Association mapping for resistance to powdery mildew in oriental tobacco (*Nicotiana tabaccum* L.) germplasm

Reza Darvishzadeh[1,2*], Ashkan Basirnia[2], Hamid Hatami Maleki[3], Morad Jafari[1,2]

[1]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Urmia University, P.O. Box 165, Km 12 Nazlou Road, Urmia, Iran. Tel: 04432752741, E-mail: r.darvishzadeh@urmia.ac.ir, ashkan.basirnia@yahoo.com, m.jafari@urmia.ac.ir.
[2]Institute of Biotechnology, Urmia University, Shahid Beheshti St., Urmia, Iran. Tel: 04433440199.
[3]Departrment of Agronomy and Plant Breeding, Faculty of Agriculture, University of Maragheh, Maragheh, Iran. Tel: 04137276008, E-mail: hatamimaleki@yahoo.com.
[*]Corresponding author, E-mail: r.darvishzadeh@urmia.ac.ir, Tel: 04432752741.

ABSTRACT

Powdery mildew caused by *Erysiphe cichoracearum* is an important fungal disease which threatens tobacco (*Nicotiana tabacum* L.) production. The objective of this study was to determine DNA markers linked to genomic regions associated with resistance to powdery mildew in tobacco through the association mapping approach. Seventy tobacco genotypes were fingerprinted using 26 simple sequence repeat (SSR) primer pairs which were distributed in several chromosomes of tobacco. A total number of 66 alleles were detected by 26 SSR primers with an average of 2.53 alleles per locus. Based on population structure analysis, the studied genotypes were classified into three groups including Chopogh, PD and Tikolak genotypes. Ou of 325 marker pairs, 6.15% showed a significant level of linkage disequilibrium (LD) ($P<0.01$). The mean of D´ for all marker pairs was 0.268. Using susceptibility data of the 70 genotypes against fungal agent, three loci (pt30034, pt30008 and pt30159) from linkage groups 22, 11 and 14b of tobacco reference linkage map were identified to be associated with the gene(s) controlling resistance to powdery mildew. The identified markers could be good candidates for marker assisted selection in powdery mildew disease resistance breeding programs. However, like any other quantitative trait, there is a requirement to assess the quality of the obtained quantitative trait loci (QTLs) before marker assisted selection (MAS) become a viable proposition.

Keywords**:** Linkage disequilibrium, mixed linear model, *Nicotiana tabacum*, powdery mildew**,** simple sequence repeat.

INTRODUCTION

Tobacco, genus *Nicotiana spp*. from family *Solanaceae*, is one of the most important industrial crops and *Nicotiana tabacum* L. is the most cultivated species in the world (Ren and Timko, 2001). Many challenges affect cultivation of tobacco in different areas. Among them, powdery mildew caused by *Erysiphe cichoracearum* DC (Smeeton and Ternouth, 1992) is an important sporadic disease in tobacco-producing countries (Shew and Lucas, 1991). When the disease is severe, powdery mildew can completely cover the leaf surface and render the leaf unusable. The major symptoms of powdery mildew are abundant tiny whitish mycelia produced by the causal fungus in the outer surface of the leaves (Shoemaker and Shew, 1999). In severe contaminations, losses in yield and quality

of tobacco could be around 30% to 80% (Darvishzadeh *et al.,* 2010). However, fungicides can provide effective control of this disease, but must be care to avoid excessive residues on the harvested leaf. According to previous studies, there is high genetic variability for resistance to powdery mildew in tobacco (Smeeton and Ternouth, 1992; Darvishzadeh *et al.,* 2010).

Recently, molecular tools especially molecular markers have revolutionized plant breeding activities. Evaluation of genomic diversity through DNA markers and identifying tightly linked markers to gene/ locus of interest offer an opportunity for breeders to apply marker assisted selection (MAS) in their breeding programs. There are some reports in tobacco that used bi-parental breeding populations such as backcrosses, F_2 intercrosses, or recombinant inbred lines (RILs) for mapping markers linked to loci controlling disease resistance (Tong *et al.,* 2012; Vontimitta and Lewis, 2012). Vontimitta and Lewis (2012) by using an SSR map developed on doubled haploid population coming from a cross 'Beinhart-1000 × Hicks' detected two major QTLs for resistance to *Phytophthora nicotianae* that explained 25.4 and 20.4% of phenotypic variation. Tong *et al.* (2012) by using an extensive SSR map constructed on F_2 population coming from a cross between two flue-cured tobacco cultivars (‘Changbohuang’ (CBH) and ‘Jinyehuang’ (JYH)) detected 3 QTLs for brown spot resistance in tobacco which explained 86% of phenotypic variation.

However, due to few recombination events happening in the bi-parental populations, the associated markers are not often tightly linked to the loci of interest (Myles *et al.,* 2009). Hence, association mapping approach, also known as linkage disequilibrium (LD) mapping, which use ancestral recombination events in germplasm collections or natural populations to make marker-phenotype associations (Abdurakhmonov and Abdulkarimov, 2008; Parisseaux and Bernardo, 2004) has been introduced. This method attains a higher resolution because of the use of all meiosis accumulated in the breeding history and linkage disequilibrium (LD). Identification of markers associated with important traits via association mapping has been reported in several plants species including maize (Thornsberry *et al.,* 2001), barley (Ivandic *et al.,* 2003; Kraakman *et al.,* 2004; Kraakman *et al.,* 2006), hexaploid wheat (Breseghello and Sorrels, 2006), chickpea (Saeed *et al.,* 2013) and long life-span forest plants (Wilcox *et al.,* 2007). However, there is narrow information about utilization of association mapping in tobacco. Lately, Fricano *et al.* (2012) applying SSR markers could classify tobacco accessions to six main clades which correspond to "Oriental", "Flue-Cured", "Burley", "Dark", "Primitive", and "Other" classes. Regarding to Fricano *et al.* (2012), pattern of LD was clearly dependent on the population structure and therefore, tobacco population structure must be considered in association analysis.

The aims of the present study were to characterize population structure in the studied oriental and semi oriental-type tobacco germplasm using SSR markers and identify SSR markers associated with responsible genes of powdery mildew resistance by general and mixed linear models (GLM and MLM) procedures.

MATERIALS AND METHODS

Plant material and phenotypic data

A population of 100 oriental and semi-oriental tobacco genotypes was evaluated for their reaction to *Erysiphe cichoracearum* DC that causes tobacco powdery mildew by natural infection at Urmia Tobacco Research Centre. Tobacco genotypes were planted in a simple square lattice design with two replications. Each plot consisted of three rows of 5 m length, with a spacing of 65 × 20 cm. To guarantee homogeneous inoculation, susceptible line (Basma S. 31) was planted throughout the experiment. The fungus diagnosis was made by direct observation of white mycelium over the leaf surface. The disease severity was determined following the scale presented by Fisher (1992) in CORESTA information bulletin, which measured the percentage of tissue affected by powdery mildew in tobacco leaves (1= few spots, 2= 1-5%, 3=5-25%, 4=25-50%, 5>50% leaf area covered).

Genotypic data

Genomic DNA was extracted from the leaves of the 70 genotypes out of 100 (Table 1) following the method described by Doyle and Doyle (1987). Concentrations of the DNA samples were deter-

mined by spectrophotometer (BioPhotometer 6131; Eppendorf, Hamburg, Germany) at 260 nm. The quality of the DNA was checked by running 1 µg DNA on 0.8% (w/v) agarose gel in 0.5X TBE buffer (45mM Tris base, 45 mM boric acid, 1mM EDTA pH 8.0). Twenty-six SSR primer pairs out of 278 from (Table 2) the tobacco SSR database (Blinder *et al.,* 2007 and 2011) were used for DNA fingerprinting. The choice of SSR markers was based on their known genetic locations to obtain near-uniform coverage of the tobacco genome and clarity of produced bands (Bindler *et al.,* 2007 and 2011). Polymerase chain reaction was carried out using the method described by Darvishzadeh *et al.* (2013). Briefly, The reaction mixture contained 2.5mM of each primer (Table 2), 0.4 Unit of Taq DNA polymerase (Cinna Gen Inc., Tehran, Iran), 100µM of each dNTP (BioFluxbiotech, http://biofluxbiotech.com), 2µl 10X PCR buffer, 2mM $MgCl_2$ (CinnaGen, Tehran, Iran), ddH_2O and 25ng template DNA in a 20µl volume. Amplification was carried using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) for 35 cycles consisting of a denaturation step at 94 ºC for 1 min, annealing at 55 ºC for 1 min and an extension step at 72 ºC for 1.5 min. An initial denaturation step at 94 ºC for 4 min and a final extension step of 10 min at 72 ºC were also included. The reaction products were mixed with an equal volume of formamide dyes (98% formamide, 10Mm EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and resolved on a 3% (w/v) agarose gel in 0.5X TBE buffer. The gels were stained with 1.0 µg ml^{-1} ethidium bromide then were photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA).

Data analysis

The SSR data were scored as co-dominant markers in order to distinguish homozygotes and heterozygotes genotypes for each locus. The levels of gene diversity were calculated using Power Marker 3.25 software (http://powermarker.net). Population structure was analyzed using a model-based Bayesian approach in the software Structure 2.3.4 (Pritchard *et al.,* 2000). Five independent runs were performed, setting the number of sub populations (K) from 1 to 10, burn in time and MCMC (Markov Chain Monte Carlo) replication number both to 100,000, and a model for admixture and correlated allele frequencies. The K value was determined by the log likelihood for each K; Ln P(D) = L(K) (Rosenberg *et al.,* 2002). Since the distribution of Ln P(D) did not show a clear number of true K, delta K (ΔK) based on the second order rate of change in the likelihood (Evanno *et al.,* 2005) was used alternatively to identify a clear peak to represent the true K value. Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (Pritchard *et al.,* 2000). Association mapping was performed to analyze marker-trait association by structured association approach using ancestry coefficient (Q values) estimates as covariate in a general linear model (GLM) by using TASSEL 2.1 software. Multiple testing corrections were performed by adjusting maker probability values for multiple test runs, by a permutation test in the TASSEL 2.1 software. The association analysis was also performed using mixed linear model (MLM) approach in TASSEL 2.1 accounting for population structure and kinship relatedness (Q+K model). We used TASSEL 2.1 to estimate kinship coefficients based on SSR markers. Linkage disequilibrium analyses (LD) were conducted in TASSEL 2.1.

RESULTS AND DISCUSSION

Genotypic variation, population structure and linkage disequilibrium

Genotypes used in this study differed considerably for powdery mildew resistance (Table 1). Among 70 tobacco genotypes used in the experiment, 15 genotypes were rated moderately or partially resistant to powdery mildew. The most resistant genotypes were from RILs population.

However some lines selected from our Chopogh and water pipe tobacco landraces showed high resistant reaction to disease that reveal the great importance of local landraces in tobacco breeding programs. Detailed information about the levels of phenotypic variation and resistance to

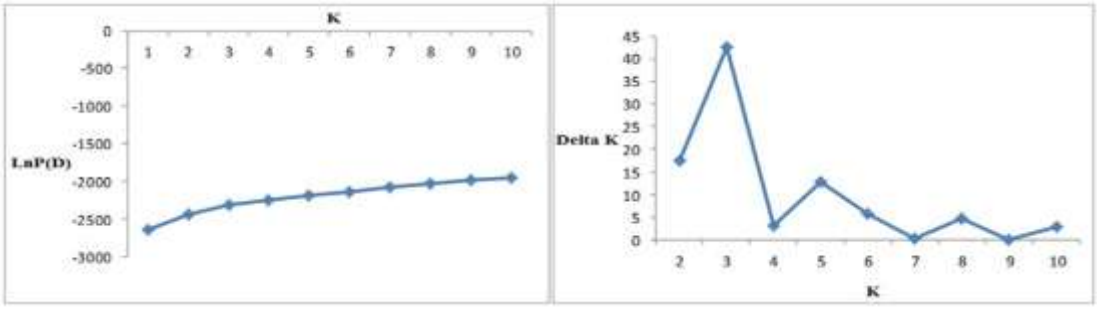

Figure 1. Bilateral charts to determine the optimal number of K (K=3) identified by Structure program.

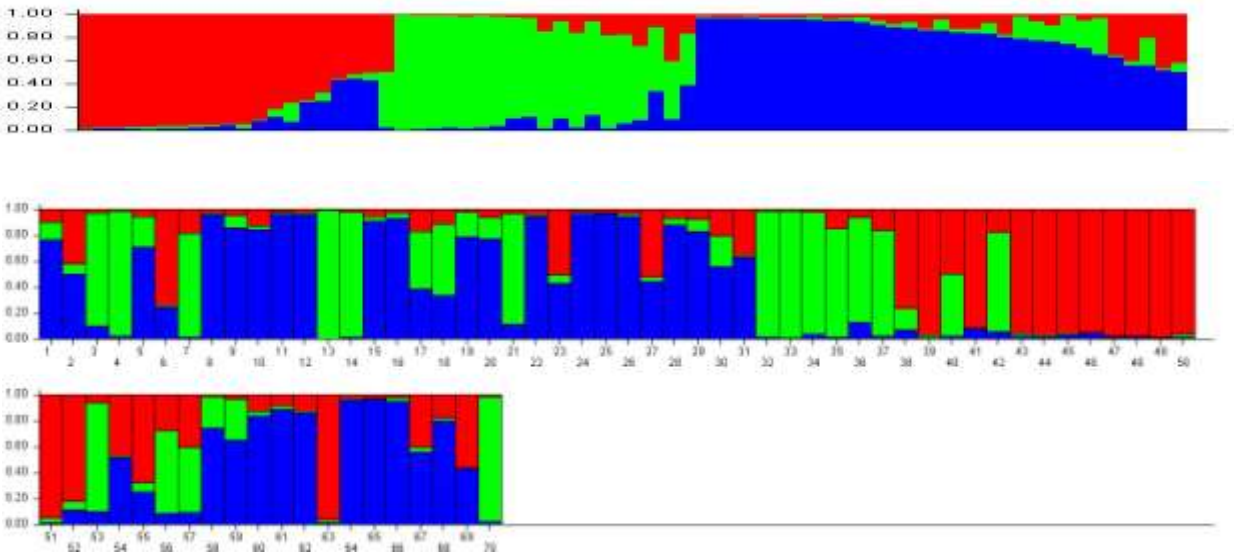

Figure 2. Genetic relatedness of the 70 genotypes of oriental and semi oriental tobacco with the 26 simple sequence repeats (SSRs) as analyzed by the Structure program. Numbers on the y-axis indicate the membership coefficient. The color of the bar indicates the three groups identified through the Structure program. Red: PD (RIL) lines; Green: Tikolak lines; Blue: SPT (Chopogh) lines. Accessions with the same colour belong to the same subgroup.

Table 1. List of selected oriental-type tobacco genotypes and their origin.

Code	Genotype name	Origin	Disease severity score (Darvishzadeh *et al.*, 2010)
G01	Kharmanli 163	Turkey	5
G02	Nevrokop	Bulgaria	5
G03	Trabozan	Turkey	4.5
G04	Krumovgraid	Bulgaria	4.5
G05	Basma Seres 31	Greece	4.5
G06	Triumph	Yugoslavia	5
G07	Xanthi	Greece	5
G08	Matianus	Iran	1.5
G09	Immni 3000	CORESTA collection	5
G10	Melkin 261	CORESTA collection	4
G11	Tyk-Kula	CORESTA collection	5
G12	ss-289-2	CORESTA collection	5
G13	Ohdaruma	CORESTA collection	2
G14	Ploudive 58	Bulgaria	5
G15	Line 20	CORESTA collection	5
G16	T-B-22	CORESTA collection	5
G17	Ts 8	CORESTA collection	5
G19	F.K.40-1	Iran	3
G20	Pz17	CORESTA collection	5
G22	K.B	CORESTA collection	5
G23	G.D.165	Bulgaria	5
G24	H.T.1	CORESTA collection	5
G25	Kramograd N.H.H. 659	Bulgaria	5
G26	T.K.23	CORESTA collection	5
G27	Alborz23	Iran	5
G29	[1]Mutant 3	Iran	1.5
G30	[1]Mutant 4	Iran	5
G31	Pobeda 1	Russian	1
G32	Pobeda 2	Russian	5
G35	Samsun 959	Turkey	5
G36	Samsun dere	Turkey	5
G41	[2]C.H.T.209.12e	Iran	5
G42	C.H.T.209.12e×F.K.40-1	Iran	5
G43	C.H.T.266-6	Iran	5
G44	C.H.T.283-8	Iran	4.5
G45	C.H.T.273-38	Iran	5
G46	Basma 12-2	Iran	5
G47	Basma 16-10	Iran	3
G48	Basma 104-1	Iran	1
G49	Basma 181-8	Iran	5
G51	Izmir	Turkey	5

Code	Genotype name	Origin	Disease severity score (Darvishzadeh *et al.*, 2010)
G52	[3]P.D.324	Iran	1
G53	P.D.325	Iran	2.5
G55	P.D.328	Iran	1
G56	P.D.329	Iran	1
G57	P.D.336	Iran	1
G58	P.D.345	Iran	1
G59	P.D.364	Iran	1
G61	P.D.371	Iran	1
G62	P.D.381	Iran	1
G63	[4]SPT 403	Iran	4
G64	SPT 405	Iran	5
G65	SPT 406	Iran	5
G66	SPT 408	Iran	5
G67	SPT 409	Iran	4
G68	SPT 410	Iran	5
G69	SPT 412	Iran	5
G71	SPT 420	Iran	5
G72	SPT 430	Iran	5
G73	SPT 432	Iran	5
G74	SPT 433	Iran	5
G75	SPT 434	Iran	5
G76	SPT 436	Iran	5
G77	SPT 439	Iran	5
G78	SPT 441	Iran	5
G80	SPT 413	Iran	1
G82	[5]Jahrom 14	Iran	5
G84	L 16	Bulgaria	5
G91	L 17	Bulgaria	1.5
G92	C.H.T.269-12e	Iran	5

[1]Early-maturing mutants developed in Urmia Tobacco research center. [2]The"C.H.T." lines known as Tikolak are semi oriental tobacco developed in Tirtash Tobacco Research Centre (Iran). [3]The "PD" lines are recombinant inbred lines (RILs) derived from the cross between Basma Seres 31 and Dubec 566 in Urmia Tobacco Research Centre. [4]The "SPT" lines known as "Chopogh" tobacco are inbred lines selected from local landraces by single-plant or pure-line selection method in Urmia Tobacco Research Centre. [5]The "Jahrom" line is Iranian water pipe's tobacco line selected from local landraces by single-plant or pure-line selection method in Urmia Tobacco Research Centre. CORESTA: Cooperation Center for Scientific Research Relative to Tobacco, Paris, France (http://www.coresta.org/).

Table 2. Primer sequences, linkage groups and position of the SSR loci applied to 70 tobacco genotypes

Primer	Forward sequence 5´→3´	Reverse sequence 5´→3´	Linkage group (LG)	Band size (bp)	Number of alleles	Position (cM)
PT30021	CATTTGAACATGGTTGGCTG	CTCAACTCTCGTCGCTCTTG	4	224	3	22.7
PT30132	CCTAACAGCATTTGCTACCCA	GATGGACAAGAGTGGCCTTT	10	216	2	65.7
PT30202	TCGAAACCTCGAGGACAGTT	TATCCAAATCTCCAAAGCCC	7	225	2	0.0
PT30159	GCATGCATATGAACATGGGA	TTTGACATCTCTACTCTTCCGTTT	14b	197	2	8.1
PT30027	CCGAGAGTTGCATTTGAATTT	AGGGTTCTACGCAAGAGATTG	13	168	3	98.6
PT30285	CATCATGGCAAGTCACCATC	TGCTGGAAATTAGCGAGGTT	18	177	3	55.3
PT30324	TGCTCTGCGTTAGAACAGGA	CGACGAGAGAAGATTAGTGAAAGA	12	151	2	133.5
PT20343	GGAACACCACCACCATAA	GGAGCTCAGGTTCCAATG	4	322	3	0.0
PT30075	CGATCGGGTCGTTACACAAT	CCCATCAGGTTGTTGGGTTA	11	195	3	12.9
PT30241	AAGTCTCGTGTGGTTGCTTT	AAAGGGCAATGTGTCTAGCTC	15	199	2	0
PT30061	TCGTCCATTTCTTTCTCTCTCA	CATAAATAGTTGCTCATTCAATCG	11	182	3	21.0
PT30110	TTGTACGTTCCTCGCTGATG	GGCCGACAATAAAGTGGCT	21	213	2	0.0
PT30034	TCCTCCAACCAAACTCAAGC	TTTCTGTTCGCGTTTCAAAT	22	228	3	0.1
PT30250	GAACACACGTTCGTCATTGG	ATAAGTCCCTTTAATTTAATTGCG	10	177	2	90.7
PT30292	AAGACAGATTGGTGCGGAAC	AGCACTTGGACAGGCGAATA	7	156	2	50.4
PT30139	ACAACAACTACGTTAGTGTGAGAA	TCATGTGTGCCAAGCTCTT	-	186	3	-
PT30260	GGTAGGGTGGAACAAATTTATCA	AATATGGTCTATGCCCGCAA	8a	225	2	0.8
PT30067	AAGCCTGGTCAGTTATCCCA	ATTCGCACCACTTAATCCCA	2	204	3	29.2
PT30126	GTGATTCCAGCGGAAGACAT	TTCGAAATAAGTACCTAGAGTCGG	10	208	3	0.0
PT30008	CGTTGCTTAGTCTCGCACTG	GGTTGATCCGACACTATTACGA	11	192	3	39.9
PT30165	ACCTCTGTGGCCGTAAGCTA	CCTCTACTTCAACAGGGTAAGAAA	19	224	2	0.0
PT30014	TGCCGTGTAAATTTCATTTGG	AGGATTCCTAACGTGTATTATGTTCT	11	205	3	76.7
PT30046	GATAGGTAGATTATCCTCTGCAACA	GGTGCTAGCAACATCATCAA	13	214	3	29.9
PT30172	AAACAACGTCGAAGCATTTG	ACGCATGAAATTGTAAGGGC	4	216	3	36
PT30205	GGTCGATCCACAATTTAAACG	GCACTTGCTCCTTTGTACCC	3b	193	2	40.5
PT30094	GGGTCATGCGTTCGAATTAT	AACAAGAACGACGGTTACGC	18	214	2	0.0

Genetic distance from the upper telomere estimated according to framework genetic linkage map of tobacco (Bindler *et al.,* 2007)

Table 3. Table represents three loci associated with responsible gene(s) of resistance to powdery mildew using GLM and MLM procedures as well as the primer sequences, linkage group and genetic location of each locus (Blinder *et al.,* 2007)

Locus	Linkage group	Sequence	Genetic location (CM)	GLM			MLM
				Marker P-value	Adjusting marker P-value	Marker R-square	Marker P-value
Pt30034	22	Forward: GACGAAACTGAGGATATTCCAAA Reverse: TGGAAACAAAGCCATTACCC	0.1	0.0100	0.0190	0.1046	0.0102
Pt30008	11	Forward: CGTTGCTTAGTCTCGCACTG Reverse: GGTTGATCCGACACTATTACGA	39.9	0.0286	0.0819	0.1160	0.0311
Pt30159	14b	Forward: GCATGCATATGAACATGGGA Reverse: TTTGACATCTCTACTCTTCCGTTT	8.1	0.0014	0.0020	0.1189	0.0037

cM: centimorgan, GLM: general linear model, MLM: mixed linear model.

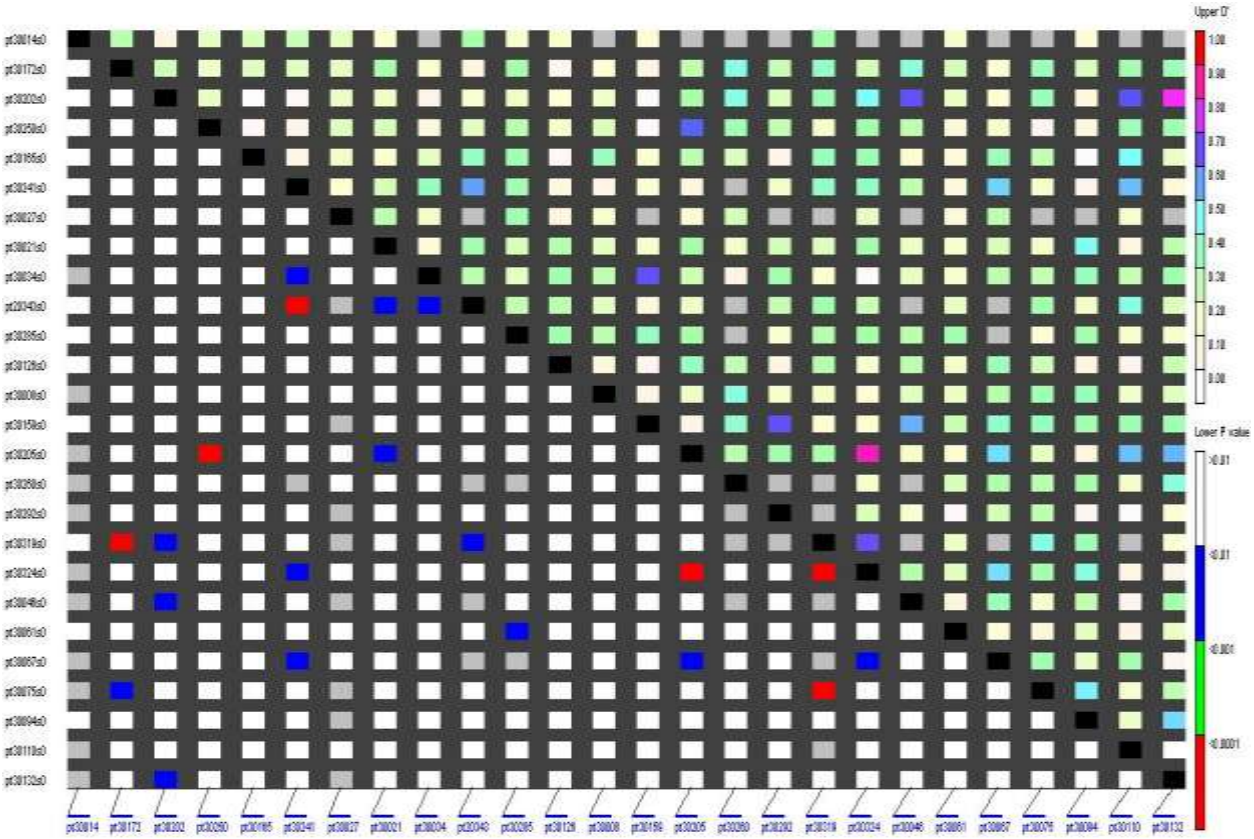

Figure 3. LD plot of the 26 pairs of SSR markers. The upper diagonal shows the linkage disequilibrium rates (D′) among each pairs of SSR loci. The lower diagonal shows the levels of significance between each pairs of SSR loci. 6.15% of the 325 pair SSR loci showed significant levels of linkage disequilibrium (P≤0.01).

disease is available from Darvishzadeh *et al.* (2010).

In this study, the 26 microsatellite loci (SSRs) were used to assess genetic diversity among the 70 oriental and semi oriental-type tobacco genotypes. A total number of 66 alleles were detected by the 26 SSR primer pairs with an average of 2.53 alleles per locus. Gene diversity ranged from 0.33 to 0.66 that shows the probability of variation of two alleles between two individuals.

Regarding the SSR data in the present research, there was an acceptable genetic variation among the studied tobacco genotypes which is accommodated with previous reports in tobacco (Devalavia *et al.*, 2010; Darvishzadeh *et al.*, 2013). According to the literature, successful association mapping in a given species requires creation of an appropriate germplasm collection with phenotypic and underlying genetic variation for the traits of interest, while the rate of LD decay and genetic relatedness determine the mapping resolution in the association mapping population (Zhu *et al.*, 2008; Sharbel *et al.*, 2000).

To dedicate the genetic structure of the association panel, a model-based Bayesian approach in the Structure software was used to subdivide inbred lines into the corresponding subgroup. Based on the ΔK values, the studied genotypes were subdivided into three subgroups (Figure 1). These subgroups matched with three groups in our association panel including: Chopogh, PDs and Tikolak (Figure 2). The 81% of PDs, 71% of SPTs and 70% of Tikolaks were assigned to the corresponding subgroups, and the remaining ones were categorized into the "mixed" subgroups based on their Q values. Incorporating population structure (Q) in association analyses could minimize the false-positive trait-markers association (Yu and Buckler 2006; Yu *et al.*, 2006)

As mentioned above, presence of LD is a prerequisite for association mapping (Sorkheh *et al.*,

2008). Linkage disequilibrium (LD) is defined as a non-random association of alleles at separate loci located on the same chromosome (Mackay and Powell, 2007). LD has been used in association mapping (Zhu *et al.*, 2008) and to locate quantitative trait loci (QTL) or major genes, based on the co-segregation of specific marker alleles and traits (Rafalski and Morgante, 2004). Several statistics have been developed for the quantification of LD. Choosing the appropriate LD measures depends on the objective of the study, and one performs better than other in particular situations and cases (Abdurakhmonov and Abdukarimov 2008). D′ is one of the most commonly used measures of LD. The D′ value ranged from 0.01 to 0.82 with the average value of 0.268 (Figure 3). In the collection under investigation, 6.15% of the 325 SSR primer pairs showed a significant level of linkage disequilibrium (LD) ($P<0.01$) (Figure 3). As expected, the LD in this germplasm encouraged us to do association analysis on this association panel.

Marker-trait association

In the present study, association mapping approach showed that three loci (pt30034, pt30008 and pt30159) from linkage groups 22, 11 and 14b of tobacco reference map (Blinder *et al.*, 2007) showed significant associations ($P\leq0.0100$, $P\leq0.0286$ and $P\leq0.0014$) with the gene(s) controlling resistance to powdery mildew in oriental and semi oriental-type tobacco genome (Table 3). Adjusting maker probabilities in multiple testing corrections shows a non-significant association ($P\leq0.0819$) between locus pt30008 with the trait of interest in the GLM procedure (Table 3). As well as to reduce false positives for the detection of association between the marker and the trait, MLM (Mixed Linear Model) procedure based on population structure and their relative kinship using (Q + K) model was applied in Tassel 2.1 software. Similar results were seen using MLM procedure and the three loci showed significant associations ($P\leq0.0102$, $P\leq0.0311$ and $P\leq0.0037$) with the gene(s) controlling the trait of interest (Table 3).

Nowadays, it is possible to localize loci or markers related to resistance to disease in tobacco. Mapping a nucleotide sequence underlying a specific trait offers an opportunity for tobacco breeders to apply marker assisted selection (MAS) in breeding programs. Several studies have been carried out to identify QTLs controlling disease resistance genes such as bacterial wilt (Nishi *et al.*, 2003), *Phytophthora nicotianae* (Vontimitta and Lewis, 2010) and brown spot (Tong *et al.*, 2012) in tobacco. However, there are few study via bi-parental and or association mapping for identifying powdery mildew resistance genes in tobacco. Recently, Li *et al.* (2011) using 127 F_2 and $F_{2:3}$ populations from the cross between 'Taiyan 7' and 'Burley 21' identified one QTL with R^2 value of %11.63 on the constructed genetic linkage map covering 3483 cM of tobacco genome. However, there are many reports about application of association mapping in other plants belong to family *Solanaceae* such as potato (D'hoop *et al.*, 2008) and tomato (Ranc *et al.*, 2012).

In this study, a low range of LD was observed between markers. Low LD indicates that marker density in the study is not enough for detecting QTLs in the genome. However, similar to findings of Shehzad *et al.* (2009) some markers still captured the signal of QTL even in such a density. Our study demonstrated a significant potential of LD-based association mapping for the identification of powdery mildew resistance genes in tobacco which is in parallel with the findings of Fricano et al. (2012). However, like any other quantitative trait, there is a requirement to assess the quality of the obtained QTLs before MAS becomes a viable proposition. To obtain unbiased estimates of QTL effects and to determine the magnitude of bias of the predictive power, different approaches have been suggested. Cross-validation has been shown to perform well for linkage mapping (Utz *et al.*, 2000) but also for association mapping (Liu *et al.*, 2013; Würschum and Kraft, 2014).

REFERENCES

Abdurakhmonov I. Y., and Abdukarimov A. (2008). Application of association mapping to understanding the genetic diversity of plant germplasm resources. *International Journal of Plant Genomics*, 2008: 574927.

Bindler G., Vander Hoeven R., Gunduz I., Plieske J., Ganal M., Rossi L., Gadani F., and Donini P. (2007). A microsatellite marker based linkage map

of tobacco. *Theoretical and Applied Genetics,* 114: 341–349.

Bindler G., Plieske J., Bakaher N., Gunduz I., Ivanov N., Van der Hoeven R., Ganal M., and Donini P. (2011). A high density genetic map of tobacco (*Nicotiana tabacum* L.) obtained from large scale microsatellite marker development. *Theoretical and Applied Genetics,* 123: 219–230.

Breseghello F., and Sorrells M. (2006). Association mapping of kernel size and milling quality in wheat (*Triticum aestivum* L.) cultivars. Genetics, 172: 1165-1177.

Darvishzadeh R, Alavi R., and Sarrafi A. (2010). Resistance to powdery mildew (*Erysiphe cichoracearum* DC.) in oriental and semi-oriental tobacco germplasm under field conditions, *Journal of Crop Improvement,* 24: 122 - 130.

Darvishzadeh R., Mirzaei L., Hatami Maleki H., Laurentin H., Alavi S. R. (2013). Genetic variation in oriental tobacco (*Nicotiana tabacum* L.) by agro-morphological traits and simple sequence repeat markers. *Revista Ciência Agronomica*, 44: 347-355.

Davalieva K., Maleva I., Filiposki K., Spiroski O., and Georgi D. (2010). Genetic variability of Macedonian tobacco varieties determined by microsatellite marker analysis. *Diversity*, 2: 439-449.

D'Hoop B. B., Paulo M. J., Mank R. A., Van Eck H. J., and Van Eeuwijk F. A. (2008). Association mapping of quality traits in potato (*Solanum tuberosum* L.). Euphytica, 161: 47–60.

Doyle J. J., and Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19: 11-15.

Evanno G., Regnaut S., and Goudet J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* 14: 2611-2620.

Fricano A., Bakaher N., Del Corvo M., Piffanelli P., Donini P., Stella A., Nikolai V., Ivanov N. V., and Pozz C. (2012). Molecular diversity, population structure, and linkage disequilibrium in a worldwide collection of tobacco (*Nicotiana tabacum* L.) germplasm. *BMC Genetics*, 13: 18.

Ivandic V, Thomas W. T. B., Nevo E., Zhang Z., and Forster B. P. (2003). Associations of simple sequence repeats with quantitative trait variation including biotic and abiotic stress tolerance in *Hordeum spontaneum*. *Plant Breeding*, 122: 300-304.

Kraakman A. T. W., Niks R. E., Van der Berg P. M. M. M., Stam P., and Van Eeuwijk F. A. (2004). Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. *Genetics,* 168: 435-46.

Li H. L., Chen M. X., Zhou D. X., Chen S. H., Tao A. F., Li Y. K., Ma H. B., Qi J. M., and Guo Y. C. QTL Analysis of Six Important Traits in Tobacco (*Nicotiana tabacum* L.). *Acta Agronomica Sinica,* 37: 2011: 1577-1584.

Liu W., Gowda M., Steinhoff J., Maurer H. P., Würschum T., and Longin C.F., Cossic F., and Christoph J. (2011). Association mapping in an elite maize breeding population. Theoretical *and Applied Genetics,* 123: 847–858.

Mackay L., and Powell W. (2007). Methods for linkage disequilibrium mapping in crops. *Trends in Plant Science,* 12: 57-63.

Myles S., Peiffer J., Brown P. J., Ersoz E. S., Zhang Z., Costich D. E., and Buckler E. S. (2009). Association mapping: Critical considerations shift from genotyping to experimental design. *The Plant Cell,* 21: 2194-2202.

Nishi T., Tajima T., Noguchi S., Ajisaka H., and Negishi H. Identification of DNA markers of tobacco linked to bacterial wilt resistance. *Theoretical and Applied Genetics*, 2003: 106: 765‑770.

Parrisseaux B., and Bernardo R. (2004). In silico mapping of quantitative trait loci in maize. *Theoretical and Applied Genetics,* 109: 508-514.

Pritchard J. K., Stephanes M., Rosenberg N. A., and Donnelly P. (2000). Association mapping in structured populations. *American Journal of Human Genetics,* 67: 170-181.

Rafalski A., and Morgante M. (2004). Corn and humans: Recombination and linkage disequilibrium in two genomes of similar size. *Trends in Genetics,* 20: 103-111.

Ranc N., Muños S., Xu J., Le Paslier M. C., Chauveau A., Bounon R., Rolland S., Bouchet J. P., Brunel D., Causse M. (2012). Genome-wide association mapping in tomato (*Solanum lycopersicum*) is possible using genome admixture of *solanum lycopersicum* var. cerasiforme. *Genes Genomes Genetics,* 2: 853–864.

Ren N., and Timko M. P. (2001). AFLP analysis of genetic polymorphism and evolutionary relationships among cultivated and wild *Nicotiana* species. *Genome*, 44: 559-571.

Rosenberg N. A., Pritchard J. K., Weber J. L., Cann H. M., Kidd K. K., Zhivotovsky L. A., and Feldman M. W. (2002). The genetic structure of human populations. *Science*, 298: 2381-2385.

Saeed A., Darvishzadeh R., and Basirnia A. (2013). Simple sequence repeat markers associated with agro-morphological traits in chickpea (*Cicer arietinum* L.). *Zemdirbyste-Agriculture,* 100: 433-440.

Sharbel T. F., Haubold B., and Mitchell-Olds T. (2000). Genetic isola-tion by distance in Arabidopsis thaliana: Biogeography and postglacial colonization of Europe. *Molecular Ecology*, 9: 2109-2118.