IDENTIFICATION OF NEW GENETIC SOURCES FROM SEA BEET TO IMPROVE SUGARBEET RESISTANCE TO CERCOSPORA LEAF SPOT

Chenggen Chu[1], Muhammad Massub Tehseen[2], Rachael Claire Poore[1], Melvin D. Bolton[1], Mohamed F.R. Khan[3], Mike Metzger[4], and Xuehui Li[2]

[1]USDA-ARS, Edward T. Schafer Agricultural Research Center, Fargo, ND 58102, [2]Department of Plant Science, North Dakota State University, Fargo, ND 58108, [3]Extension Sugarbeet Specialist, North Dakota State University & University of Minnesota, and [4]Minn-Dak Farmers Cooperative, Wahpeton, ND 58075

Introduction

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola* Sacc., is the most widespread foliar disease in sugar beet (*Beta vulgaris* L.). Significant losses can occur under warm and humid environments with yield losses as high as 42 - 50% (Verreet et al., 1996). Application of fungicide and growing resistant cultivars are two main measures for controlling the disease but using host resistance would be more effective with a lower cost. Vogel et al. (2018) found that recent breeding efforts have made CLS resistant cultivars comparable to susceptible ones in terms of yield performance, consequently, the resistant cultivars thus have a relatively better economic performance since no fungicide needs to be applied.

Many studies were conducted to identify germplasms resistant to CLS and some accessions of *Beta vulgaris* spp. *maritima*, the wild ancestor of sugar beet, were found to have a high level of resistance and were used as a source of CLS resistance (Leuterbach et al., 2004). Our findings in the last year also indicated that a cluster of 355 *B. maritima* accessions showed a further genetic distance to sugarbeet and have much greater potential for improving CLS resistance and broadening the genetic base (Tehseen et al., 2022).

In this research, we evaluated all available *B. maritima* accessions and a few germplasm lines from other wild relatives for resistance to CLS, and then used genotype data through SNPs covering the whole genome of sugarbeet to identify genomic regions associated with the resistance based on genome-wide association study (GWAS).

Materials and methods

A total of 602 *B. vulgaris* accession from NPGS (National Plant Germplasm System) and USDA-ARS sugarbeet genetics program at Fargo, ND were used and planted in Foxhome, MN for field evaluation of resistance to Cercospora leaf spot. The accessions included 582 lines from wild beet *B. maritima* and the remaining 20 genotypes from subspecies *B. macrocarpa* (10 lines), *B. atriplicifolia* (4 lines), *B. patula* (2 lines), *B. macrorhiza* (1 lines), *B. palonga* (1 line), *Patellifolia procumbens* (1 line) and *P. webbiana* (1 line). Accessions were collected from 25 countries and were divided into seven regions of the world **(Table 1)**. Whereas nine accessions had no geographic information available.

Table 1. List and origin of wild beet accessions used in the current study with their putative geographic regions.

Region	Countries (no. of lines)	Total
Africa	Egypt (25), Morocco (31), Tunisia (1)	57
Asia	China (1), India (3), Israel (1)	5
Northern Europe	Denmark (21), Ireland (47), Jersey Island (1), UK (106)	175
Southern Europe	Croatia (1), Cyprus (1), Greece (56), Italy (103), Portugal (6). Spain (11), Turkey (5)	183
Western Europe	Belgium (3), France (141), Germany (2), Netherlands, (2), Guernsey Island (1)	149
Eastern Europe	Poland (1), Russian Federation (2)	3
North America	United States	21

Field evaluation of CLS resistance was conducted as randomized complete block designs with two replications included. The two-row plots were 10 feet long, with 22-inch row spacing and 8 – 10 inches for plant space within a row. The trial was planted on May 27th, 2022, in Foxhome, MN. Inoculation was performed on July 8th and repeated after two weeks. Disease ratings were made on September 12th using a 0 – 9 scale with 0 as immune (no CLS spots), 1 – 3 as resistant (a few scattered spots to some dieback on lower leaves), 4 - 6 as moderately resistant/susceptible (increasing amounts of dead and disease tissue on several to most plants of the row), and 7 - 9 as susceptible (diseased leaf has 50 - 100% of area necrosed on most plants of the row) (Ruppel & Gaskill, 1971).

For genotyping all accessions using GBS platform, approximately 0.1 g of fresh leaf tissue was collected from 7 – 10 plants of each accession and was dried in a freeze drier 35EL (SP Scientific, Inc., Warminster, PA, USA) for 72 hrs. Dried tissues were ground using a homogenizer (SPEX, Inc., Metuchen, NJ, USA). Genomic DNA was extracted from dried tissue using a DNA purification system (KingFisher, Inc., Falls Church, VA, USA), and DNA samples were fragmented by co-digestion using restriction enzymes *NsiI* and *BfaI* to produce DNA fragments. Barcoded adapters were ligated to DNA fragments from each accession to identify fragments generated from each individual accession. GBS sequencing libraries were constructed according to Hilario et al. (2015) by PCR amplification of barcode ligated DNA using a 96-plex plate followed by purification and quantification of the PCR product before sequencing. An Illumina HiSeq 2000 sequencing system (Illumina, Inc., San Diego, CA, USA) was used to sequence about 150 base pairs at both ends of fragments. The obtained fragmental sequences were anchored to the reference sugarbeet genome sequence assembly EL10.2 of sugarbeet line EL10 (McGrath et al., 2022) and compared among accessions to identify genome-wide SNPs through reference-based Tassel pipeline (Glaubitz et al., 2014). Raw SNP data were filtered by removing SNPs with a missing data rate of over 20%, followed by genotype imputation through the computer program Beagle (v5.0) (Browning & Browning, 2007) that achieved a data-missing rate of 0% and only the bi-allelic SNPs were kept.

For analyzing population structure in the *B. maritima* and other wild beet accessions, the computer program STRUCTURE (v.2.3.4) that implements model-based Bayesian cluster analysis was used, and 10 independent replicates for each putative subpopulation ranging from k = 2 –10 under the admixture model was assessed using a burn-in period of 50,000 and 50,000 Markov Chain Monte Carlo (MCMC) replications. To infer the optimal clusters/sub-populations, the best K value representing the optimum number of sub-populations was estimated as Delta K (ΔK) based on the change in the log probability of data between successive structure iterations using Structure Harvester (https://taylor0.biology.ucla.edu/structureHarvester/). In addition, the discriminant analysis of principal components (DAPC) that implemented using the R package "adegenet" was also used to verify results from the program STRUCTURE.

GWAS was carried out using a R package GAPIT (Genome Association and Prediction Integrated Tool) (Lipka et al., 2012). Briefly, a standardized mixed linear model (MLM) (Yu et al., 2006) was used as $y = X\beta + Qv + u + e$,

where y is the vector of observed phenotypes, X is the vector of SNP markers, β is the marker fixed effects vector to be estimated, Q is the population matrix derived from PCA analysis, v is the vector of fixed effects due to population, u is random effects vector and e is the residual vector. The variance of u is estimated as Var (u) = $2KVg$, where K is the kinship matrix derived from individuals based on the proportion of shared alleles and Vg is the genetic variance. K matrices were generated using TASSEL v 5.0 (Bradbury et al., 2007).

Results & discussion

CLS evaluation

The wild beet germplasm showed high variation for response to CLS in the crop season of 2022 (Fig. 1). Out of the 602 wild beet accessions planted, 236 (39%) showed a resistance response with disease ratings of 3 or less included 17 accessions having near immune reaction. A total of 274 (45%) accessions showed moderately resistant to moderately susceptible reaction type, and these accessions could be pivotal for further detecting quantitative trait loci (QTL) of CLS resistance. A total of 33 wild beet accessions were found susceptible to CLS with disease ratings of 7 to 9. In addition, a total of 59 accessions could not be evaluated in the field this year due to the tiny size of plants or matured too early with no green leaves at time of disease rating. Overall, CLS evaluation from this year indicated the high levels of resistance in *B. maritima* and proved the concept of using wild beet as resistance source for sugarbeet improvement. Utilization of *B. maritima* accessions also has the benefit of increasing genetic base of sugarbeet.

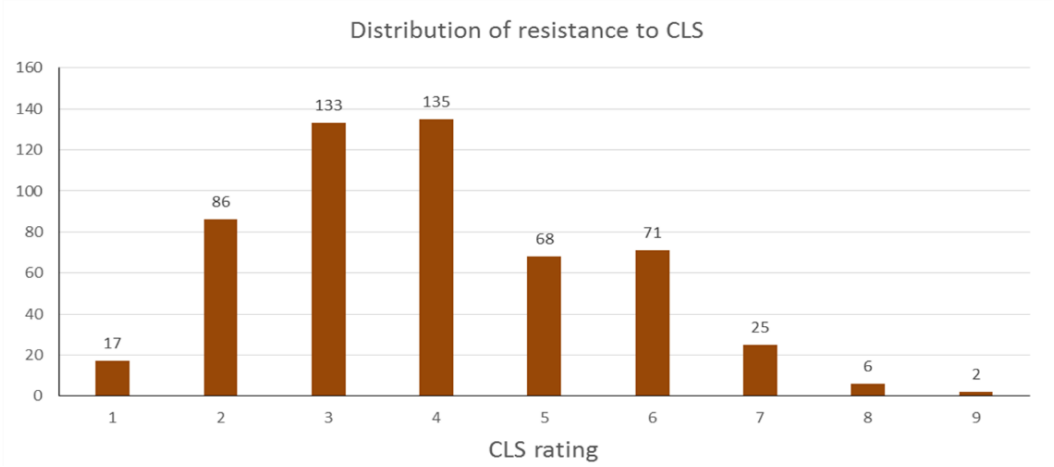

Fig. 1. Distribution of resistance to Cercospora leaf spot (CLS) in wild beet accessions evaluated in Foxhome, MN in 2022.

Based on origin of the *B. maritima* accession used in the evaluation, the highest number of resistant lines were collected from Italy (54), followed by France (53) and United Kingdom (35) including England and Wales. It is also noted that amongst the countries with more than 20 accessions, the highest percentage of resistant was observed in lines collected from Denmark where 66% of the accessions showed resistant response followed by Italy with 52% of the genotypes resistant to CLS (Fig. 2).

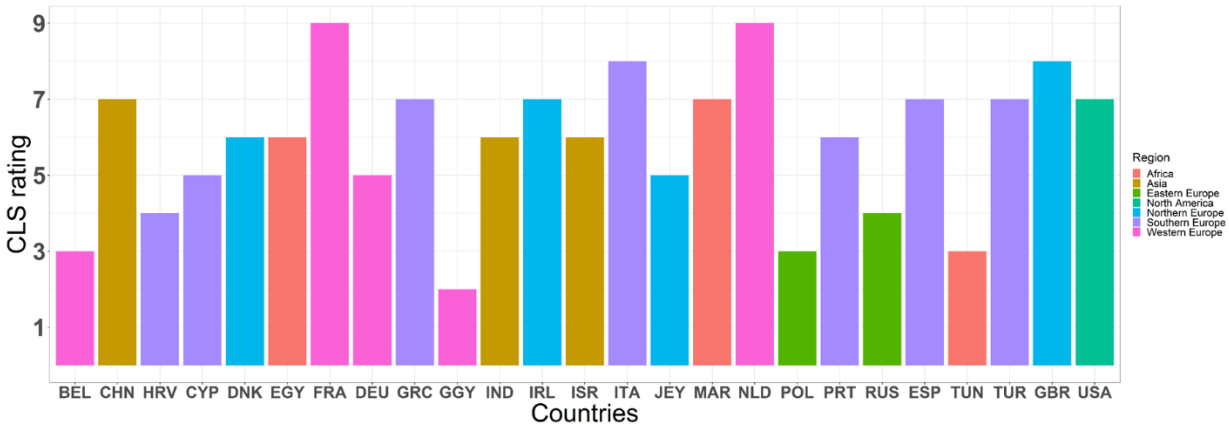

Fig. 2. Distribution of resistance to Cercospora leaf spot (CLS) in wild beet accessions based on geographic location of accessions collected. CLS evaluation was conducted in Foxhome, MN in 2022.

Genotypic data

A set of 520K raw SNPs were generated by the GBS platform. After the initial QC based on missing percentage and filtration of minor allele frequency (MAF) greater than 5%, a set of 147,764 markers were selected and distributed across all nine chromosomes (**Fig. 3**). The maximum number of SNPs were observed on chromosomes 6 (19,140) and 5 (19,115), and chromosome 9 had the minimum SNPs (14,277). The average density of markers across the whole genome was 3.81 markers per kb. The lowest density was observed on chromosome 5 (4.07 marker/kb), whereas the highest density was on chromosome 1 (3.54 markers/kb).

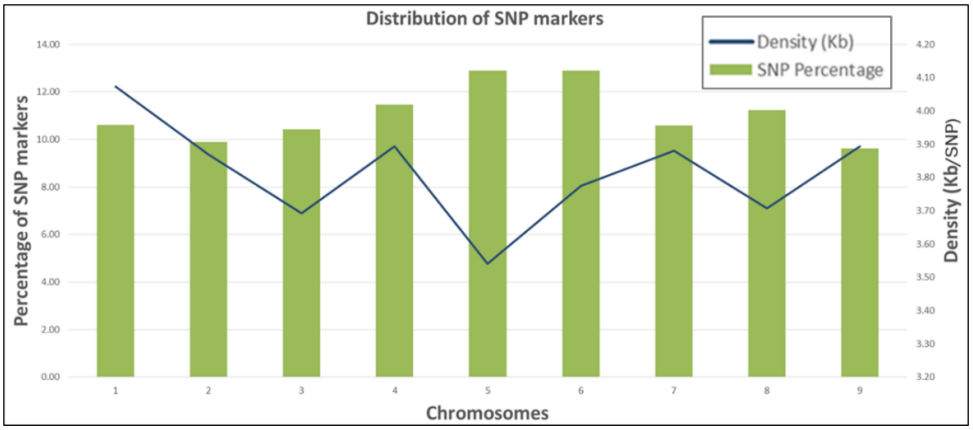

Fig. 3. Distribution of SNP markers across the genome.

Population structure

The STRUCTURE program identified 5 sub-populations in the whole *B. maritima* germplasm used in the current study with majority of accessions in the two sub-populations (Fig. 4). The sub-populations were mostly admixed though lines from the country tend to be closer to each other (Fig. 5).

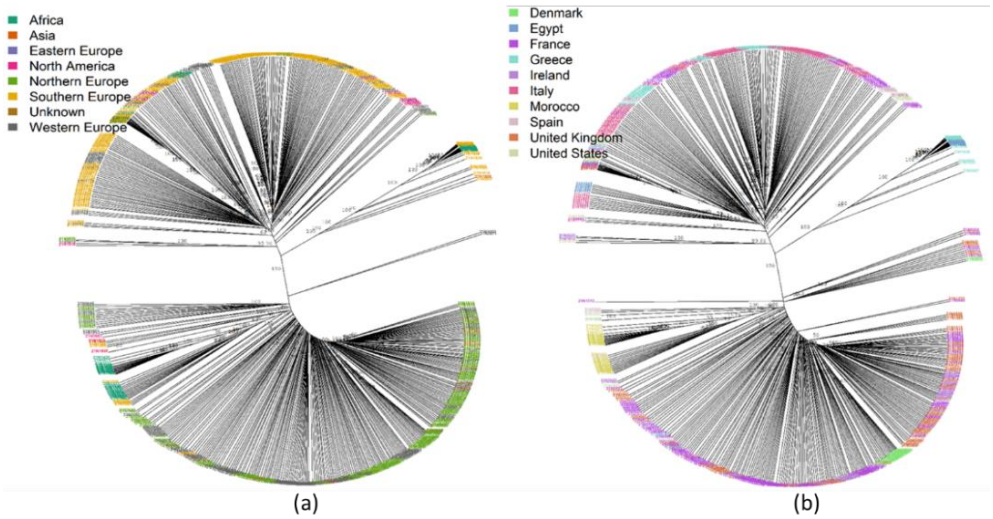

Fig. 4. Population structure of 602 wild beet accessions. (a) regional-based. (b) country based.

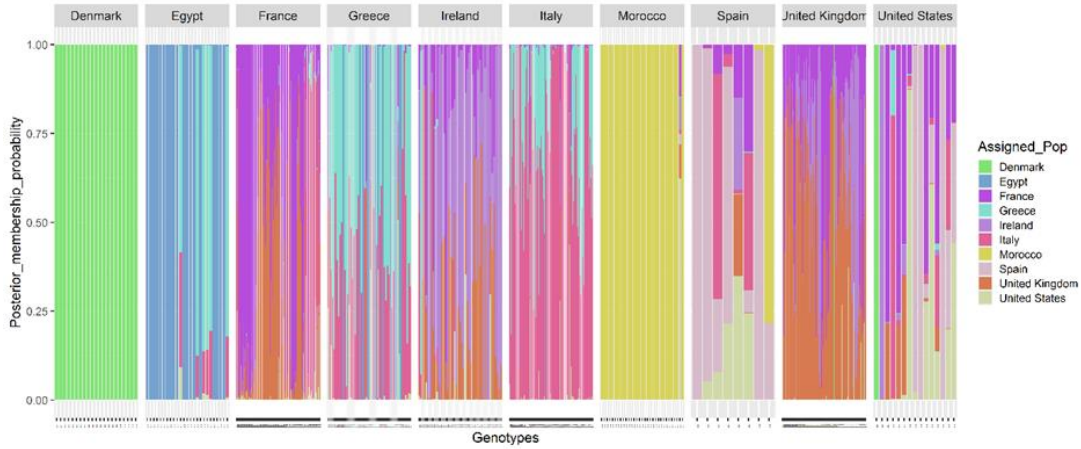

Fig. 5. Population structure of 602 wild beet accessions to show admixture and similarity of accessions from the same country.

Genome-wide association study (GWAS)

A total of 15 SNPs from all nine chromosomes were found significantly associated with CLS resistance based on the threshold of $P < 0.0001$. The highest number of significant markers were detected on the chromosome 8, followed by chromosome 2. The chromosomes 1, 3, 4, 5, 6, and 7 all harbored a single SNP associated with the resistance. Each marker explained 3 to 7% of the total phenotypic variation **(Table 2 and Fig. 6)**.

Table 2. Genomic regions significantly associated with resistance to CLS in wild beet accessions.

SNP	Allele	Chromosome	pseudo-molecule position*	LOD	Favorite allele	Effect	PVE (%)#
S1_8696154	T/C	1	8,696,154	4.1	T	-0.46	3.51
S2_26158536	G/C	2	26,158,536	4.5	G	-0.53	2.84
S2_39167159	G/A	2	39,167,159	4.3	G	-0.59	3.53
S2_39167176	G/A	2	39,167,176	4.3	G	-0.59	3.53
S3_31075893	T/A	3	31,075,893	4.4	T	-0.55	2.51
S4_11695614	A/G	4	11,695,614	4.2	A	-0.45	3.11
S5_52583161	A/C	5	52,583,161	4.2	A	-0.44	2.05
S6_5240439	A/G	6	5,240,439	4.4	G	-0.76	2.85
S7_32060319	T/G	7	32,060,319	4.2	T	-0.53	3.97
S8_38260846	T/C	8	38,260,846	5.3	T	-0.48	4.34
S8_1230157	A/T	8	1,230,157	4.5	T	-0.43	2.87
S8_21922678	G/A	8	21,922,678	4.3	G	-0.73	3.09
S8_14178686	T/C	8	14,178,686	4.1	C	-0.38	3.22
S9_6019498	G/T	9	6,019,498	4.4	T	-0.78	3.18
S9_6024336	G/T	9	6,024,336	4.1	T	-0.70	2.79

*pseudo-molecule position is according to McGrath et al. (2022).
#PVE = phenotypic variation explained

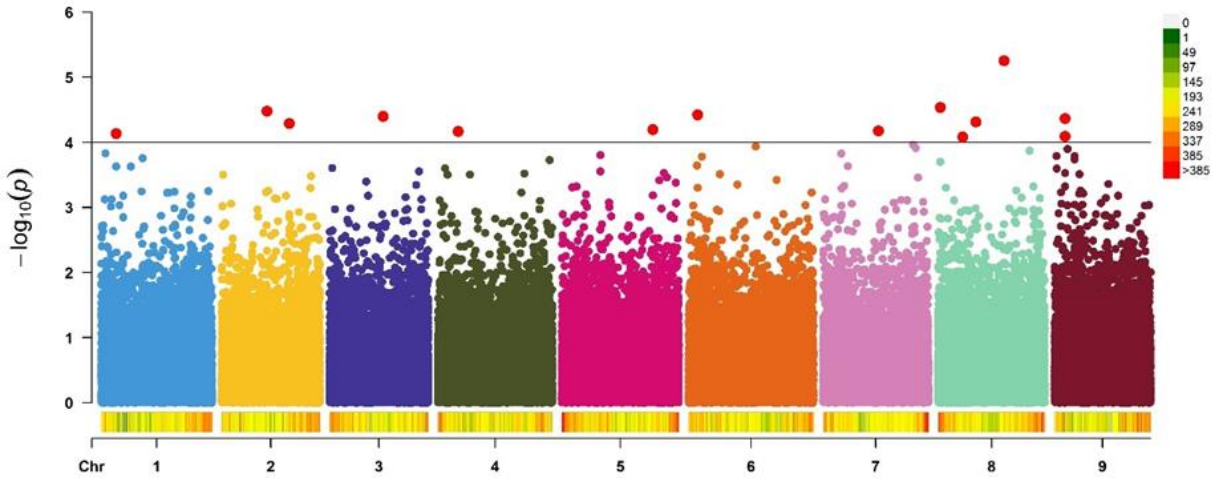

Fig. 6. Manhattan plots of GWAS showing genomic regions significantly associated with resistance to CLS in wild beet accessions.

Similar to our report, the significant markers to CLS resistance have been reported on all nine chromosomes (Weiland & Koch, 2004). Previously, Five QTL on chromosomes 1, 2, 3 and 9 with the phenotypic variability ranging from 7% - 18.3% (Nilsson et al., 1999); Seven QTL on five chromosomes with minor and major effects (Schäfer-Pregl et al., 1999); four minor effect QTL on three chromosomes (Koch et al., 2000); four QTL on four different chromosomes i.e. 3, 4, 7 and 9 explaining phenotypic variance ranging from 6.2% to 25.1% (Setiawan et al., 2000) and four QTL, two major and two minor on four chromosomes (Taguchi et al., 2011). CLS resistance is

quantitative and polygenic with 4-5 genes involved in disease expression (Nielsen et al., 1997; Smith & Gaskill, 1970).

Candidate gene predictions and function annotations

Candidate genes with their putative proteins/enzymes associated with significant loci from GWAS were predicted using the Phytozome_13 database available at https://phytozome-next.jgi.doe.gov (McGrath et al., 2022). The reference genome was screened 2.5kb up and downstream the significant markers with putative functions that could be related to the trait were selected as candidates. Putative genes in 15 genomic regions were scanned and resulted in 16 genes according to sequence assembly EL10.2 **(Table 3)**.

Table 3. Candidate genes with putative proteins/enzymes

Gene	Chromosome	SNP	Protein/Enzyme
Bevul.1G028900	1	S1_8696154	Zinc finger, CCHC-type
Bevul.2G092000	2	S2_26158536	RNA-binding protein with serine-rich domain 1 (RNPS1)
Bevul.2G129400	2	S2_39167159	NAC DOMAIN CONTAINING PROTEIN 38
Bevul.2G129900	2	S2_39167176	Leucine rich repeat (LRR)
Bevul.3G145000	3	S3_31075893	MLO-LIKE PROTEIN
Bevul.4G062900	4	S4_11695614	Protein kinase domain
Bevul.5G170000	5	S5_52583161	disease resistance protein RPS2 (NB-ARC & LRR)
Bevul.6G031400	6	S6_5240439	F-box-like protein
Bevul.7G103000	7	S7_32060319	Glycoside hydrolase, Pectin lyase fold/virulence factor
Bevul.8G106900	8	S8_38260846	Zinc finger, CCHC-type
Bevul.8G010000	8	S8_1230157	MYB
Bevul.8G008200	8	S8_1230157	F-box domain proteins
Bevul.8G081100	8	S8_21922678	programmed cell death protein 5 (PDCD5, TFAR19)
Bevul.8G064200	8	S8_14178686	F-box domain
Bevul.8G037100	9	S9_6019498	ACYL CARRIER PROTEIN/ZINC FINGER PROTEIN
Bevul.8G035800	9	S9_6024336	RIBOSOMAL PROTEIN S6 KINASE

Among these genes, 10 were annotated for functional proteins directly involved in plants disease resistance and defense mechanism. The proteins related to these genes included *Leucine-rich repeat (LRR) domains, Protein kinase domain, F-box domain Proteins, NAC domain containing protein, disease resistance protein RPS2 (NB-ARC & LRR), Zinc finger C2H2-type proteins domain,* and *programmed cell death protein 5 (PDCD5, TFAR19)*. While the remaining 6 genes were reported to play key roles in plants defense via controlling signaling and regulatory pathways in plants. The putative proteins/enzymes related to these candidate genes include *RNA-binding protein with serine-rich domain 1 (RNPS1), MLO like protein, Glycoside hydrolase family Pectin lyase fold/virulence factor, MYB encoding protein, Acyl carrier protein/Zinc finger protein,* and *Ribosomal protein s6 kinase proteins.*

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