

**Cultivated potato (*Solanum tuberosum*) is susceptible to many pests and pathogens, but the most important threat to potato production is, so far, the late blight disease, caused by the oomycete *Phytophthora infestans.* Resistance genes from the wild *Solanum* sp. have been used by breeders to develop late-blight-resistant cultivars. Two sets of Black differentials potato genotypes (*R1, R2, …, R11*) were used to identify a new marker for resistance gene *R11* of potato to late blight. RAPD polymorphic bands were isolated, cloned, and converted into SCAR primers. By amplification of genomic DNA with SCAR primers followed by enzymatic digestion with *Hinf*I restriction enzyme, and verified by Southern blotting, a marker of *R11* resistance gene of potato to *Phytophthora infestans* was identified.**

**Keywords:** *Phytophthora infestans*, *Solanum tuberosum*, RAPD, SCAR, Southern blotting.

## INTRODUCTION

Even after more than 175 years since its first epidemic, causing the famous Irish famine (1840[s]), *Phytophthora infestans* is still a fearsome threat to potato crop. It is considered a reemerging disease, that still outbreaks until these days and threaten the potato production worldwide (Fry et al., 2015; Fones et al., 2020; Yuen, 2021). It attacks all the parts of the plant (the leaves, stem, and tubers). *P. infestans* is a redoubtable pathogen for tomato crop too; it can also infect other *Solanaceae* species such as eggplants, pepper, and others more. Crop losses and costs of late blight control exceed the multibillion euro amount (Razukas et al., 2007).

Late blight control involves an excessive use of chemicals. Generally more than 12 applications are effectuated to insure a sufficient yield. The environmental cost and the appearance of isolates resistant to fungicide are two important issues for chemical control of late blight. Breeding for late blight resistance is an environment friendly option. Traditional potato breeding for late blight resistance was based on introgression of resistance genes from the wild relative species *Solanum demissum*. Eleven *R* genes (*R1, R2, ..., R11*) were used for potato breeding. However, *P. infestans* pathogen was able to overcome this resistance.

Marker assisted selection is developed to overcome the difficulties encountered by conventional breeding (Francia et al., 2005). In formal plant breeding, based on phenotypic evaluation, breeders spend considerable effort and time in refining the crosses and looking after the desired characters (Ranade et al., 2001). Molecular

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markers are not affected by the environmental conditions and they are detectable in any stage of plant development (Kumar, 1999; Francia et al., 2005).

There are two types of molecular markers; dominant and codominant. The first group includes Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length polymorphism (AFLP) and so on, are relatively easy to use and inexpensive (Ghislain et al., 2006). The second group includes Simple Sequence Repeat (SSR), Restriction Fragment Length Polymorphism (RFLP), and many more. Generally, a codominant marker is preferred because the heterozygotes can be detected in backcross generations (Mackill, 2007). RAPD technique is a PCR based technique and it allows the detection of the polymorphism after amplification of randomly chosen DNA region using single arbitrary primer. DNA fragments which can be amplified by RAPD are less than ~5000 base pairs (Smith, 2005). Ranade et al. (2001) outlined three majors advantages of RAPD markers: 1) consist of universal set of primers that can be used and screened in a short period of time; 2) it is not necessary to isolate and clone the DNA probes; 3) small amount of DNA is required. RAPD technique is also inexpensive when it is compared with other molecular marker technologies, it is easy and quick (McGregor et al., 2000; Kumari and Thakur, 2014).

However, RAPD markers have two major disadvantages: they have low reproducibility and generally are not transferable (Bardakci, 2001; Collard et al., 2005). But it is used for genotyping and molecular breeding for several species (Pop et al., 2010; Alam, et al., 2014; Rajesh et al., 2014; Mhuka et al., 2016/2017; Olawuyi and Azeez, 2019), and still found useful (Costa et al., 2016). The transformation of RAPD markers to Sequence characterized amplified region (SCAR) markers is an option to overcome these disadvantages. Sun et al. (2020) used SCAR markers transformed from RAPD markers to discriminate tobacco cultivars. SCAR markers are specific, simple and reliable even at the intraspecific level (Feng et al., 2018; Val et al., 2020) and efficient in gene detecting, Zhang and Panthee (2021) used SCAR markers successfully to detect several genes in tomato.

In this paper, we aimed to develop a simple and inexpensive molecular marker that could be used to detect the gene *R11* for resistance of potato to *P. infestans*. We started from RAPD markers then transformed into type SCAR (Sequence Characterized Amplified Regions) markers. The SCAR markers were not specific so we used the restriction enzyme to generate more polymorphism and search for the specific bands.

## MATERIAL AND METHODS

A total of 26 potato genotypes each one harbouring a single R gene for potato against *P. infestans*, and 2 genotypes *R0* (no *R* genes). These genotypes constituting the so called the Black differentials (*R1, R2, R3, R4, R5, R6, R7, R9, R10,* and *R11*). The first set was kindly sent to us by the National Institute of Research and Development for Potato and Sugar Beet Braşov. The second set was sent to us by the United States Department of Agriculture - Agricultural Research Service (USDA - ARS). The potato plants were grown under controlled conditions in greenhouse.

Potato genomic DNA was isolated from fresh potato leaves and it was performed according to Lodhi et al. (1994) modified by Pop et al. (2003) protocol. The DNA quantification and quality check were performed using the Nanodrop™ Spectrophotometer. The DNA concentration was adjusted to 30 ng/µl prior to amplification.

***Amplification with RAPD primers:*** twenty-two RAPD primers were used for an initial primer testing on potato DNA (Table 1). PCR reaction were performed in a total volume of 25 µl, containing 5 µl Buffer (10mM tris Cl-Go Taq Green Master mix, Promega-pH 9), 3 µl $MgCl_2$, 0,5 µl dNTP, 0,2 µl Taq polymerase (Go Taq Flexi - Promega), 1,6 µl primer (Table 1), 2 µl DNA and up to the total volume we added sterile free DNase/RNase water. PCR condition were:

1 cycle: 94°C for 30 seconds;

45 cycles: 94°C for 30 seconds,
34°C for 1 minute,
72°C for 1 minute;
1 cycle: 72°C 4 for minute and 4°C for 24 h.

The amplification product were separated in 0,8% agarose gel and revealed in UV light.

*Table 1*. Primers used for DNA amplification

| Primer | Sequence | Primer | Sequence |
|---|---|---|---|
| OPA-11 | 5'-CAATCGCCGT-3' | 70-04 | 5'-CGCATTCCGC-3' |
| OPA-16 | 5'-AGCCAGCGA-3' | 70-08 | 5'-CTGTACCCCC-3' |
| OPA-17 | 5'-GACCGCTTGT-3' | Mic-07 | 5'-TGTCTGGGTG-3' |
| OPA-18 | 5'-AGGTGACCGT-3' | Mic-13 | 5'-TTCCCCCCAG-3' |
| OPA-20 | 5'-GTTGCGATCC-3' | Mic-14 | 5'-TGAGTGGGTG-3' |
| OPC-04 | 5'-CCGCATCTAx-3' | 595 | 5'-GTCACCGCGC-3' |
| OPC-08 | 5'-TGGACCGGTG-3' | 270 | 5'-TGCGCGCGGG-3' |
| OPC-9 | 5'-CTCACCCGTC-3' | 534 | 5'-CACCCCCTGC-3' |
| OPC-13 | 5'-AAGCCTCGTC-3' | 563 | 5'-CGCCGCTCCT-3' |
| OPC-20 | 5'-ACTTCGCCAC-3' | 594 | 5'-AGGAGCTGGC-3' |
| 70-03 | 5'-ACGGTGCCTG-3' | 570 | 5'-CCCCCCTAAT-3' |

***Isolation of polymorphic bands:*** the polymorphic bands were isolated from the gel, the DNA from bands was re-amplified using the same primer, separated by electrophoresis in 0.8% agarose gel and isolated from gel in order to obtain a single well individualized band.

***DNA cloning and insert verification:*** the selected bands were cloned into GM2163 *E. coli* strain. The cloning and genetic transformation was performed with Sticky-End Cloning protocol (Fermentas) and Transformaid™ Bacterial Transformation System (Fermentas), according to the manufacturer instructions.

The insert verification was made by plasmidial DNA isolation and PCR amplification with specific primer for the vector pJET1 and pJET1R vector: 5'-GCC TGA ACA CCA TAT CCA TCC-3' and 5'-GCA GCT GAG AAT ATT GTA GGA GAT C-3', respectively. The amplification was performed using the following conditions: 1 cycle at 94°C for 30", 35 cycles at 94°, 55.5°, 72°C for 30", 1', and 1', respectively, 1 cycle at 72°C for 4 minutes, and the last step was at 4°C for 24 h.

***Sequencing:*** the plasmids with the good insert were sequenced by Microsynth, Austria.

***SCAR Primer construction based on band sequences:*** based on the sequences obtained, a set of SCAR primers were constructed using the Blast program from the NCBI. The primers obtained and tested are listed in Table 2.

***Primer testing***: potato DNA from different genotypes was amplified with these primers. PCR amplification was:

1 cycle: 94°C for 3 minutes;
35 cycles: 94°C for 1 minute,
64°C for 1 minute,
72°C for 1 minute;
1 cycle: 72°C for 7 minutes.

***Enzymatic digestion:*** the amplification products were digested using several restriction enzymes: *Eco*RI, *Hinf*I, *Hind*I, *Alu*I, *Rsa*I, *Msp*I, *Hae*III. The digestion products were separated in 0.8% agarose gel. The PCR mixture consisted in: $H_2O$: 7.75 µl, buffer: 1.5 µl, enzyme: 0.75 µl, and PCR product: 5 µl, incubated for 2 h at 37°C. The reaction was stopped by incubation at 65°C for 15 minutes.

***Southern blotting:*** potato DNA was isolated from potato leaves of the genotypes *R0* and *R11*, and then digested with the restriction enzyme. The fragments obtained were separated in agarose gel 0.8% (overnight at 20 V), then transferred to a nylon membrane.

The fixation was realized by UV radiation for 4 minutes. The probe was prepared based on the band 33.2.2 originated from the genotype *R11*. The DNA fragment was labeled using Biotin DecaLabel DNA Labeling Kit (Fermentas) following the manufacturer instructions. The detection of the hybridization was performed using the Biotin Chromogenic Detection kit, according to manufacturer instruction.

*Table 2*. SCAR primers obtained based on the sequences of RAPD polymorphic bands

| Primer | Sequence | *R gene* |
|---|---|---|
| 2.2.1 | 5'-CCC ACG CCA CCT ACA ACG GTT-3'<br>5'-CAG CAC CGG GGC CAG ATT CA-3' | *R5* |
| 1.2.4 | 5'-GGG CTC TCC AGA TCT TCT AGG TGG T-3'<br>5'-GCC CCG GTG CTG CTT ACG AA-3' | *R5* |
| 1.2.3 | 5'-CTG GCC CCG GTG CTG CTT AC-3'<br>5'-GGC TCT CTG GTC GCA CAG TCG-3' | *R6* |
| 2.2.2 | 5'ACG CCA CCT ACA ACG GTT CCT GA-3'<br>5'-CAC GCA CCT AGC TGA GAT GAA AGC-3' | *R6* |
| 3.2.1 | 5'-CCC ACG CCA CCT ACA ACG GT-3'<br>5'-CGG TCC GTG ATA CTG GTC GCG-3' | *R1* |
| 5.2.2 | 5'-ACA AGA ACT CAA TTG GCG ACA AGC T-3'<br>5'-ACG CCC TAA ATG CAA GAA GCA TCA-3' | *R1* |
| 9.2.2 | 5'-GGC TCG GGG AAA TGC GGA CC-3'<br>5'-CGA GCG CCC ACC GAT ATG GG-3' | *R4* |
| 9.2.1 | 5'-GGT CAA GCG GTG CTC G T-3'<br>5'-CAC TCG CCA CAC ATG TAC TAC GCC-3' | *R4* |
| 5.2.3 | 5'- GAG CCT CGT TGA GCC AAA GAT AGG C-3'<br>5'- TGG GCA CGC AAC GTG TTG AAG-3' | *R1* |
| 29.2.1 | 5'-CCC ACG CCA CCT ACA ACG GTT GTT C-3'<br>5'-CTG AAA CAC CTC GCG GCC CT-3' | *R1* |
| 28.2.2 | 5'-GCT GAC CCT GAC CAC GGC TTC-3'<br>5'-CTA GTC AGG GCC GCG AGG TGT-3 | *R1* |
| 25.2.2 | 5'-GAA AAA CGC CAG CAA CGC GGC-3'<br>5'- TGC GTA TTG GGC GCT CTT CCG-3' | *R2* |
| 32.2.1 | 5'-ATG GCA CAA GGG GGC GCT AAG A-3'<br>5'-TTG ATC CTC CGC GTG CCC CTA-3' | *R3* |
| 31.2.3 | 5'-CCA CGC CAC CTA CAA CGG TTC C-3'<br>5'-AAC CCG CGA CCA AGT CCA CAG-3' | *R5* |
| 30.2.2 | 5'-AAG GCC GCC GAA GGA ATC CG-3'<br>5'-GCC GCC AGA TCT TCC GGA TGG-3' | *R10* |
| 32.2.3 | 5'-CGG CCT ACC CGG AAT GCG TC-3'<br>5'-GGC CGC CAG ATC TTC CGG ATG-3' | *R10* |
| 33.2.2 | 5'-GTG GTG GCA GCG GCA ATT GC-3'<br>5'-ACC GTG CCA AGT GGC TGC AC-3' | *R11* |
| 2.2 | 5'-CGA CTC GAC GAC TGG TTC GACGA-3'<br>5'-GCA CGA ATC TCC GTT CGC CGG-3' | *R2* |

## RESULTS AND DISCUSSION

In this study, 22 decamer primers (RAPD) were screened out of which 16 primers successfully amplified the genomic DNA of the potato Black differential sets used in this study. Each potato genotype harbors one *R* gene (*R1, R2, R3*, …, *R11*), the exception is made by the 2 genotypes (1 and 15) harboring no R genes. Four of these primers (MIC-14, 270 BC, 563 BC, and 594 BC) were able to generate rich and useful DNA polymorphism (Table 3, Figure 1).

*Table 3*. Primers that shown polymorphism with potato DNA

| Primer | Sequence |
|---|---|
| MIC-14 | 5`-TGAGTGGGTG-3` |
| 270 BC | 5`-TGCGCGCGGG-3` |
| 563 BC | 5`-CGCCGCTCCT-3` |
| 594 BC | 5`-AGGAGCTGGC-3` |

Thirty four polymorphic bands were identified as potential markers for different resistance genes carried by potato genotypes used for this study. These bands were isolated from the agarose gel (Figure 1).

Each one of these bands was isolated from the agarose gel, and re-amplified with the same primer (Figure 2).

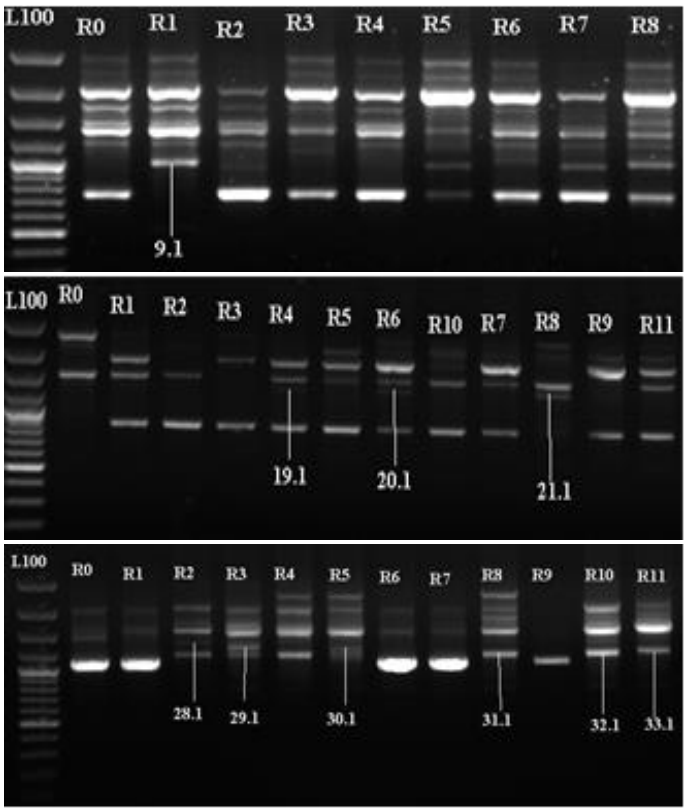


*Figure 1*. Polymorphism obtained with the primer 270 (up), Mic-14 (middle), and 594 (down). The numbers 9.1, 19.2 etc. represent the bands isolated from the gel.

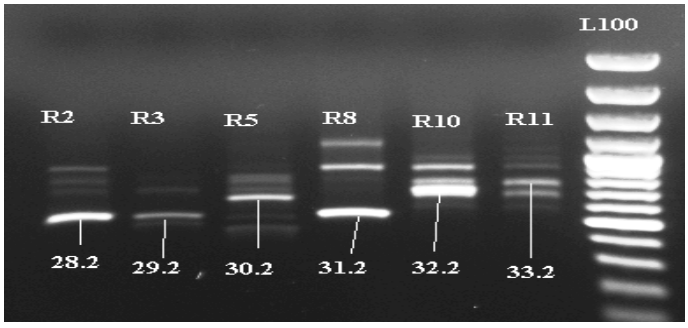


*Figure 2*. First re-amplification product of DNA recuperated from bands with 594 primer

Because the bands were so close to each other, after re-amplification, several new bands were identified. The bands of interest are again isolated from the gel and re-amplified with the same primer until obtaining a clean single band (Figure 3). When a single clean band is obtained, it is cloned using pJET vector, and then introduced into *E. coli*. In order to verify the fragment insertion, the plasmids from *E. coli* are isolated, and the DNA amplified with specific primer for the vector pJET. If the obtained band had the same weight of the original band, the plasmids were sent to sequencing. Based on their sequences, SCAR primers were elaborated. A total of 18 SCAR primers were generated (Table 2).

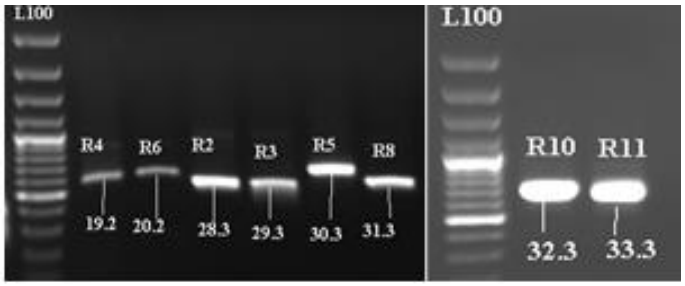


*Figure 3*. Second re-amplification products of DNA recuperated from band with 594 primer

Some genotypes generated more SCAR primers than others. *R1* generated 4 primers, 3 primers were obtained from the sequence of the polymorphic bands generated by the genotypes *R5*, 2 primers from the genotypes: *R2*, *R4*, *R6*, and *R10*. From the genotypes *R3* and *R11* we were able to elaborate only one primer from each one of them (Table 2).

Then these SCAR primers were used to amplify the potato DNA from the 2 Black differential sets. The obtained electrophoresis profiles did not discriminate between the resistance genes and nor the genotype *R0* (Figure 4). The band is omnipresent for all the potato genotypes. So far, the developed SCAR primers were not able to discriminate between the resistant and the susceptible genotypes. As a result, they could not be used as a marker for any *R* gene.

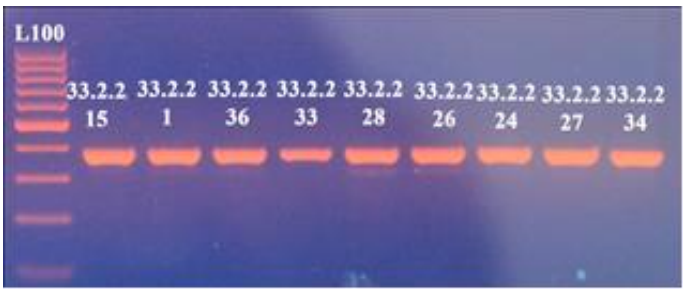


*Figure 4.* Amplification products obtained with 33.2.2 primers at susceptible potato (1 and 15) and resistant potato to late blight. 33.2.2 represents the primer constructed from *R11*, 15 and 1: *R0*, 36: *R11*, 27: *R3*

To overcome this issue, the band 33.2.2 (obtained from the R11 genomic DNA) was digested with different restriction enzymes (*Eco*RI, *Hinf*I, *Hind*I, *Alu*I, *Rsa*I, *Msp*I, *Hae*III). After digestion, the DNA was re-amplified again. The primer 33.2.2 was the only one able to generate useful polymorphism when combined with the restriction enzyme *Hinf*I (Figure 5).

DNA fragments obtained after digestion with *Hinf*I revealed the polymorphism of restriction sites contained by each fragment. All potato genotypes, with the exception of *R11* (Figure 5: 36) have 2 restriction sites (the enzymatic digestion generated 3 bands for 100, 300 and the original band of 400 bp). *R11* have only one restriction site (resulting in two bands of 100 and 300 bp), and *R3*

(Figure 5: 27), does not contain any restriction site.

After molecular hybridization, Southern blotting detected the *R11* only in the genotype *R11*. Southern blotting corrected the amplification of other genotypes by the primer 33.2.2. It means that the fragment from where the primer 33.2.2 was constructed was specific for the *R11* gene.

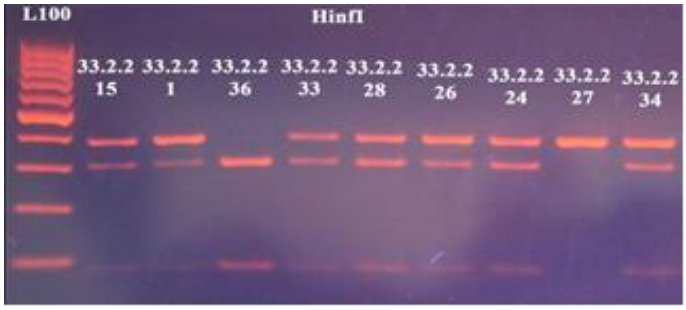


*Figure 5*. Restriction fragments of 33.2.2 amplification products with *Hinf*I restriction enzyme. 33.2.2 primers at susceptible potato (1 and 15) and resistant potato to late blight. 33.2.2 represents the primer constructed from *R11*, 15 and 1: *R0*, 36: *R11*, 27: *R3*. L100: Ladder 100 pb (Fermentas).

Late blight caused by *P. infestans* is the most devastating disease on potato. Breeding for late blight resistance is one of the most environment friendly control methods of plant pathogens. Marker-assisted selection (MAS) is time saving, the most efficient and accurate method in plant breeding (Roychowdhury et al., 2014).

In this study, we aimed to develop a molecular marker for the resistance gene *R11* of potato to late blight. Potato resistance to late blight is controlled by major *R* genes. It is also controlled by QTLs (Bradshaw et al., 2006a).

The enzymatic digestion with *Hinf*I showed that the DNA fragment 36 (*R11*) does not contains only one restriction site, compared to the other genotypes harboring 2 restriction sites each one, with the exception of 27 (*R3*), which is free of restriction sites. This could be explained by the fact that R11 is an allelic version of *R3* complex locus (Huang, 2005). The *R11* gene is probably situated on the chromosome 11 with other genes (*R5, R6, ..., R10*) (Huang, 2005; Bradshaw et al., 2006b; Hein et al., 2009).

The primer 33.2.2 obtained originally from an *R11* potato genotype amplified all potato genotypes tested (*R0, R1, ..., R11*). The conversion of RAPD markers to SCAR, increases the specificity of the marker. SCAR markers improve the reproducibility and reliability of PCR assays, and therefore their utility increases for many applications (Busconi et al., 2006). In our case, the conversion into SCAR of RAPD marker decreases the polymorphism obtained after amplification. One band was obtained for all the genotypes, with the same weight. This monomorphism could be explained by the fact that the majority of resistance genes contain the highly conserved fragments (NBS-LRR), the majority of resistance genes are part of the family of Nucleotide Binding Site/Leucine Rich Repeat (NBS-LRR) (Hammond-Kozack and Parker, 2003) in which also potato *R* genes are included. NBS fragments are highly conserved regions in resistance genes (van der Linden et al., 2004). The amplification by 33.2.2 of *R0* genotypes lead us to think that the fragment amplified by 33.2.2 contains or has parts of analogues of resistance genes. Also another explanation is that the primer 33.2.2 is really part of *R11*, but it is not specific. So the construction of other primers based on the same sequence of DNA isolated from the genotype *R11*, and from where the primer 33.2.2 is constructed is highly indicated. The situation with monomorphic SCAR amplification was also encountered by other authors (Kelly and Miklas, 1998; Horesjsi et al., 1999; Gupta et al., 2006). SCAR primer may contain ubiquitous sequences in multiple genomics

regions that may result in mismatched primer annealing (Gupta et al., 2006). To overcome this problem we exploited the restriction enzyme *Hinf*I polymorphism.

Using this technique, we were able to identify a marker for *R11* (Figure 6). However, it does not allow us to determine the distance between the marker and gene in question. The determination of the distance between the gene and the marker is possible only with two additional techniques: Near-Isogenic Lines (NIL) or by Bulk Segregant Analysis (BSA). Bradshaw et al. (2006b) used BSA combined with AFLP to identify a marker for *R11* (PAG/MAAG_172.3), with a distance of 8.5 cM. Other techniques also are available such as NBS profiling (van der Linden et al., 2004). RAPD technique is still attractive by its simplicity, easiness inexpensiveness in comparison to other technique (McGregor et al., 2000). Combined by the transformation into SCAR markers, the reproducibility issue is resolved. In our study, the SCAR markers were discriminatory between the resistance genes. The use of restriction enzyme *Hinf*I resolved this issue by generating less diversity for *R11* gene (only 2) compared to the rest of the set (3). For the gene *R3*, *Hinf*I did not have any restriction site, so the same band was obtained. It could be, also considered as a marker for *R3*.


*Figure 6*. Nylon membrane hybridized with 33.2.2 probe.
The hybridization took place at approximately 1600bp

Even though, resistance from *S. demissum* (*R1*, *R2*, …, *R11*) are taught to be easily overcome by the pathogen, they are still useful in late blight resistance breeding especially by gene pyramiding. Vossen et al. (2016) showed that *R8* provide a strong resistance response and it is found in the cultivars Sarpo Mira with other *R* genes. Taoutaou et al. (2019) showed that gene pyramiding of the genes (*R1*, *R2*, *R3*, and *R4*) caused a delay of disease manifestation in the potato genotype with the four *R* genes compared to potato genotype with the gene *R1* only, even though both genotypes were sensitive to *P. infestans* race used in their study.

## CONCLUSIONS

By using RAPD technique and the transformation of RAPD markers into SCAR markers, and after enzymatic digestion a marker for resistance gene *R11* of potato to late blight was identified. The primary polymorphism was not detected after the amplification of potato DNA with the primer 33.2.2, but the digestion with *Hinf*I revealed secondary polymorphism. The Southern

blotting confirms the specificity of the primer 33.2.2 to the resistance gene *R11*.

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