UDC 575.630
https://doi.org/10.2298/GENSR1701077S
Original scientific paper

GENETIC CHARACTERIZATION AND MOLECULAR MAPPING OF NOVEL CHLOROPHYLL DEFICIENCY GENE IN AIR-CURED TOBACCO (*Nicotiana tabacum* L.)

Zeinalabedin SHAHADATI-MOGHADDAM[1,2], Danial KAHRIZI[3], Elham KAZEMI[*4]

[1]Tirtash Research and Education Center, Behshahr, Mazandaran, Iran
[2]Department of Plant Breeding and Biotechnology, Sari Agricultural Sciences and Natural Resources University (SANRU), Mazandaran, Iran
[3]Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran
[4]Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

Shahadati-Moghaddam Z., D. Kahrizi, E. Kazemi (2017): *Genetic characterization and molecular mapping of novel chlorophyll deficiency gene in air-cured tobacco* (*Nicotiana tabacum* L.). - Genetika, Vol 49, No.1, 77 -86.

This study was performed to genetical and morphological investigation of a novel chlorophyll deficiency gene in tobacco leaf. One low chlorophyll content (LCC) variety (Urumieh 2) and high chlorophyll content (HCC) variety (Burley Ree 103) from the Burley type was crossed and the F_2 generation was grown on the field. One hundred plants were selected, contained low and high chlorophyll content. These plants were sampled and DNA was extracted. Sixty RAPD primers were tested on parents, LCC and HCC Bulks based on Bulk Segregant Analysis (BSA). Chi-square test confirmed the monogenic segregation. Regresion analysis showed that there was strong relationship between greenness degree and chlorophyll contents. Four primers (OPE17, OPC09, OPB08 and OPR02) showed polymorphism and after the test on 97 samples from the F_2 generation two markers were selected (OPB08-1050 and OPC09-1900). That showed 15.9 and 10.8 CM distance from chlorophyll locus respectively.

Keywords: BSA, chlorophyll, RAPD, tobacco

INTRODUCTION

Chlorophyll is a photosynthetic pigment and an essential component of the plant photosystem which located in chloroplast. Chloroplasts evolved through the integration of free-living photosynthetic prokaryotic organisms into eukaryotic hosts, following an endosymbiotic

Corresponding author: Elham Kazemi, Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran, Tel: +98 918 3322235, Fax: +98 383 3832 10 83, email: ekazemi2002@yahoo.com

relationship. However, the gene complement of these endosymbionts has since been redistributed between chloroplast and nucleus (MARTIN *et al*., 2002).

Chlorophyll biosynthesis pathway has several intermediates. Glutamic acid is attached to a tRNA molecule and converted to 5-aminolevulinic acid (ALA). Two molecules of ALA are then condensed to form porphobilinogen (PBG), which ultimately form the pyrrole rings in chlorophyll. The next phase is the assembly of protoporphyrin IX from four molecules of PBG. Magnesium is inserted by magnesium chelatase into the center of the porphyrin. Then belong several steps chlorophyll is synthesis involved NADPH, light and enzymes such as chlorophyll synthetase (BEALE, 1999; REINBOTHE and REINBOTHE, 1996). So numerous genes are involved in various aspects of chlorophyll accumulation, but existence of yellow leaf mutants in plants showed that there are key genes to control chlorophyll content.

Leaf color variation is one of the common visible morphological traits in higher plants, which is correlated with the chlorophyll content (RICHARDSON *et al*., 2002), and the change in leaf color is usually caused by nuclear genes involved in chlorophyll metabolism (SUZUKI *et al*., 1997). Studies on inheritance, mapping, and cloning of genes controlling leaf color in yellow-leaf mutants have been conducted in several crops (DE JONG *et al*., 1998; HUDSON *et al*., 1993; NOTHNAGEL and STRAKA, 2003; RUNGE *et al*., 1995; WANG and ZHANG, 2008; WU *et al*., 2007).

ELSHAFEI *et al*. (2013) investigated leaf chlorophyll content, flag leaf senescence and cell membrane stability traits in wheat (*Triticum aestivum* L.) under water-stressed condition and introduced 5 SRAP markers for chlorophyll content locus with 10.4 to 22.7 CM distance in wheat. YUE *et al*. (2009) reported that segregation ratio of F_2 population in sunflower fits in 3:1 and selected a TRAP marker for chlorophyll content locus with 4.2 cM distance. WANG *et al*. (2008) finded three SSR markers linked with single dominant gene (Gc) controls the trait of high chlorophyll content in rice with 0, 0.58 and 1.18 cM distance.

There are yellow –leaf types in air-cured tobacco containing mutant genes such as burley21. Inheritance and genetic control of leaf color in burley types is digenic and recessive (DAVIS and NIELSEN, 1999). However, SALAJI (2000) transferred a novel mutant gene into burley types from oriental type and introduce some varieties called Urumieh. In this report genetical and morphological study of this gene was performed and RAPD markers were tested for linkage map.

MATERIALS AND METHODS

Population Development

Urumieh 2, low chlorophyll content (LCC) cultivar, has been crossed with Burley Ree 103, a high chlorophyll content (HCC) cultivar, in 2012 at the Tirtash Research and Education Center (Iran). The F_1 was grown and selfed to produce F_2 population. F_2 plant and their progeny, F_3 families, were used for genetic analysis in 2014. Two hundred of F_2 plants were randomly tested by Yates-correction Chi-square for distribution of green and yellow leaf (3:1).

Chlorophyll Evaluation

Greenness degree, a parameter related to leaf chlorophyll content (RICHARDSON *et al*., 2002), was measured for each plant with a hand-held chlorophyll meter (SPAD-502, Konica Minolta Sensing Inc.) as a mean in three location on the cutter leaf. At the flowering stage, five plants each from the two parents, five F_1 plants, and five plants from the two groups of the F_2 population (one group with green leaf and the other group with yellow leaf) were sampled for chlorophyll content measurement using a spectrophotometer with the following procedures.

Approximately 20 mg of cutter leaf tissue from each plant was sampled for chlorophyll extraction with 1 mL of 85% acetone in a micro test tube at 60°C overnight. The extract was measured at wavelengths of both 645 and 663 nm with a Lambda EZ201 spectrophotometer (Perkin Elmer). Chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated using MACKINNEY'S (1941) specific absorption coefficients as reported by CHORY *et al*. (1989).

chlorophyll a (mg/g)=12.72(A_{663})-2.59(A_{645})×V/1000×W

chlorophyll b (mg/g)=22.88(A_{645})-4.68(A_{663})×V/1000×W

total chlorophyll (mg/g)= chlorophyll a + chlorophyll b

V= Final volume of chlorophyll extract in 85% acetone (ml)

W= fresh weight of leaf (g)

In addition, the ratios of chlorophyll a to chlorophyll b (chlorophyll a/b) were also calculated. Significant differences among the parents, F_1 hybrids, and the yellow and green F_2 plants were estimated by analysis of variance with the levels of 5% and 1%.

Genomic DNA Manipulation and RAPD primers

Total genomic DNA was isolated from about 100 mg (fresh weight) leaf tissue sampled from individual plants of the parental lines and the F_2 population according to DELLAPORTA *et al*. (1983). DNA concentration was determined with a Lambda EZ201 spectrophotometer (Perkin Elmer) and adjusted to approximately 10 ng/mL for PCR amplification.

Sixty random decamers were screened from Operon kits (Table 1) for selection of RAPD markers linked with chlorophyll content gene.

Table 1. Operon kits random decamers and their sequenses

Primer	Sequenses	Primer	Sequenses	Primer	Sequenses
OPA-06	GGTCCCTGAC	OPI-04	CCGCCTAGTC	OPQ-01	GGGACGATGG
OPA-09	GGGTAACGCC	OPI-07	CAGCGACAAG	OPQ-05	CCGCGTCTTG
OPA-13	CAGCACCCAC	OPJ-04	CCGAACACGG	OPQ-06	GAGCGCCTTG
OPB-07	GGTGACGCAG	OPJ-05	CTCCATGGGG	OPR-02	CACAGCTGCC
OPB-08	GTCCACACGG	OPJ-14	CACCCGGATG	OPR-04	CCCGTAGCAC
OPB-10	CTGCTGGGAC	OPK-04	CCGCCCAAAC	OPR-06	GTCTACGGCA
OPC-02	GTGAGGCGTC	OPK-08	GAACACTGGG	OPS-03	CAGAGGTCCC
OPC-05	GATGACCGCC	OPK-09	CCCTACCGAC	OPT-09	CACCCCTGAG
OPC-09	CTCACCGTCC	OPK-17	CCCAGCTGTG	OPU-13	GGCTGGTTCC
OPC-19	GTTGCCAGCC	OPK-20	GTGTCGCGAG	OPU-19	GTCAGTGCGG
OPD-02	GGACCCAACC	OPL-04	GACTGCACAC	OPV-07	GAAGCCAGCC
OPE-01	CCCAAGGTCC	OPM-03	GGGGGATGAG	OPV-08	GGACGGCGTT
OPE-13	CCCGATTCGG	OPM-12	GGGACGTTGG	OPV-10	GGACCTGCTG
OPE-17	CTACTGCCGT	OPM-16	GTAACCAGCC	OPW-03	GTCCGGAGTG
OPG-02	GGCACTGAGG	OPN-03	GGTACTCCCC	OPX-11	GGAGCCTCAG
OPG-07	GAACCTGCGG	OPN-14	TCGTGCGGGT	OPX-17	GACACGGACC
OPH-04	GGAAGTCGCC	OPO-06	CCACGGGAAG	OPZ-07	CCAGGAGGAC
OPH-13	GACGCCACAC	OPP-13	GGAGTGCCTC	OPZ-09	CACCCCAGTC
OPH-19	CTGACCAGCC	OPP-14	CCAGCCGAAC	OPAE-02	TCGTTCACCC
OPI-01	ACCTGGACAC	OPP-16	CCAAGCTGCC	OPAE-07	GTGTCAGTGG

Bulked Segregant Analysis

The bulked segregant analysis strategy (MICHELMORE *et al*., 1991) was used for establishment of the linkage relationship between the phenotype and the polymorphic RAPD markers. Two bulked DNAs, LCC and HCC bulks (equal amounts of DNA from eight plants in each bulk), were established from F_2 (non segregated F_3). PCR was performed on parental DNAs, LCC and HCC bulks with RAPD primers and were tested in electrophoresis to screen polymorphic RAPD markers. Polymorphic markers between the two bulks were then confirmed by surveying in two groups of F_2 individuals. Selected markers were run on 97 F_2 plants for map construction. Genetic distances between linked markers (LOD>3.0) were calculated with the computer program Mapmaker/EXP3.0 (LANDER *et al*., 1987) using KOSAMBI'S (1944) mapping function.

RESULTS

Heredity of leaf color

The two parental lines displayed an obvious difference in leaf color, which can be easily assessed in field (Figure 1). All F_1 hybrid plants expressed the green phenotype which indicated that yellow leaf color is recessive to green. There was two separate groups in F_2 population as yellow and green leafed. In 200 plants of the F_2 population, 158 were green leafed and 42 were yellow leafed. Yates-correction Chi-square test (YATES, 1934) confirmed the monogenic segregation ratio of 3:1 in significance level 5% (χ^2 =1.82).

Figure 1. Apearence of yellow leafed plants in F_2 population

Relationship of leaf color and chlorophyll contents

Chlorophyll contents analysis revealed a significant difference in the contents of chlorophyll a, chlorophyll b, and total chlorophyll among the two parental lines and their progenies (Table 2). The green-leafed parent, F_1 and green-leafed F_2 group had much more greenness degree, chlorophyll a, chlorophyll b and total chlorophyll than the yellow-leafed parent and yellow-leafed F_2 group. However F_1 were in intermediate green and yellow leafed plants in chlorophyll b. There was no significant difference in chlorophyll a/b. The contents of

chlorophyll a, chlorophyll b and total chlorophyll in the yellow-leafed F_2 group were reduced by 62.5%, 60.0%, and 61.9%, respectively, in comparison with those in the green-leafed progenies.

Regresion analysis showed that there was strong relationship between greenness degree and chlorophyll contents (R^2> 0.97) but there was not any correlation between greenness degree and chlorophyll a/b (Figure 2). Therefore we can estimate chlorophyll a and chlorophyll b on greenness degree at 97% certainty and total chlorophyll at 98% certainty.

Table 2. Chlorophyll contents in the parents, F_1hybrid, the yellow-leafed and green-leafed plants in the F_2 population

Genotype	Greenness degree	Chl a† (mg/g)	Chl b (mg/g)	Chl t (mg/g)	Chl a/b
GLP*	61.7±3.22[a]	2.02±0.19 [a]	0.72±0.06 [a]	2.74±0.24 [a]	2.80±0.13 [a]
YLP	25.7±1.75[b]	0.85±0.08 [b]	0.30±0.02 [c]	1.15±0.09 [b]	2.83±0.18 [a]
F_1	60.2±2.44 [a]	1.95±0.11 [a]	0.66±0.03 [b]	2.61±0.12 [a]	2.93±0.22 [a]
GLF_2	62.3±2.20 [a]	2.09±0.21 [a]	0.71±0.04 [a]	2.81±0.25 [a]	2.92±0.15 [a]
YLF_2	24.8±2.11 [b]	0.78±0.07 [b]	0.28±0.02 [c]	1.07±0.09 [b]	2.73±0.11 [a]
CV	5.1	9.3	6.9	8.2	7.0

Values with different letters are significantly different at P<0.001 according to Duncan's test.

* GLP= green-leafed parent, YLP= yellow-leafed parent, GLF_2= green-leafed F_2 group, YLF_2= yellow-leafed F_2 group

† Chl a= chlorophyll a, Chl b= chlorophyll b, Chl t= Total chlorophyll

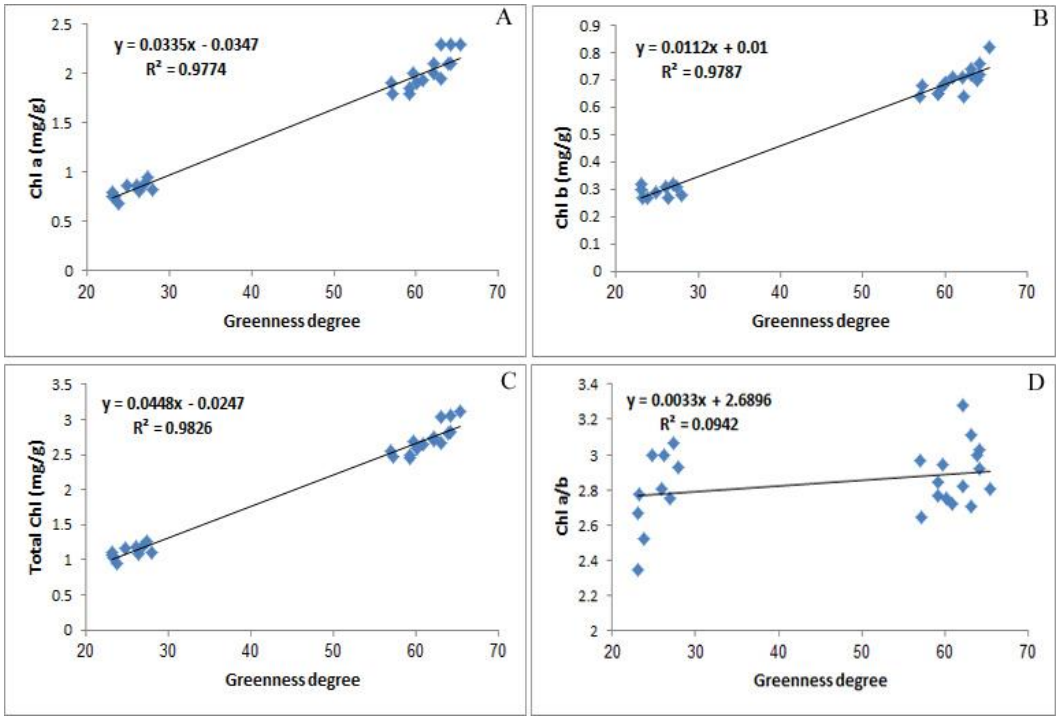

Figure 2. Regression plots of greenness degree with chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and chlorophyll a/b (D) in 25 plants (parents, F_1, two groups F_2)

Primer selection based on BSA

Parental DNAs, LCC and HCC bulks were tested with 60 RAPD primers and 4 primers, showed polymorphism, (OPE17, OPC09, OPB08 and OPR02) were selected. Individual F_2 groups test denied OPE17. Finally three primers OPC09, OPB08 and OPR02 were selected and were investigated on 97 genotypes randomly chosen F_2 plants for map construction. (Figure 3). Three bands 1050-bp, 1900-bp and 750-bp were linked to yellow leafed in repulsion phase from OPB08, OPC09 and OPR02 respectively. These markers appear in green leafed plants.

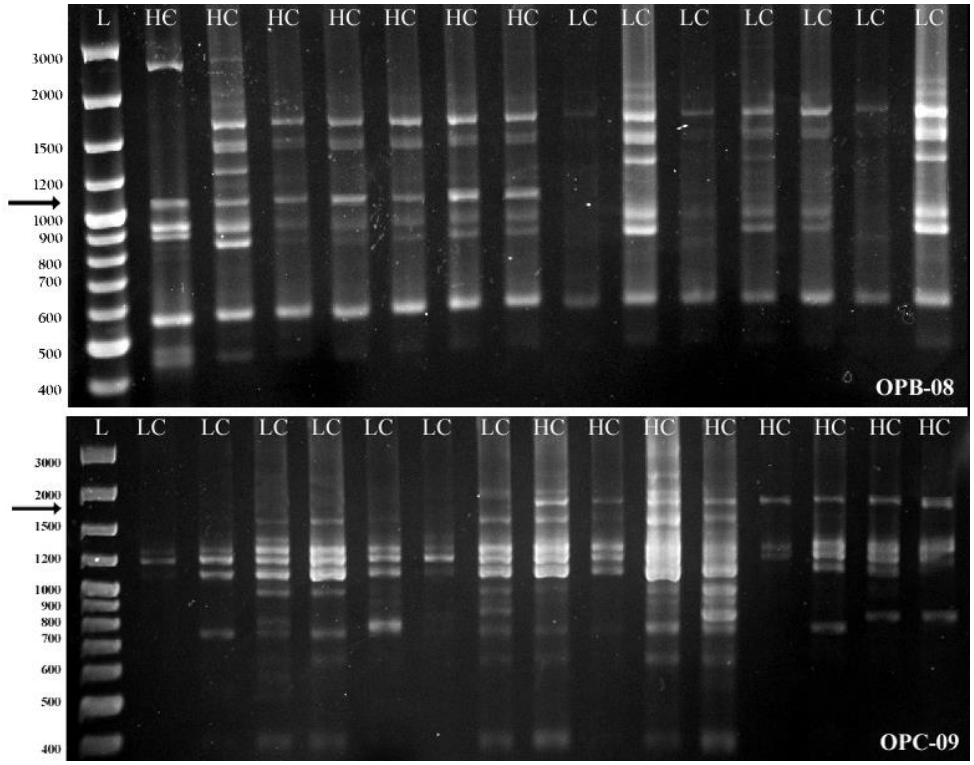

Figure 3. PCR products OPB08 and OPC09 in F_2 individuals. Apearence OPB08-1050 and OPC09-1900 markers in high chlorophyll (HC) and low chlorophyll (LC) content plants

Linkage analysis

Investigation of map construction based on KOSAMBI'S (1944) mapping function showed that markers OPB08-1050, OPC09-1900 and OPR02-750 were located in 15.9, 10.8 and 19.3 cM genetic distance with chlorophyll deficiency gene (Figure 4).

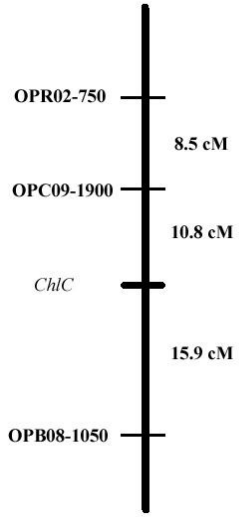

Figure 4. Genetic map of the chlorophyll content gene (*ChlC*) and its linked RAPD markers. Numbers are genetic distances between loci in cM.

DISCUSSION

High chlorophyll content in tobacco leaf is one of the quality reduction factors in cured leaf. Because all leaf pigments should be disintegrated in curing and high chlorophyll content creates problem in this process (DAVIS and NIELSEN, 1999; TSO, 1991). So one of the breeding strategy is decreasing of leaf chlorophyll content in order to precocity and improve the quality of cured leaf. Detection of chlorophyll deficiency genes and their utilization well be so useful.

There are many reports for identifying and mapping chlorophyll content genes in different crops (EFRATI *et al*., 2005; JIANG *et al*., 2012; LIU *et al*., 2007; WANG and ZHANG, 2008; YIN *et al*., 2010; YUE *et al*., 2009). Though reports show that the genetic control of leaf color and chlorophyll content in the burley type of tobacco is digenic (DAVIS and NIELSEN, 1999). However, our study indicated that the gene which transferred from the oriental type to burley is a new recessive gene controls the chlorophyll content. To map development of this gene it is necessary to production of NILs and using other markers such as SSR for fine detecting and chromosome finding.

Regression slop in chlorophyll a (0.033) was more than chlorophyll b (0.011) which showed that there was more diversity in chlorophyll a than chlorophyll b. But chlorophyll deficiency gene is not more effective in chlorophyll a than chlorophyll b. Because regression between greenness degree and chlorophyll a/b was not significant. Therefor content of chlorophyll a and b was changed in same ratio approximately (Figure 2).

Since both the RAPD markers and green leafed trait are dominant, therefore repulsion phase between markers and chlorophyll deficiency gene can be useful. Because we can be assured that there is recessive gene and low chlorophyll content in the absence of the bands (there is not dominant gene).

Abbreviations

LCC: low chlorophyll content; HCC: high chlorophyll content; *ChlC* : Chlorophyll content gene
BSA: Bulk Segregant Analysis; RAPD: Random Amplified Polymorphic DNA;GLP: green-leafed parent;YLP: yellow-leafed parent;Chl a: chlorophyll a;Chl b: chlorophyll b

ACKNOWLEDGEMENTS

The authors are thankful to Zagros Bioidea Co., Razi University Incubator and Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) for all supports.

Received April 22nd, 2016
Accepted September 18th, 2016

REFERENCES

BEALE, S.I. (1999): Enzymes of Chlorophyll Biosynthes. Photosynth. Res., *60*: 43-73.

CHORY, J., C.A. PETO, M. ASHBAUGH, R. SAGANICH, L. PRATT, F. AUSUBEL (1989): Different Roles for Phytochrome in Etiolated and Green Plants Deduced from Characterization of Arabidopsis thaliana Mutants. Plant Cell, *1(9)*: 867-880.

DAVIS, D.L. and M.T. NIELSEN (1999): Tobacco: Production, Chemistry and Technology: Wiley-Blackwell.

DE JONG, H., L.M. KAWCHUK, V.J. BURNS (1998): Inheritance and mapping of a light green mutant in cultivated diploid potatoes. Euphytica, *103*: 83-88.

DELLAPORTA, S.L., J. WOOD, J.B. TICKS (1983): A plant molecular DNA minipreparation version 2. Plant Molecular Biology Reporter, *1*: 19-21.

EFRATI, A., Y. EYAL, I. PARAN (2005): Molecular mapping of the chlorophyll retainer (cl) mutation in pepper (Capsicum spp.) and screening for candidate genes using tomato ESTs homologous to structural genes of the chlorophyll catabolism pathway. Genome, *48(2)*: 347-351. doi: 10.1139/g04-119

ELSHAFEI, A. A., M. SALEH, A.A. AL-DOSS, K.A. MOUSTAFA, F.H. AL-QURAINY, , M.N.BARAKAT (2013): Identification of new SRAP markers linked to leaf chlorophyll content, flag leaf senescence and cell membrane stability traits in wheat under water-stressed condition. Australian Journal of Crop Science, *7(6)*: 887-893.

HUDSON, A., R. CARPENTER, S. DOYLE, E.S. COEN (1993): Olive: a key gene required for chlorophyll biosynthesis in Antirrhinum majus. EMBO J, *12(10)*: 3711-3719.

JIANG, S., X. ZHANG, F. ZHANG, Z. XU, W. CHEN, Y. LI (2012): Identification and fine mapping of qCTH4, a quantitative trait loci controlling the chlorophyll content from tillering to heading in rice (*Oryza sativa* L.). Journal of Heredity, *103(5)*: 720-726.

KOSAMBI, D.D. (1944): The estimation of map distances from recombination values. Ann. Eugenet., *12*: 172-175.

LANDER, E.S., P. GREEN, J. ABRAHAMSON, A. BARLOW, M.J. DALY, S.E. LINCOLN, L.A. NEWBERG (1987): MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, *1(2)*: 174-181.

LIU, W., Y. FU, G. HU, H. SI, L. ZHU, C. WU, Z. SUN (2007): Identification and fine mapping of a thermo-sensitive chlorophyll deficient mutant in rice (*Oryza sativa* L.). Planta, *226(3)*: 785-795.

MACKINNEY, G. (1941): Absorption of light by chlorophyll solutions. Journal of Biological Chemistry, *140*: 315-322.

MARTIN, W., T. RUJAN, E. RICHLY, A. HANSEN, S. CORNELSEN, T. LINS, . . . D. PENNY (2002): Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci USA, *99(19)*: 12246-12251.

NOTHNAGEL, T. and P. STRAKA (2003): Inheritance and mapping of a yellow leaf mutant of carrot (*Daucus carota*). Plant Breeding, *122*: 339-342.

REINBOTHE, S. and C. REINBOTHE (1996): The regulation of enzymes involved in chlorophyll biosynthesis. Eur. J. Biochem., *237(2)*: 323-343.

RICHARDSON, A.D., S.P. DUIGAN, G.P. BERLYN (2002): An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol., *153*: 185-194.

RUNGE, S., B. VAN CLEVE, N. LEBEDEV, G. ARMSTRONG, K. APEL (1995): Isolation and classification of chlorophyll-deficient xantha mutants of Arabidopsis thaliana. Planta, *197(3)*: 490-500.

SALAJI, M.S. (2000): Precocious Burley. Paper presented at the Bull. Spec. CORESTA, Lisbon.

SUZUKI, J.Y., BOLLIVAR, D.W., C.E. BAUER (1997): Genetic analysis of chlorophyll biosynthesis. *Annu Rev Genet, 31*, 61-89.

TSO, T.C. (1991): Production, Physiology and Biochemistry of Tobacco Plant: Ideals Inc.

WANG, A.Y. and C.Q. ZHANG (2008): QTL mapping for chlorophyll content in maize. Yi Chuan, *30(8)***:** 1083-1091.

WANG, F., G. WANG, X. LI, J. HUANG, J. ZHENG (2008): Heredity, physiology and mapping of a chlorophyll content gene of rice (*Oryza sativa* L.). J. Plant Physiol., *165(3)*: 324-330.

WU, Z., X. ZHANG, B. HE, L. DIAO, S. SHENG, J. WANG, J. WAN (2007): A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. Plant Physiol, *145(1)*: 29-40.

YATES, F. (1934): Contingency table involving small numbers and the $\chi 2$ test. Journal of the Royal Statistical Society, *1(2)*: 217-235.

YIN, Z., F. MENG, H. SONG, X.HE, X. XU, D. YU (2010): Mapping quantitative trait loci associated with chlorophyll a fluorescence parameters in soybean (*Glycine max* L. Merr.). Planta, *231(4)*: 875-885.

YUE, B., X. CAI, B. VICK, J. HU (2009): Genetic characterization and molecular mapping of a chlorophyll deficiency gene in sunflower (*Helianthus annuus*). J. Plant Physiol., *166(6)*: 644-651.

GENETIČKA KARAKTERIZACIJA I MOLEKULARNO MAPIRANJE NOVOG GENA ZA DEFICITARNOST HLOROFILA KOD DUVANA (*Nicotiana tabacum* L.)

Zeinalabedin SHAHADATI-MOGHADDAM[1, 2], Danial KAHRIZI[3,4], Elham KAZEMI[4]

[1]Tirtash istraživački i obrazovni centar, Behshahr, Mazandaran, Iran
[2]Departman za oplemenjivanje biljaka i biotehnologiju, Sari Univerzitet za poljoprivredne nauke i prirodne resurse (SANRU), Mazandaran, Iran
[3]Departman za poljoprivredu i oplemenjivanje biljaka, Poljoprivredni fakultet, Razi Univerzitet, Kermanshah, Iran
[4]Medicinsko biološki istraživački centar, Kermanshah Univerzitet medicinskih nauka, Kermanshah, Iran

Izvod

U radu su obavljena genetička i morfološka proučavanja novog gena sa deficitom hlorofila u listu duvana. Jedan varijetet (Urumieh 2) sa niskim sadržajem hlorofila (LCC) i jedan sa visokim sadržajem hlorofila (HCC), varijetet (Burley Ree 103), ukršteni su i F_2 generacija je gajena u polju. Odabrano je 100 biljaka kod kojih je urađena ekstrakcija hlorofila. 60 RAPD prajmera je testirano na roditeljima, LCC i HCC, analizom grupnih uzoraka (BSA). Testom χ^2 je potvrđena monogenska segregacija. Regresiona analiza je pokazala veliku međuzavisnost nijanse zelene boje i sadržaja hlorofila. Četiri prajmera (OPE17, OPC09, OPB08 i OPR02) su pokazala polimorfizam i posle testa na 97 uzoraka iz F_2 generacije odabrana su dva markera (OPB08-1050 i OPC09-1900). Kod njih je utvrđena udaljenost od 15.9 odnosno 10.8 CM kod hlorofilskih lokusa.

Primljeno 22. IV. 2016.
Odobreno 18. IX. 2016.