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مقاله پژوهشی

ژن‌های مقاومت به بیماری سفیدک سطحی سیب در ژنوتیپ‌های وحشی سیب ایران

مهدی جمالوند[۱]، بهرام شریف نبی[۱*]، پریسا رحیمی[۱] و فریبا قادری[۲]

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چکیده

سیب (*Malus domesticca* Borkh)، یکی از ارزشمندترین گیاهان خانواده *Rosaceae* است که به دلیل ارزش غذایی و اقتصادی بالا، یکی از محصولات کشاورزی با اهمیت محسوب می‌گردد. بیماری سفیدک سطحی سیب که در اثر قارچ *Podosphaera leucotricha* ایجاد می شود، یکی از بیماری های مهم سیب است که سالانه خسارت کمی و کیفی فراوانی به محصوی سیب وارد می کند. در سال‌های اخیر، استفاده از ارقام مقاوم جهت ایجاد مقاومت به بیماری سفیدک پودری سیب مورد توجه واقع گردیده است؛ به این منظور جهت ردیابی ژن‌های مقاومت *Pl1*، *Pld*، *Plbj* و RGAs به قارچ *P. leucotricha* روی ژنوتیپ‌های وحشی سیب ایران، از مناطق مختلف استان‌های آذربایجان غربی، گلستان، فارس، چهار محال و بختیاری و اصفهان، نمونه برداری شد. حضور دامنه‌های محافظت شده NBS-LRR، موتیف AAA از خانواده بزرگ موتیف‌های P-loop NTPase در پروتیین NBS-LRR-like و نیز دامنه‌ی محافظت شده NB-ARC در ناحیه N-tetminal، و همچنین ناحیه غنی از اسید آمینه لوسین در ناحیه C-terminal در دامنه LRR که توسط RGAs کد می‌شود، در نمونه‌های مذکور، مورد تایید واقع شد. ژن مقاومت *Pl1* در تمامی ژنوتیپ ها به جز یک موردردیابی شد. ژن مقاومت *Pld* تنها در سه نمونه از ۴۷ نمونه جمع آوری شده و ژن مقاومت *Plbj* در ۱۳ نمونه از تمامی ژنوتیپ های وحشی جمع آوری شده از مناطق سردسیر استان های اصفهان و آذربایجان غربی وجود داشت. توالی این ژن در بانک ژن جهانی ثبت گردید. ردیابی ژن های مقاومت و آنالوگ های آن می تواند در ایجاد ارقام مقاوم سیب به بیماری در آینده کمک شایانی نماید.

کلیدواژه: *Malus*، *Podosphaera leucotricha*، ژن‌های مقاومت، آنالوگ ژن های مقاومت

* مسئول مکاتبات، پست الکترونیکی: sharifna@iut.ac.ir

۱. گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه صنعتی اصفهان.

۲. گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه یاسوج.

Apple powdery mildew resistance genes in Iranian wild apple genotypes

M. Jamalvand[1], B. Sharifnabi[1*], P. Rahimi[1], and F. Ghaderi[2]

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Abstract

Apple (*Malus domestica* Borkh) is one of the most valuable agricultural products in the Rosaceae family because of its high nutritional and economic value. Powdery mildew of apple which is caused by *Podosphaera leucotricha*, is one of the primary fungal diseases with an extensively negative impact on the quality and quantity of apple production. In recent years, the use of resistant cultivars for controlling powdery mildew of apple has been considered. For this purpose, the candidate resistance genes *Pl1, Pld, Plbj*, and *RGA* (Resistance Gene Analogs) were detected in 47 genotypes of wild apples collected from West Azerbaijan, Golestan, Fars, Chaharmahal va Bakhtiari and Isfahan provinces. The presence of NBS-LRR conserved domains and P-loop NTPase motif in NBS-LRR-like protein, which encode by the *RGA* in N-terminal, were confirmed in wild apple (*Malus orientalis*) genotypes of Iran. *Pl1* resistance gene was detected in all collected samples in this research except one. *Pld* gene just was observed in three out of the 47 samples, and the *Plbj* resistance gene was detected in just 13 samples of all wild genotypes collected from cold areas of Azerbaijan and Isfahan provinces; the sequences of this gene was introduced to the Global database. Detection of resistance genes and *RGAs* can help in developing resistant apple varieties in future.

Keywords: *Podosphaera leucotricha*, *Malus*, Resistance genes, RGA

* Corresponding Author, Email: sharifna@iut.ac.ir
1. Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Iran.
2. Department of Plant Protection, Faculty of Agriculture, Yasouj University, Iran.

Introduction

Apple Powdery mildew (PM) is a major disease of apple trees caused by *Podosphaera leucotricha* (Ell. and Ev.), which is reported from all apple-growing regions of the world (Wurms & Chee 2011). Powdery mildew of apple is distributed in large parts of Iran, including West and East Azerbaijan, Khorasan, Tehran, and Isfahan provinces (Ershad 2009).

The disease agent produces a white cover of fungal mycelium on the leaf surface called "primary mildew" in Spring (Marine *et al.* 2010) and severely damages apple leaves, flowers, fruits, buds, shoots and twigs (Xu 1999). Currently application of fungicides is the conventional method for controlling of PM and up to 15 sprays per year were usually applied at intervals of 7, 10 or 14 days between the green cluster stage and the end of shoot growth in the past years (Wurms & Chee 2011), which is cost-effective and also follows severe human and environmental risks. Besides, these fungicides also can cause the formation of new resistant strains of fungi (Marine *et al.* 2010). So different mathematical models have been developed based on the various aspects of apple powdery mildew epidemiology (Xu 1999). Alternative methods have been investigated to substitute chemical control. However, the development of durable pyramided resistant cultivars is considered as one of the competent control methods, but breeding new cultivars is a long and arduous process. Identifying the resistance genes in wild cultivars has led to the development of sustainable resistance cultivars and is one of the most important goals of durable apple production systems. Some resistant genes are identified, and it is easier to search for the resistance sources in wild apple cultivars (Caffier & Parisi 2007). The small-fruited Asiatic *Malus* spp. is the origin of several dominant powdery mildew resistance genes (Patzak *et al.* 2011). PCR molecular marker-assisted breeding (MAB) is a beneficial approach to identify the resistance genes within apple germplasm collections and could be used to increase the number of sources for disease resistance in breeding programs (Patzak *et al.* 2011). The SCAR (OPAT20-450) marker was identified and resulted in the development of the *pl1* gene (Markussen *et al.* 1995). Then N18-SCAR was used for the *pl2* gene, which had a dominant resistance to powdery mildew (Caffier & Laurens 2005). The application and development of the SCAR markers for *Pld* and *Plw* represents a successful combination of a multiplex reaction and a reason for the success of pyramidal resistance (James et al. 2004). The advantage of the microsatellite markers (SSRs) is that the alleles are replicated uniquely and are linked to resistance genes (Maric *et al.* 2010). The genomic scan based on SSR showed that the *Plbj* gene with CH02a10 and CH02c11 primers was the first dominant gene of powdery mildew resistance, which is located on the tenth apple linkage group (Dunemann & Schuster 2009). Also, the pieces of the literature indicated that wild apple is the source of resistance to powdery mildew (Sestras *et al.* 2011), which can be mentioned as *Pl1* gene from *M. robusta*, *Pl2* gene from *M. zumi, Pld* gene from D12 (Gelvonauskis & Gelvonauskiene 2003), *Plm* gene from Mildew Immune Selection, *Plw* gene from ornamental apple species (James & Evans 2004), *Pla* gene from Aotea 1 and *Plbj* gene from *M. baccata* var *jacki*. Besides, other cultivars have been identified as resistant cultivars such as *M. hupensis, M. sargentii, M. baccata* var. *mandshurica*, and *M. sieboldii* (Dunemann & Schuster 2009). The results of the marker analysis showed the combination of homozygous and pyramided resistance against apple scab (*Rvi6* and *Rvi4*), the resistance locus against powdery mildew (*Pl2*), and the FBF7 fire blight resistance QTL in progeny plants (Kellerhals *et al.* 2013). In another study, three PCR molecular markers for the powdery mildew resistance genes *Plw, Pl1* and *Pld* were used in a group of 279 apple cultivars from the Czech collection of apple genetic resources and the results proved the presence of one or two powdery mildew resistance genes in the small-fruited cultivars *Malus evereste*, Golden Gem, Sprengeri and Hilleri, and in the larger fruited cultivars Hagloe Crab, Borovinka and Tita Zetei (Patzak *et al.* 2011). It should be noted that there is the potential for new PM races to overcome the host resistance (Caffier & Parisi 2007).

In general, apple powdery mildew disease is widespread in semi-arid areas, and Iran is also located in such a climate. Therefore, the detection of resistance genes such as *Pl1, Pld, Plbj*, and *RGAs* to PM is the main objective of this research, using polymerase chain reaction-based molecular markers, especially the SCAR marker.

Figure 1. Geographic locations of *Malus* genotypes sampling sites in different provinces used in this study. Each site is indicated by a black dot. West Azerbaijan province (1, Kilah Shin Oshnavieh mountain, 2, Mirabad Oshnavieh forest, 3, Bochan forest), Isfahan province (4, Hamestan valley, 5, Chalcherane forest), Fars province (6, Bil Dasht Arjan mountain) and Golestan province (7, Zarrin Gol Aliabad forest, 8, Late Nowruz Galikesh forest).

Materials and Methods

Sampling

Leaf samples of Iranian wild apple genotypes were collected from different regions of Isfahan, Golestan, Fars, and West Azerbaijan province. The samples were transferred to the laboratory in the sterile packets for further analysis (Figure 1).

Molecular resistance marker analysis to Podosphaera leucotricha

Total DNA was extracted from fresh wild apple leaf samples by Murray and Thompson (1980)'s method (Murray and Thompson 1980). Three PCR molecular markers of resistance genes (*Pl1, Pld, plbj*) and Disease Resistance Gene Analogs (*RGAs*) to apple powdery mildew *P. leucotricha* were used for molecular analyses (Table 1).

The PCR reaction was performed in a total volume of 20 µl using thermal cycle consisting 5 min 96°C (initial denaturation step), followed by 35 cycles of 1 min at 94°C, 45 s at 56°C, 1 min at 72 °C, and with a final extension of 72°C for 8 min. The PCR products were scored for the presence or absence in each sample and recorded by the number of base pairs for each PCR molecular marker based on the size measured with 100 bp ladder (Sinaclon, Cat. No. SL7111).

Sequencing of PCR products and data analysis

The amplicon part of the genes was cloned into the pTG19-T (Vivantis, Selangor DE, Malaysia) and pJET1.2/blunt plasmids (Thermo Scientific CloneJET PCR Cloning Kit #K1231) for TA cloning steps and transferred to *Escherichia coli* MC1061 (Sambrook & Russel 2001) for direct sequencing. *E. coli* MC1061 was used for plasmid manipulations and cloning steps. Cloning of the genes was confirmed by direct colony PCR reaction by specific primers.

Fifty microliter of the desired product from the plasmid extraction was sent to Macrogen Inc. South Korea. The obtained sequences were compared with the sequences in the Gene Bank (NCBI) using the Blast search tool (Altschul *et al.* 1997, Hall 1999). The sequences were aligned using the software MAFFT v.7.043b (Katoh & Standley 2013), and phylogenetic analyzes were performed

Table 1. PCR molecular markers for detection of powdery mildew resistance genes

Marker primers	PCR type	Detected gene	Primer sequence (5'-3')	Size (bp)	Reference
OPAT20	RAPD	*Pl1*	F:GTTTAGCGGTTTAGGTGCTTGTG R: AACTCCTTGATTTCTCCTATTGTT	450	Markussen *et al.* 1995
EMDM 01	SCAR	*Pld*	F:GAATTCACTTGTGCTAAG R:GGAAAGAAAGACCAAAATAAACG	90	James *et al.* 2004
PLBJ	SCAR	*plbj*	F:GAATTCAAACTCCAAATGCC R:AGGGGTCTTTGTGTTTCTGG	80-118	Dunemann & Schuster 2009
OLE 1121/1122		*RGA*	F:GGWATGGGWGGWRTHGGWAARACAC- R:ARNWYYTTVARDGCVARWGGVARWCC	500	Baek & Choi 2013

Table 2. Characteristics of *Malus* species isolates used in phylogenetic analysis

Host	Location	Gene Bank accession numbers
Malus baccata	South Korea	HQ399045 [a]
M. baccata	South Korea	HQ399009 [a]
M. prunifolia	South Korea	AF516641 [a]
M. floribunda	New Zealand	DQ644416 [a]
M. floribunda	New Zealand	DQ644355 [a]
M. domestica	South Korea	XM_017327535 [a]
M. domestica	South Korea	XM_017324579 [a]
M. domestica	Hungary	FJ477235 [a]
M. domestica	Hungary	FJ477234 [a]
Pyrus bretschneideri	South Korea	XR_001952453 [a]
Pyrus bretschneideri	South Korea	XM_018645443 [a]
Arabidopsis thaliana	South Korea	NM_001198458 [a]
M. orientalis	Iran	MK405519 [b]
M. orientalis	Iran	MK405520 [b]
M. orientalis	Iran	MK405521 [b]
M. orientalis	Iran	MK405522 [b]
M. orientalis	Iran	MK405523 [b]
M. orientalis	Iran	MK405524 [b]
M. orientalis	Iran	MK405525 [b]

[a] Cited from Bank and Choi 2013
[b] From this study

using the distance method. The distance matrix of aligned sequences was evaluated by Kimura 2-parameter (Kimura 1980) and the phylogenetic tree was constructed using the Maximum Parsimony (MP) method with the Close-Neighbor-Interchange algorithm (Nei 1973) and a predetermined pattern using MEGA 6.0 (Molecular Evolutionary Genetics Analysis, ver 4.0) with 1000 bootstrap (Tamura *et al.* 2007). Sequences of different species of *Malus* spp. were registered at the Gene Bank (Table 2).

Results and Discussion

A total of 44 wild apple genotypes were collected from different regions of Iran which most of them belonged to *M. orientalis* and *M. floribunda* (Table 3). *M. orientalis* -the only wild species of Iranian apple- were collected from different regions. This species has leaves with 4-6×1.5-3.5 cm dimensions, underlying crusty surface, and spherical fruits with 2-3 cm long and 2.5-3 cm width, small and sour taste. *M. orientalis* species naturally grows in north and northwest forests, as well as Alborz heights to Zagros country sides (Mozaffarian 2005).

The apple samples collected from Isfahan University had elliptical and stretched leaves with 5-7 cm long, as well as small spherical red fruits and was identified as *M. floribunda*. The origin of this species is Japan and known as Japanese ornamental apple. The height of the apple tree reaches to 10 meters. Another characteristic of *M. floribunda* is pink to red flowers as well as fluffy pedicel

Table 3. Sources and location of *Malus* spp. from Iran different places

Isolate code	Location of sampling	Genotype	Common name
pd1, pd2, pd3	Hamestan Valley	*Malus* sp.	**Sour apple**
Pd4	Zarrinq Gol Aliabad forest	*M. orientalis*	**Wild apple**
pd5, pd6, pd7, pd8	Late Nowruz Galikesh forest	*M. orientalis*	**Wild apple**
pd9, pd10, pd11, pd12	Isfahan University	*M. floribunda*	**Ornamental apple**
pd13, pd14	Bil Dasht Arjan Mountain	*M. orientalis*	**Ornamental apple**
pd15,pd16	Kilah Shin Oshnavieh Mountain	*M. orientalis*	**Wild apple**
pd17,pd18	Chalcherane forest	*M. orientalis*	**Wild apple**
pd19, pd20, pd21, pd22, pd23, pd24, pd25, pd26, pd27, pd28, pd29, pd30, pd31	Mirabad Ashnavieh forest	*M. orientalis*	**Wild apple**
pd32,pd33,pd34,pd35,pd36, pd37, pd38, pd39, pd40, pd41, pd42, pd43, pd44	Bochan forest	*M. orientalis*	**Wild apple**

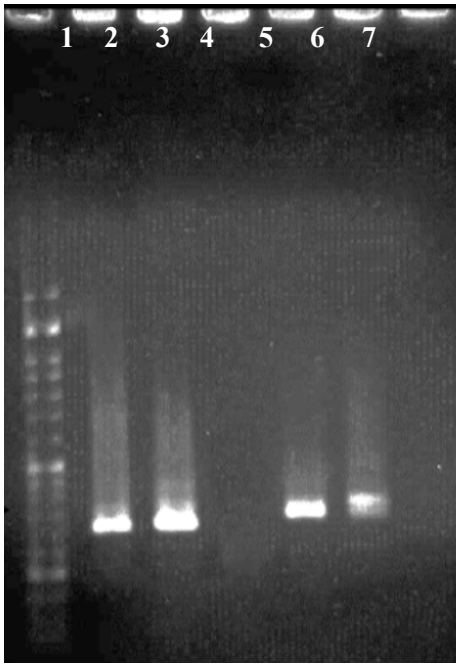

Figure 2. The 450 bp DNA fragment generated with the primer OPAT20 (Operon) which is linked to the *Pl1* gene was excised from a native agarose gel. 1: 50 bp ladder, 2: H2, 3: H3, 4: H4, 5: B1, 6: B2, 7: Negative control.

(Mozaffarian 2004).

Only three genotypes (*pd1*, *pd2*, and *pd3* isolates) were collected from the Hamestan valley in Isfahan province. These genotypes were known as sour apples by local people, but it was not possible to determine their species accurately, due to the fruitlessness at sampling time.

Detection of resistance genes to the P. leucotricha

The presence of resistance genes to *P. leucotricha* was studied in several wild Iranian apple genotypes by using several proprietary SCAR markers. The results showed that OPAT20, EMDM, PLBJ-SCAR, and OLE 1121/1122 primer pairs were detectable for *Pl1*, *Pld*, *plbj*, and *RGA* resistance genes in different cultivars.

OPAT20 primer pairs were used to detect the *Pl1* resistance gene, which designed in 1995 by converting RAPD to SCAR marker (Markussen *et al.* 1995, Dunemann *et al.* 2007) and amplified a 450 bp amplicon. Co-dominant OPA20 marker is located at 4.5 centimorgans in the distal end of the linkage group 12 of the *Pl1* resistance gene (Markussen *et al.* 1995, Dunemann *et al.* 2007). The results showed that a 320-350 bp fragment was amplified in all collected samples in this research except H4 (Figure 2).

The Blast aligning of this amplicon just confirms the relationship to apple and pear mRNA. In addition to a Disease Resistance Gene Analogs (*RGAs*), which is located at 12 cM from the *Pl1* gene, *Pld* as another resistance gene to powdery mildew and *vg* and *vb*, as two resistance genes to apple scab are present in this region (Dunemann *et al.* 2007). The *Pl1* gene was isolated from OP3762 cultivar with the origin of widespread species *Malus robusta* for the first time (Markussen *et al.* 1995). This gene was detected in all comprehensively studied genotypes even in the red delicious domestic cultivar. Regard to this fact that OPA20/SCAR marker and RGA gene which coded NBS-LRR protein are linked to *Pl1* resistance gene, suggest that this general oligogenic resistance inhibit the necrotic spots caused by the powdery mildew agent (Korban & Riemer 1990, James *et al.* 2004). The presence of the *Pl1* gene on the linkage group 12, with *Pld* and *RGA* genes, should create sustained resistance to apple powdery disease, but the emergence of new fungal rac-

es would break its resistance (Dunemann *et al.* 2007).

For the detection of the *Pld* resistance gene, EMDM primer pairs were used to amplify a 90 bp amplicon. This gene was detected in Ros1, GerdB4, Challa samples. James *et al.* (2004) identified the first set of molecular markers linked to the gene for mildew resistance, *Pld* by bulked-segregant approach, and to test different microsatellites, amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) primers in a progeny segregating for *Pld.* The map of most tightly linked markers to *pld* was prepared by segregations of the markers in the resistant and susceptible bulks, and three AFLP, one RAPD, and two microsatellite markers were identified. EMDM01 was designed by converting AFLP to the SCAR marker (James *et al.* 2004). This marker is located at five centimorgans of the *pld* gene at the distal end of Linkage Group 12 (Gelvonauskis & Gelvonauskiene 2003). In comparison to *Pl1* and *Pl2*, this gene has a higher resistance level to apple powdery mildew disease (James *et al.* 2004). In the same research on Czech Apple (*Malus* × *domestica*), *PI-w, PI-l*, and *PI-d* were not detected in all used world apple cultivars (Patzak *et al.* 2011). In the recent study was performed on genetic diversity and population structure of the Caucasian wild apple (*Malus orientalis* Uglitzk.) in the Hyrcanian forest (Iran) and its resistance to apple powdery mildew, *Pld* gene just was observed in nine out of the 14 sampling sites (Amirchakhmaghi *et al.* 2018).

The *Plbj* resistance gene was identified from wide genotype *M. baccata* for the first time and detected by PLBJ-SCAR primer pairs by converting the AFLP marker to SCAR (Dunemann & Schuster 2007). This marker is located at seven centimorgans of the *Plbj* gene in linkage group 10 and induce resistance to apple scab, too (Dunemann & Schuster 2007). This primer was amplified an 80-118 bp amplicon. In GerdA3, GerdA4, GerdC5, GerdC6, GerdA1, GerdB2, GerdB3, GerdB6, Chal1a, Chal2a, Mir1, Mir2, and Mir6 samples and all wild genotypes collected from cold areas of Azerbaijan and Isfahan provinces, just an 80 bp fragment was amplified, sequenced and registered in global database.

The family of resistance gene analogs (*RGAs*), which is one of the largest gene families in plants with a nucleotide-binding site (NBS) domain, assigned the most significant number of disease resistance genes. Perazzolli *et al.* (2014) identified 868 *RGAs* in the genome of the apple (*Malus*×*domestica* Borkh.) cultivar 'Golden Delicious' (close to 1.51% of the total number of predicted genes for this cultivar). Proteins codified by *RGAs* can be further classified to toll/interleukin-1 receptor (TIR) or N-terminal coiled-coil (CC) and BED finger (Bed) (Meyers *et al.* 2005). The predicted proteins have protected domains and motifs that play a pivotal role in resistance to plant disease agents. NBS-LRR is a receptor of kinase and plays a role in the signal pathways (Baek & Choi 2013). TIR domain is similar to the Toll protein of *Drosophila melanogaster* and known as Interleukin-1 receptor protein in mammals. This protein plays a role in the innate immune system. The second subfamily non-TIR or coiled-coil motif belongs to structural protein and involved in the regulation of the genes and transcription factors expression (Sekhwal *et al.* 2015). To detect the RGA resistance gene, a primer pair of 1121/1122 OLE was used, which amplify a 500 bp amplicon. RGA was not detected in H3 and N2a apple samples and B2 and PN1 pear samples (Figure 3). However, the 500 bp band of amplified fragment was visualized in some samples and sent for sequencing to Macrogene Company, South Korea.

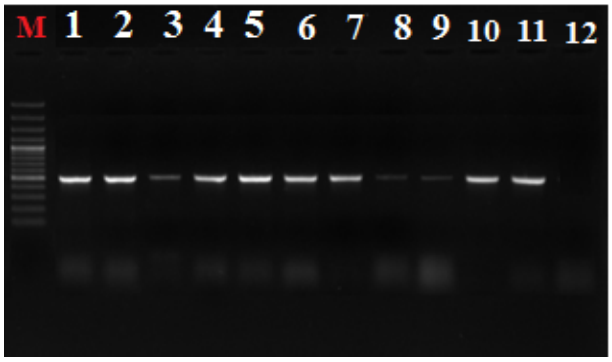

Figure 3. The 500 base pair amplicon corresponding to 1121/1122 OLE (*RGA* gene) is amplified (M: marker 100, 1: pd32, 2: pd33, 3: pd34, 4: pd35, 5: pd36, 6: pd37, 7: pd38, 8: pd39, 9: pd40, 10: pd41, 11: pd42, 12: control)

Phylogenetic analysis

The resulted sequences of *RGA* genes were aligned with two apple genotypes from South Korea (*M. baccata* 2-6, 6-12 and 6-21), *M. floribunda* from New Zealand, and analyzed with CLC Se-

quence Viewer 7.0.2 software and the phylogenetic

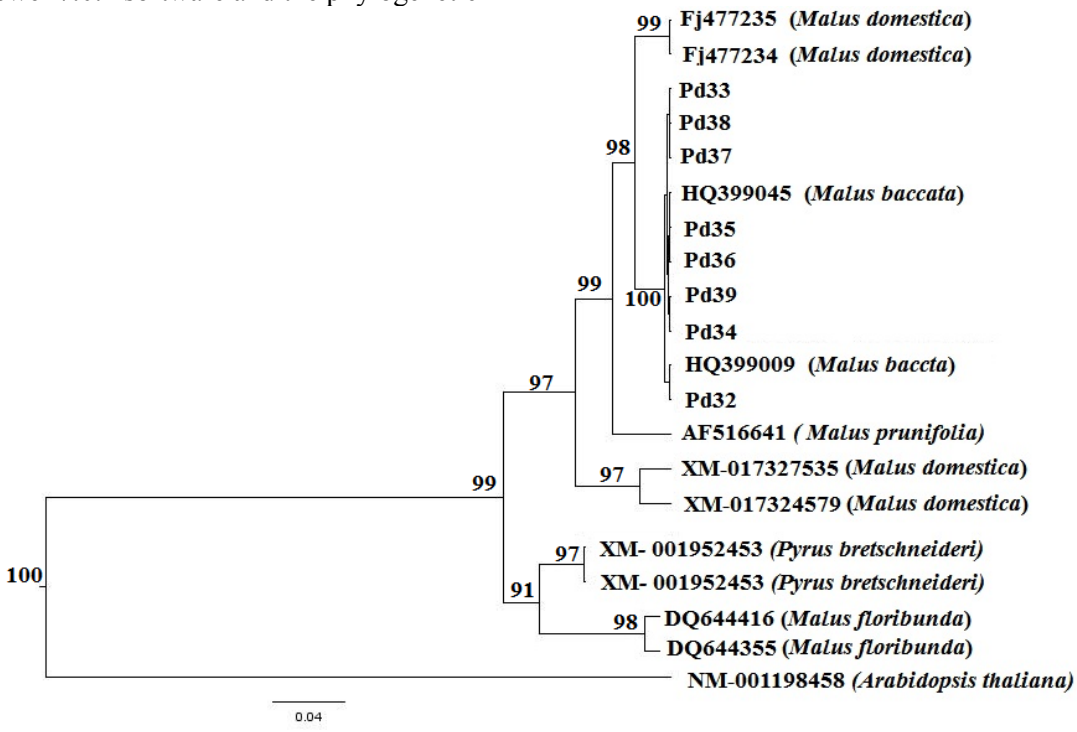

Figure 4. A phylogenic tree of 20 taxa of *Malus* spp. and *Pyrus* inferred from RGA: Bootstrap values > 50% (1000 replicates) of Maximum Parsimony (MP) analysis is shown above the branches (Bar indicates the nucleotide substitution in MP analysis). *Arabidopsis thaliana* is out group. Accession numbers from Gene Bank are shown in parenthesis.

tree was made by DNAMAN 4.0 with 1000 bootstrap (Figure 4). *Pyrus communis* and *Arabidopsis thaliana* were used as outgroup. The phylogenetic tree was made by the Maximum Parsimony (MP) method based on the RGA gene sequence and indicated that the studied samples in this study belonged to one clade. Regarding the Maximum Parsimony (MP) tree, all sequences of this clade were grouped by *M. baccata* (HQ399045 and HQ399009) genotypes from South Korea with 100% validation. In other words, they formed a monophyletic group (Figure 4). It can be concluded that differences between the sequences of plant species are related to the different reactions to various phytopathogen races.

To confirm the DNA sequence of GerdA5-1 samples, its translation was analyzed by global protein database EMBOSS (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) and from 6 frames. The first which lack of stop codon was selected and analyzed in Blast database which confirmed the existence of the P-loop NTPase (P-loop containing Nucleoside Triphosphate Hydrolases) domain in the NBS-LRR-like protein in the Korean genotype. Therefore, due to the high percentage of similarity in the nucleotide aligning and regarding to the place of GerdA5 genotype toward to Korean genotype in the phylogenetic tree, it is possible that this motif can be found in the protected domains of Iranian genotypes.

All the aligned sequences had NBS and LRR domains, but in *M. domestica* which coded *RGA9* protein, the TIR domain was found in addition to these domains. These proteins play a role in the pathogen diagnosis pathways and plant defense signaling system.

Conclusion

Powdery mildew causes economic losses each year and the use of resistant apple cultivars is the best way to control this fungus. It is necessary to identify and evaluate valuable sources of resistance within broad local cultivars to have an effective resistance breeding program. The use of marker-assisted selection is an excellent approach for the

identification of resistance genes and the creation of resistant cultivars. SCAR primer pairs were used in this study to detect *Pl1*, *Pld,* and *Plbj* resistance genes in 47 Iranian full apple genotypes. The Iranian apple genetic resources showed a wide variability of resistance genes to powdery mildew diseases. *Pld* and *Plbj* genes were detected in the samples collected from the cold region of Isfahan and Azerbaijan. *RGA* was amplified in some samples, and the phylogenetical analysis confirmed the results. Our results are in a striking echo with Patzak *et al.* (2011) and Amirchkhmaghi *et al.* (2018), who showed a few numbers of cultivars bearing resistance genes to powdery mildew. Regarding to this fact that *Pld*, *Plbj* and *RGA* were detected in samples collected from the cold area, these genes should be considered in apple breeding programs for generation of resistant cultivar with two or more resistance sources to overcome the evolution of pathogen virulence. The markers *Pl1*, *Pld*, and *Plbj* that were considered in this study should also be screened across the Iranian wild apple gene pool, and more effort should be made to unravel the genetic basis of powdery mildew resistance.

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References

Altschul S. F., Madden T. L., Schäffer A. A., Zhang J., Zhang Z., Miller W. and Lipman D. J. 1997. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. Nucleic Acids Research 25:3389-3402.

Amirchakhmaghi N., Yousefzadeh H., Hosseinpour B., Espahbodi K., Aldaghi M. and Cornille A. 2018. First insight into genetic diversity and population structure of the Caucasian wild apple (*Malus orientalis* Uglitzk.) in the Hyrcanian forest (Iran) and its resistance to apple scab and powdery mildew. Genetic Resources and Crop Evolution 65:1255–1268.

Baek D. E. and Choi C. 2013. Identification of resistance gene analogs in Korean wild apple germplasm collections. Genetics and Molecular Research 12: 483-493.

Caffier V. and Parisi L. 2007. Development of apple powdery mildew on sources of resistance to *Podosphaera leucotricha*, exposed to an inoculum virulent against the major resistance gene *Pl-2*. Plant Breeding 126:319–322.

Caffier V. and Laurens F. 2005. Breakdown of *Pl2*, a major gene of resistance to apple powdery mildew, in a French experimental orchard. Plant Pathology 54:116–124.

Dunemann F. and Schuster M. 2009. Genetic characterization and mapping of the major powdery mildew resistance gene *Plbj* from *Malus baccata jackii*. Acta Horticulturae 814:791-798.

Dunemann F., Peil A., Urbanietz A. and Garcia–Libreros T. 2007. Mapping of the apple powdery mildew resistance gene *Pl1* and its genetic association with an NBS-LRR candidate resistance gene. Plant Breeding 126:476-481.

Ershad D. 2009. *Fungi of Iran*. Iranian Research Institute of Plant Protection, 539Pp.

Gelvonauskis B. and Gelvonauskiene D. 2003. Inheritance of resistance to powdery mildew and apple blotch in progenies of scab-resistant apple cultivars. Biologija 1:73-76.

Hall T. A. 1999. "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT". Nucleic Acids Symposium Series 41:95-98.

James C. M and Evans KM. 2004. Identification of molecular markers linked to the mildew resistance genes *Pld* and *Plw* in apple. Acta Horticulturae 663: 141-146.

James C.M., Clarke J.B. and Evans K. M. 2004. Identification of molecular markers linked to the mildew resistance gene *pld* in apple. Theoretical and Applied Genetics 106:178–183

Katoh K. and Standley D. M. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Molecular Biology and Evolution 30:772–780.

Kellerhals M., Baumgartner I. O., Leumann L., Frey J.E. and Patocchi A. 2013. Progress in Pyramiding Disease Resistances in Apple Breeding. Acta Horticulturae 976, 487-491.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–120.

Korban S. S. and Riemer S. E. 1990. Genetics and histology of powdery mildew resistance in apple. Euphytica 48:261-267.

Maric S., Lukic M., Cerovic R., Mitrovic M. and Boškovic R. 2010. Application of molecular markers in apple breeding. Genetika 2:359-375.

Marine S. C., Yoder K. S. and Baudoin A. 2010. Powdery mildew of apple. *The Plant Health Instructor*. Virginia Polytechnic Institute and State University, USA.

Markussen T., Kruger J., Schmidt H. and Dunemann F. 1995. Identification of PCR-based markers linked to the powdery-mildew-resistance gene *Pli* from *Malus robusta* in cultivated apple. Plant Breeding 114:530-534.

Mozaffarian V. 2005. Trees and shrubs of Iran. Farhang Moaser Publishers, Tehran, pp 1–1054.

Meyers B. C., Kaushik S. and Nandety R. S. 2005. Evolving disease resistance genes. Current Opinion in Plant Biology 8:129–134.

Murray M. G. and Thompson W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8: 4321-4326.

Nei M. 1973. Analysis of Gene Diversity in Subdivided Populations. Proceeding of the National Academy of Sciences of the United States of America 70:3321-332.

Patzak J., Paprstein F. and Henychova A. 2011. Identification of Apple Scab and Powdery Mildew Resistance Genes in Czech Apple (*Malus × domestica*) Genetic Resources by PCR Molecular Markers. Czech Journal of Genetics and Plant Breeding 47:156–165

Perazzolli M., Malacarne G., Baldo A., Righetti L., Bailey A., Fontana P., Velasco R., Malnoy M. 2014. Characterization of Resistance Gene Analogues (RGAs) in Apple (*Malus x domestica* Borkh.) and their evolutionary history of the Rosaceae family. Plos One. 9(2):e83844.

Sambrook J. and Russel D. W. 2001. Molecular cloning: A laboratory manual. Third edition. Cold Spring Harbour Laboratory Press. Cold Spring Harbour. NY.

Sekhwal M. K., Li P, Lam I., Wang X., Cloutier S., You F. M. 2015. Disease Resistance Gene Analogs (RGAs) in Plants. International Journal of Molecular Sciences 16:19248-19290.

Sestras A. F., Pamfil D., Dan C., Bolboaca S. D., Jäntschi L., Sestras R. E. 2011. Possibilities to improve apple scab (*venturia inaequalis* (Cke.) Wint.) and powdery mildew (Podosphaera leucotricha (Ell. et Everh.) Salm.) resistance on apple by increasing genetic diversity using potentials of wild species. Australian Journal of Crop Science 5: 748–755.

Tamura K., Dudley J., Nei M. and Kumar S. 2007. Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24:1596–1599.

Wurms K.V. and Chee A. 2011. Control of powdery mildew (*Podosphaera leucotricha*) on apple seedlings using anhydrous milk fat and soybean oil emulsions. New Zealand Plant Protection 64:201-208.

Xu X. M. 1999. Modeling and forecasting epidemics of apple powdery mildew (*Podosphaera leucotricha*). Plant Pathology 48:462-471.