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Original scientific paper

IDENTIFICATION OF QTLS ASSOCIATED WITH LOW CHLORIDE ACCUMULATION IN ORIENTAL TOBACCO

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The construction of linkage maps and identification of genomic regions controlling traits have great significance for plant breeders. Among different chemical traits in oriental tobacco, chloride content in leaves when it is less than 1.5% improves burning quality. In this study, genetic analysis of quantitative trait loci affecting the chloride accumulation of oriental tobacco leaf was performed using an F_2 population of a cross between two oriental tobacco genotypes, 'Basma Seres 31' and 'SPT 406', comprising 100 individuals. A normal distribution was observed for the chloride accumulation in the F_2 population. Linkage map with 23 informative microsatellite and 29 inter simple sequence repeat markers was constructed, which covered 570.8 cM of the tobacco genome. Single marker analysis, interval mapping and composite interval mapping were used to detect the putative QTLs controlling chloride accumulation. There was not any significant relation between ISSR markers and chloride accumulation. SSR marker PT30346 was found to be significantly associated with chloride accumulation through single marker analysis. Two QTLs including Chl_{IM} and Chl_{CIM} with R^2 values of 0.4 and 0.07 were identified using IM and CIM, respectively.

Key words: Chloride accumulation, Linkage map, Oriental tobacco, QTL

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INTRODUCTION

Nicotiana spp. is one of the most economically important non-food crops. Beside leaves as its economic part, seeds contain 38% of non-edible oil which could be an appropriate substitute for diesel fuel (GIANNELOS *et al.,* 2002). *Nicotiana* belongs to family Solanaceae with more than 64 species, among which *N. tabaccum* is one of the most cultivated species. It is an allopolyploid species ($2n = 48$) with basic chromosome number of $x = 12$ (KNAPP *et al.,* 2004), having the highest genome size (4500 Mbp) in the family Solanaceae (ARUMUGANATHAN and EARLE, 1991). Numerous types of tobacco are defined by different criteria such as region of production, intended use in cigar (i.e., filler, binder and wrapper) and cigarette manufacturing, method of curing (flue-, air-, sun- and fire-cured tobacco) as well as morphological and biochemical characteristics (i.e., aromatic fire-cured, bright leaf tobacco, Burley tobacco, Turkish or oriental tobacco, REN and TIMKO, 2001). Turkish or oriental tobacco is a sun-cured, highly aromatic, small-leafed type which is grown in Turkey, Iran, Greece, Bulgaria, Lebanon and the Republic of Macedonia. It has the ability to grow in low fertility soils (GULER GUMUS, 2008). In order to get an American Blend type of cigarette, it is mixed with more robust tobacco such as Virginia and Burley tobaccos.

The tobacco leaf is composed of 85-90% water, mineral matter and organic compounds. The latter may be divided into organic acids, carbohydrates and alkaloids (DAVIS and NIELSON, 1999). Among mineral nutrients, chloride is recognized as an essential micronutrient in tobacco cultivation. A small amount of chloride (below 1.5%) can improve yield and certain quality factors of oriental tobacco whereas the values greater than 2% inhibit the burning properties of tobacco (ALAVI *et al.,* 2008). Recently, large genetic variations have been reported for chloride accumulation in Iranian oriental tobacco germplasm (DARVISHZADEH *et al.,* 2011) which is controlled by both additive and non-additive genetic effects (DARVISHZADEH and ALAVI, 2011). Quantitative inheritance often results from the segregation of multiple genetic factors. Molecular tools facilitate the identification of genomic locations controlling complex traits using quantitative trait loci (QTLs) analysis (DAVAR *et al.,* 2010; ABDI *et al.,* 2012). Identification of QTLs using a molecular marker map, not only allows genetic dissection of complex traits, but also expedites transfer of QTLs through a process known as marker-assisted selection (MAS).

Saturated linkage maps have been constructed for most crops in Solanaceae. The first genetic linkage map of tobacco was interspecific (*N. plumbaginifolia* × *N. longiflora*), which constructed by using RFLP and RAPD markers and consisted of 19 linkage groups covering 1385.6 cM of tobacco genome (LIN *et al.,* 2001). NISHI *et al.* (2003) by means of 106 AFLP markers using double haploid lines of barley tobacco established 10 linkage groups with total length of 383 cM and identified one QTL for the bacterial wilt resistance. JULIO *et al.* (2006) using AFLP, ISSR, SCAR and SSAP markers on 114 recombinant inbred lines from a cross between 'ITB32' and '4K78' developed a map with a total length of 707.6 cM which included 18 linkage groups. They identified several QTLs accounting for 8-41.5% of the phenotypic variation controlling 59 agronomic, leaf quality, chemical composition and smoke properties traits on 12 linkage groups. BINDLER *et al.* (2007, 2011) identified a high saturated linkage map of flue-cured tobacco which consisted of 24 tentative linkage groups spanning 1,920 cM and 3270 cM, respectively. VONTIMITTA and LEWIS (2012) by using SSR markers and a doubled haploid population generated from a cross between cultivars 'Beinhart-1000' and 'Hicks', detected two major QTLs affecting resistance to *Phytophthora nicotianae* that explained 25.4 and 20.4% of phenotypic variation. In another study, TONG *et al.* (2012) using 213 individuals of F_2 population derived

from the cross between two flue-cured tobacco cultivars including 'Changbohuang' (CBH) and 'Jinyehuang' (JYH) and by means of SSR markers constructed extensive linkage map of tobacco. TONG *et al.* (2012) reported 3 QTLs for brown spot resistance in tobacco which explained 86% of phenotypic variation.

In the present study, we aimed to develop a genetic linkage map of oriental tobacco using F_2 individuals from the cross between two divergence oriental tobacco genotypes including 'SPT 406' and 'Basma seres 31' and to identify QTLs associated with chloride accumulation in oriental tobacco leaves.

MATERIALS AND METHODS

Mapping Population

Based on our previous study (DARVISHZADEH *et al.,* 2011), two oriental tobacco genotypes 'SPT 406' and 'Basma Seres 31', showing low and intermediate leaf chloride accumulation as well as contrasting agro-morphological performance such as days to flowering, leaf width, leaf length, number of leaf, stem height and cured leaf yield were selected for the present study. One hundred F_2 individuals were obtained by crossing 'Basma seres 31' as a maternal line and 'SPT 406' as a paternal one and selfing of their F_1 progenies.

Measurement of Chloride Accumulation

The F_2 population and their parents were transplanted in the experimental field of Urmia Tobacco Research Center in Iran with soil properties presented in Tab. 1. A sample of three leaves from upper, middle and lower regions of each F_2 plants as well as their parents were taken and sun-cured to measure leaf chloride accumulation. The percentage of chloride was determined according to DARVISHZADEH *et al.* (2011) method. Briefly, tobacco leaves were grinded to produce a fine powder; 100 mL dH_2O was added to the 1 g of the fine powder. Tubes were shaken and put on a steam bath for 30 min and then were filtered through Whatman No. 42 filter papers; 10 mL sample were taken from the filtered solution and mixed with the 50 mL dH_2O: 60 mL of the latter was used as a blank in another tube. Several drops of K_2CrO_4 agent (5 g of the K_2CrO_4 in 100 mL dH_2O) were added to the samples and blank vials. For titration, each one was mixed with 0.025 mol l71 $AgNO_3$ (4.47 g of the $AgNO_3$ in 100 mL dH_2O) so that the colour of the vials was converted from light yellow to burnt orange. The percentage of Cl was calculated, using the Eq. (1):

$$Cl = \frac{(A-B).f.35.3}{W\dfrac{(100-M)}{100}} \times 100 \qquad (1)$$

Where: A = Amount of $AgNO_3$ used for tobacco sample, B = Amount of $AgNO_3$ used for blank sample, W = Tobacco weight, M = Percentage of leaf humidity, f = Normality of the $AgNO_3$.

Table 1. Some physical and chemical properties of soil in Urmia Tobacco Research Centre (UTRC), Urmia, Iran

Soil parameters	$\overline{X}$	Soil parameters	$\overline{X}$
pH	7.6	OC (%)	0.73
Ec × 10^{-3}(dS m^{-1})	0.6	SP (%)	49.9
P (mg kg^{-1})	39.3	$CaCO_3$(%)	11.84
K (mg kg^{-1})	565	HCO_3 (%)	3.57
N (Total) (%)	0.11	Sand (%)	16
Mg (mmol c l^{-1})	1.87	Silt (%)	44
Ca (mmol c l^{-1})	1.89	Clay (%)	40
Cl (mmol c l^{-1})	0.80		

Construction of Genetic Linkage Map

Genomic DNA was extracted from single plants of the parental lines, as well as F_2 individuals, according to the method of DELLAPORTA *et al.* (1983). A total of 162 simple sequence repeat (SSR) primer pairs from tobacco SSR linkage map (BINDLER *et al.,* 2007) with 80 inter simple sequence repeat (ISSR) primers from University of British Columbia were screened for polymorphisms between two parental lines. SSR and ISSR analysis were carried out with the method described by EK *et al.* (2005) and YANG *et al.* (2007), respectively. Genotyping of F_2 individuals was accomplished using polymorphic markers. Linkage analysis was processed through the Carthagene software (GIVRY *et al.,* 2005) with the Kosambi function, minimum LOD score of 3.0 and maximum distance of 50 cM.

QTL Analysis

Single marker analysis through ANOVA method was conducted in SAS 9.1 software. Both interval mapping (IM) and composite interval mapping (CIM) were conducted by Windows QTL Cartographer 2.5 (BASTEN *et al.,* 2001). A LOD score of 2.3 resulting from permutation test was used for identifying significant QTLs.

RESULTS AND DISCUSSION

Phenotypic Data

The female parent ‘Basma Seres 31’ and the male parent 'SPT 406' differed significantly ($P<0.01$) in term of leaf chloride accumulation, using t-test (Tab. 2). Frequency distribution of F_2 individuals and their parents for chloride accumulation showed continuous normal patterns, suggesting that chloride accumulation is controlled by a polygenic system (Fig. 1). Some of F_2 progenies showed lower chloride accumulation value than their low accumulating parent (SPT 406), whereas some other progenies sh owed higher chloride accumulation values than their high accumulating parent (Basma Seres 31), indicating a transgressive segregation for the chloride accumulation (Fig. 1).

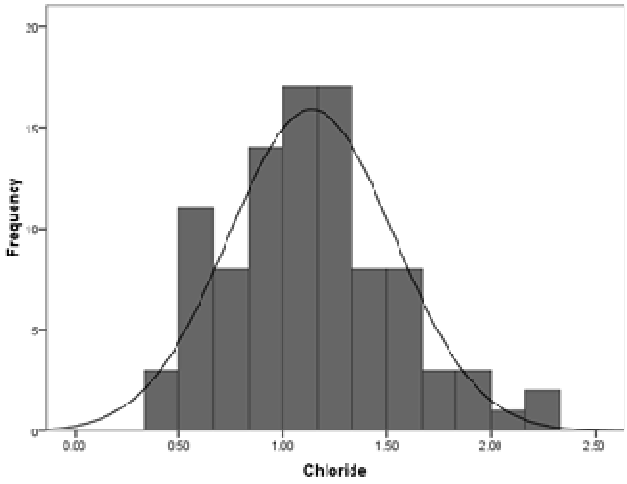

Figure 1. Frequency distribution of chloride accumulation in tobacco F_2 individuals and parental lines

Table 2. Comparison of chloride content values of tobacco parents

Trait	Genotype	Mean	Standard deviation	t-value
Chloride	'Basma seres 31'	2.08	0.27	-5.65**
	'SPT 406'	0.49	0.04	

**, Significant at 0.01 probability level

Genetic Linkage Map

Of 162 SSR primer pairs and 80 ISSR primers tested, 23 SSR primers (12.3%) and 20 ISSR primers (20%) produced clear polymorphism between parental lines which had also Mendelian segregation in population. In this study, there were low level of polymorphisms in the two classes of studied markers which were accommodated with REN and TIMKO (2001) and JULIO *et al.* (2006). Regarding genotyping results, in most cases there was one polymorph marker per each polymorphic ISSR marker. Likewise, the majority of SSR loci amplified two fragments in the tobacco genome suggesting that one fragment is being amplified from each of the two tobacco genomes (BINDLER *et al.,* 2007). However, in nearly all cases, for markers amplifying two fragments, only one fragment could be mapped due to the fact that the other fragment was monomorph in the parents. A total of 52 markers (23 SSR and 29 ISSR) produced through polymorph primers were used for construction of genetic linkage map. Of these 52 markers, 34 markers (15 SSR, 19 ISSR) assigned to 7 linkage groups and 18 markers remained unlinked. The resulting genetic linkage map of oriental tobacco is presented in Fig. 2. Linkage groups named as I to VII. The constructed genetic linkage map covered 570.8 cM of the oriental tobacco genome.

The number of markers per linkage group ranged from 2 to 12. According to literature, small linkage groups in tobacco are ordinal. In JULIO *et al.* (2006) work, seven genetic linkage groups out of 18 established linkage groups had less than 4 markers. Most of linkage groups established by HONG-BO *et al.* (2008) using SRAP and ISSR markers had two markers per linkage group. In the produce linkage map, linkage group V (LG V) has the highest number of markers (12 markers) which is the largest in term of cM size (193.9 cM; Fig. 2). Similarly, BINDLER *et al.* (2007, 2011), reported a map with the largest linkage groups of 163.1 and 199 cM. Likewise, the largest linkage group reported by HONG-BO *et al.* (2008), had 291 cM lengths with 20 markers.

In the present study, off the markers examined, the ISSRs are newly mapped markers. Considering the SSR markers position in the present map, 10 markers are common on 3 linkage groups between the current map and the most recent intraspecific map published by BINDLER *et al.* (2007) (Fig. 3). Herein, linkage groups LG I and LG II is resemble to LG 8a and LG 10 of published map reported by BINDLER *et al.* (2007), respectively. LG V is similar to both LG 3a and LG 11. Comparison of this oriental tobacco map with previous map, confirms that the linear order of markers are not maintain across the different type of tobacco although small number of markers show co-linearity (Fig. 3). Markers with a different order within the same linkage group suggest that these discrepancies are more likely to be due to chromosomal re-arrangements that occur during the evolution of oriental tobacco from a common ancestor.

QTL Analysis

Single marker analysis (SMA) through one-way analysis of variance was carried out, using marker genotypes as groups. The ANOVA establish significant linkage between SSR markers and chloride data (Tab. 3). One SSR marker (PT30346) is found to be significantly associated with chloride accumulation and the R-square value for this locus is 0.16 (Tab. 3). Two QTLs were mapped for chloride accumulation by interval mapping (Chl_{IM}) and composite interval mapping (Chl_{CIM}), respectively. These two QTLs are on LG V and LG II which explain 40% and 7% of the total phenotypic variation, respectively (Tab. 4; Fig. 2). Controversy to JULIO *et al.* (2006), the result of CIM is not similar with IM. Whereas, there is coincidence between IM results and single marker analysis and the marker PT30346 was commonly linked to chloride accumulation. JULIO *et al.* (2006) reported that one to the three QTLs is involved in control of agronomic and chemical traits of tobacco. NISHI *et al.* (2003) via two years data of tobacco bacterial wilt identified two QTLs which possess 43.8% and 34.3% of resistance variation. In the case of chloride accumulation, it is rarely measured in previous studies (except JULIO *et al.,* 2006) that could not recognize any QTL for it. Identifying markers linked to diseases resistant genes were stated in tobacco (NISHI *et al.,* 2003; VONTIMITTA and LEWIS, 2012) while there were not rather studies on complex traits such as tobacco burning quality related traits. Narrow studies about complex traits of tobacco could be probably due to the difficulty of detecting DNA polymorphisms within *N. tabacum* (JULIO *et al.,* 2006).

Table 3. Significant markers for tobacco chloride accumulation by single marker analysis

Marker	R^2	F	*Pr* > F	Adjusted *Pr* value	
PT30346	0.16	7.67	0.00087	0.00096	*

P-values are considered significant (*), if *P*-value<Bonferroni corrected P-value. R^2 is the percent of individual phenotypic variance explained by the marker

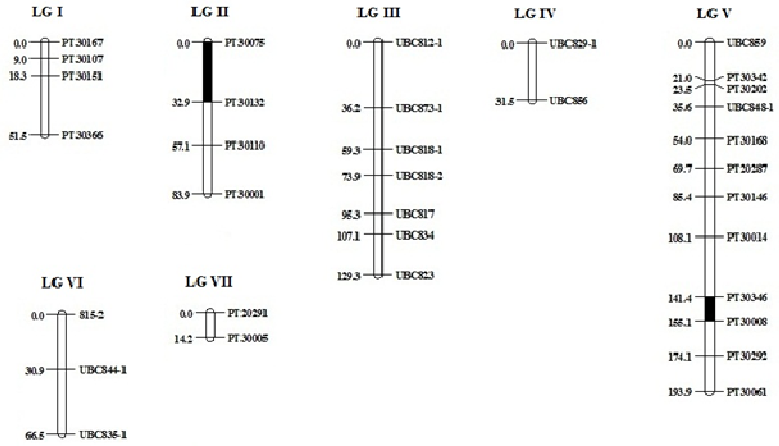

Figure 2. Linkage map of SSR and ISSR markers in tobacco F_2 population derived from the cross between 'Basma seres 31' × 'SPT 406'. Bars represent intervals associated with the QTLs

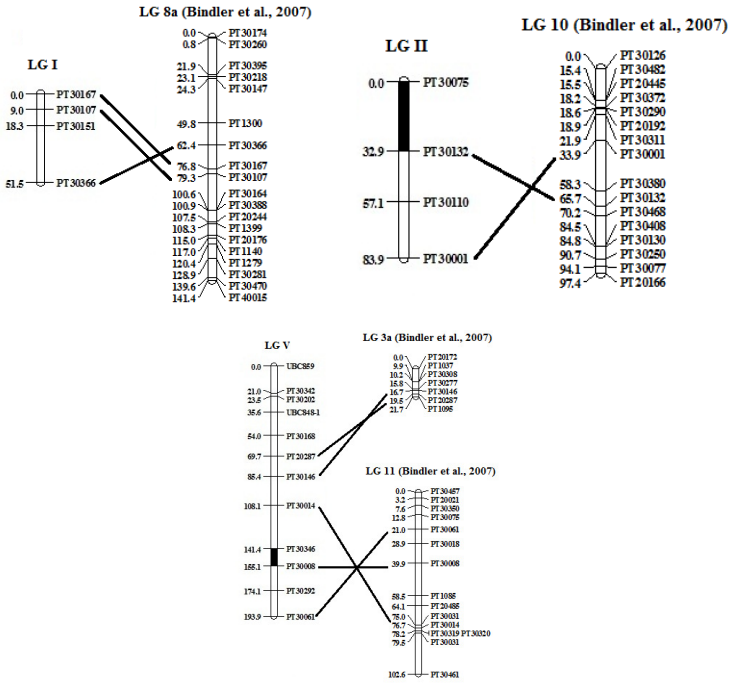

Figure 3. Comparison of the constructed linkage map with the map of flue-cured tobacco (Bindler et al., 2007) based on SSRs positions. Common markers are connected with line

Our results revealed that QTLs with major and minor effects could be involved for chloride accumulation in oriental tobacco. The additive effect of QTLs identified by IM (Chl_{IM}) and CIM (Chl_{CIM}) were -0.1 and -0.2, respectively (Tab. 4). So, alleles responsible for reducing chloride accumulation in oriental tobacco could be transferred from 'Basma seres 31' to progenies. This is not unexpected with intermediate reaction of 'Basma seres 31' to chloride accumulation. Likewise, with traits having transgressive segregation it is possible to have allele transmission by each of parental lines toward offspring. However, this could not be very likely herein because additive values of both Chl_{IM} and Chl_{CIM} were less than their dominance values (Tab. 4). Similarly, DARVISHZADEH and ALAVI (2011) reported that the general combining ability (GCA) of chloride accumulation (due to additive gene effect) was less than the specific combining ability (SCA; due to dominance gene effect) in oriental tobacco. According to Tab. 4, results of IM is coincidence with the findings of DARVISHZADEH and ALAVI (2011) and positive dominance effects of QTL identified by IM (Chl_{IM}) manifested that this dominant allele was transferred from SPT 406 as paternal genotype with low chloride accumulation to individuals. In the present study, there was also over-dominance genetic effect for chloride accumulation which is in accommodation with previous report in oriental tobacco (DARVISHZADEH and ALAVI, 2011). The latter workers using diallel mating design proved that some F_1 hybrids of oriental tobacco had chloride accumulation outside the range of their parents, having implication on the over-dominance genetic control of chloride accumulation. Here, Chl_{IM} show over-dominance effects (with d/a ratios of 5.7) for reducing chloride accumulation while Chl_{CIM} show this state (with d/a ratios of -2) for increasing chloride accumulation.

Table 4. QTLs affecting chloride accumulation (Chl) in the 100 F_2 plants derived from a cross between 2 oriental tobaccos 'Basma seres 31' and 'SPT 406'

Method	QTL	Interval	LG	Position[a]	LOD[b]	R^{2c}	a[d]	d[e]	d/al[f]
IM	Chl_{IM}	PT30346-PT30308	V	145.4	2.3	0.40	- 0.1	0.57	5.7
CIM	Chl_{CIM}	PT30075-PT30132	II	0.0	3.0	0.07	- 0.2	-0.40	-2.0

[a] Expressed in Kosambi cM, from the top of linkage group (LG), [b] likelihood that the effect occurs by linkage/ likelihood that the effect occurs by chance, [c] R^2 is the percentage of individual phenotypic variance explained by each QTL, [d] Additive gene effect of putative QTL: negative sign in additive effect indicates that the allele comes from the tobacco maternal line (Basma seres 31) and positive sign indicates that the allele is from the paternal line ('SPT 406), [e] Dominance gene effect of putative QTL and [f] Degree of dominance

CONCLUSION

The F_2 population produced from the cross between 2 oriental tobacco genotypes, including 'Basma seres 31' × 'SPT 406' had capability to map QTLs linked to other chemical properties of tobacco leaves and smoke-related traits. The resulting map is the first reported linkage map of oriental tobacco and could be improved and extended with the addition of new markers. Among the markers mapped in the 'Basma seres 31' × 'SPT 406' F_2 population, 3 linkage groups shared some common markers with already published reference map in tobacco. QTLs with major and minor effects interfere in the leaf chloride accumulation of oriental tobacco.

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IDENTIFIKACIJA QTL VEZANIH ZA NISKU AKUMULACIJU HLORIDA U ORIJENTALNOM DUVANU

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Izvod

Među različitim hemijskih osobina orijentalnog duvana sadržaj hlorida u listu niži od 1,5 % poboljšava kvalitet sagorevanja. Vršena su ispitivanja QTL odgovornih za akumulaciju hlorida biljaka F_2 generacije dobijene ukrštanjem dva orijentalna genotipa, 'Basma Seres 31' i'SPT 406', obuhvatajući 100 individua. Dobijena je normalna distribucija za akumulaciju hlorida u F_2 populaciji. Konstruisana je mapa ukopčanosti koristeći 23 informativna mikrosatelita I 29 repetitivnih sekvenci u jednostavnim sekvencama, što je pokrilo 570.8 cM genoma duvana. Analiza pojedinačnij markera, mapiranje interval i složeni interval mapiranja su korišćeni za detektovanje mogućih QTLs koji kontrolišu akumulaciju hlorida. Nije utvrđena signifikantna vezanost ISSR markera i akumulacije hlorida. Za SSR marker PT30346 je utvrđena značajna povezanost sa akumulacijom hlorida analizom pojedinačnog markera. Identifikovana su dva QTLs uključujući Chl_{IM} i Chl_{CIM} sa R^2 vrednostima 0.4 i 0.07 korišćenjem IM i CIM.

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