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LINKAGE MAPPING OF CAROTENOID CLEAVAGE DIOXYGENASE-4 FAMILY IN LENTIL GENOME

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ABSTRACT

Plant carotenoid cleavage dioxygenases (CCDs) are a family of enzymes that catalyze the carotenoids oxidative cleavage and apocarotenoids and also play a crucial role in plant development and growth. CDD4 is a member of CDDs. It has two isoforms as CCD4a and CCD4b and they encode enzymes to catalyze the cleavage of carotenoids forming pigment compounds and aroma. In this study, CDD4 genes were mapped for the first time in the linkage group of lentil. CsCCD4af was located at 102.3 cM on linkage group 3 (LG3). CCD4-P-r1-1 and CCD4-P-r1-2 were located at 75.5 cM and 82.9 cM on LG3, respectively. CCD4-P-r1-3 was located at 151.6 cM on LG5. CsCCD4a/b-r was amplified but could not mapped due to its monomorphic band profile between parents. Location of these genes on the linkage map of lentil will help breeders improve strategies in order to generating new cultivars with higher carotenoid concentration.

Keywords: Carotenoid, Carotenoid Cleavage Dioxygenase-4, Lentil, Linkage map

INTRODUCTION

Carotenoids known to be a various group and consist of more than 700 carotenoids, which act as antioxidants, photoprotectants and photosynthetic accessory pigments were synthesized by plants, bacteria and algae (Ruiz-Sola and Rodriguez-Concepcion, 2012). On the other hand, mammals cannot synthesize these carotenoids and they provide these by dietary intake. In mammals, processing of carotenoids with provitamin A activity ensures the vitamin A essential in order to differentiation of normal tissue as well as immune, organ, and visual development (Sommer and Vyas, 2012). Although, plants are the primary dietary sources of carotenoids, levels of vitamin A carotenoid in plants are inadequate to meet minimum nutritional requirements. For this reason, deficiency of vitamin A remains common in many countries (Fitzpatrick et al., 2012). In order to solve this problem, improving the crops vitamin A ingredient through molecular breeding is a critical strategy.

Plant carotenoid cleavage dioxygenases (CCDs) play a crucial role in plant development and growth (Snowden et al., 2005). They are a family of enzymes, which catalyze the carotenoids oxidative cleavage and apocarotenoids such as retinol (vitamin A), abscisic acid (ABA), strigolactones (SL) and other volatile compounds that provide to the aroma of flowers and fruits and color for attracting pollinators (Rodrigo et al., 2006; Ohmiya, 2009). Also, apocarotenoids play a significant role in various agronomic traits such as responses of biotic and abiotic stress (Vallabhaneni et al., 2010) and act as hormones (Giuliano et al., 2003).

The first group of gene defined as encoding a CCD was the *Vp*14 maize gene, which plays a significant role in ABA formation (Ahrazem et al., 2010). Apocarotenoids are widely common in nature and especially exist in ABA metabolism in higher plants. It is derived from oxidative cleavage of the 9-*cis* epoxy carotenoids 11, 12 double bond (NCEDs) (violaxanthin or/and neoxanthin) (Tan et al., 2003) and plays a significant role in responses to environmental stresses related to loss of water and in seed development (Nambara and Marion-Poll, 2005). Thus, developmental and environmental signals may manage in the ABA biosynthesis regulation in plant tissues (Rodrigo et al., 2006).

The second group of CCDs includes CCD1, CCD4, CCD7 and CCD8 (Ahrazem et al., 2010). Next to NCEDs, CCD1 is the best-studied enzyme due to it contributes to synthesis of several important volatile compounds that contribute to aroma compounds and flavor (Simkin et al., 2004b; Auldridge et al., 2006; Mendes-Pinto, 2009). Another member of CCDs is CCD4 that encodes enzymes to catalyze the cleavage of carotenoids forming pigment compounds and aroma (Ohmiya et al., 2006; Huang et al., 2009). Plants produce two CCD4 isoforms as CCD4a and CCD4b that have different chemical and biological functions in plants (Ohmiya et al., 2006; Huang et al.,

2009). The member of the CCD4 subfamily was first characterized in chrysantemum (Ohmiya et al., 2006) and the enzymatic activity was identified in saffron (Rubio et al., 2008), apple, chrysantemum, *rose*, and Arabidopsis (Huang et al., 2009). CCD1 contribute towards volatile production, whereas CCD4 control carotenoid breakdown because of their different subcellular degradation (Brandi et al., 2011). Last member of CCDs are CCD7 and CCD8 and they encode enzymes to catalyze the cleavage of carotenoids to form SL, the hormone involved in the inhibition of shoots branching (Domagalska and Leyser, 2011; Waters et al., 2012).

Lentil is the third most consumed pulse crop legume after pea and chickpea around the world and due to its high protein, carbohydrates and micronutrients content, it becomes a source of staple daily food for many humans (Wang and Daun, 2006). Carotenoids are also crucial nutrients for human health but human cannot synthesize carotenoids and they must obtain these through diet (EL-Qudah, 2009). Approximately, fifty distinct carotenoids can be metabolized into vitamin A (Krinsky and Johnson, 2005). On the other hand, about 250 million children are vitamin A-deficient around the world. Out of them, approximately 400,000 vitamin A-deficient children become blind annually and half of them dying within twelve months of losing their eyesight (Muller and Krawinkel, 2005). Because of these nutritional apprehensions of vitamin A deficiency in humans, new cultivar development, which has alleviated carotenoid concentration, has become a primary purpose of breeding strategies in many crop species such as, soybean (Zimmermann and Hurrell, 2002), rice (Paine et al., 2005), wheat (Hidalgo et al., 2006; Lachman et al., 2013) and maize (Kimura et al., 2007). Unfortunately, little information exists about carotenoids of lentils.

CCDs contain several highly conserved motifs. Conservation of exon-intron structure in orthologous genes clades, promote the utilize of gene properties as references for phylogenetic derivation (Rokas and Holland, 2000) so that the knowledge of the genomic structure is very essential for the evolutionary relationships discovery and for identify gene families (Ahrazem et al., 2010). On the other hand, given the carotenoids dietary importance and vitamin A deficiency prevalence, a better knowledge of the plant carotenoids, is required (Kim et al., 2012; Chandler et al., 2013; Gonzalez-Jorge et al., 2013). CCDs have been identified in various plant species such as Arabidopsis (Tan et al., 2003), tomato (Simkin et al., 2004a), petunia (Snowden et al., 2005), melon (Ibdah et al., 2006), orange (Rodrigo et al., 2006), carrot (Just et al., 2009), saffron (Ahrazem et al., 2010), maize (Vallabhaneni et al., 2010), rice (Vallabhaneni et al., 2010), sorghum (Vallabhaneni et al., 2010), chrysanthemum (Yoshioka et al., 2012) and grape (Lashbrooke et al., 2013). Identification of such loci will be key in order to ensuring synergistic or alternative means for changing the CCDs content of specific plant tissues. But to date CCDs genes have not been identified and mapped in the lentil genome. The aim of current study was to identify and map CCD4 genes in lentil recombinant inbred line (RIL) population named as LR39.

MATERIALS and METHODS

Plant material and DNA extraction

The cross of "PI 320937" (P1) × "Eston" (P2) was utilized in order to generated a population of 96 lentil RILs named as LR-39. This population was developed by advancing F_1 plants from the simple cross, and the RILs developed by a single seed descent from the F_2 to the F_7 generation at the University of Saskatchewan, Canada since 2001. These RILs were kindly provided by Prof Albert Vandenberg University of Saskatchewan, Canada. The RIL seeds were then amplified at the experimental station of the Department of Field Crops at Ege University, Izmir, Turkey during 2012-2013 and 2013-2014 growing seasons.

Young leaves from individuals of LR-39 RIL population and both parents of this population were harvested and placed in an aluminum foil, and finally labeled with their RIL numbers. Then, the foil was placed in liquid nitrogen. The frozen leaves were then stored in a deep freezer (-86 °C). A Qiagen (Valencia, CA, USA) DNA Isolation Kit was used to extract genomic DNA from 96 RIL individuals and the parents. The DNA purity was assessed on a 0.8% agarose gel, and a Qubit® 2.0 fluorometer (Life Technologies, US) was used to quantify the purified DNA.

DArT analysis

Protocol of Ates et al. (2016) was followed for DArT analysis.

PCR analysis of CCD4 primers

For PCR analysis, the protocol from Gedik et al. (2017) was used and nonoverlapping gene specific primers (Ahrazem et al., 2010) were surveyed for polymorphisms between parents of LR39 population (Table 1). Agarose gel electrophoresis (2%) was used with 1 x TBE buffer for 2 h in order to analyze PCR products then gel visualized via ethidium bromide staining by a G-BOX gel documentation system (Syngene, USA). Band sizes were calculated by comparison with a DNA ladder (1000 bp, Thermo Sci. Co.).

Table 1. Names of CDD4 primers, sequences, and references.

Primer name	Sequence	Orientation	Annealing	References
CsCCD4af	5′-CAATCTCAAGTATTAGCATTC-3′	Sense	46	(Ahrazem et al., 2010)
CsCCD4a/b-r	5′- CTGCTGTGACAGCAGCTCAGC-3′	Antisense	47	
CCD4-P-r1	5′-CTTGTTGATACTGATACTCTTCT-3′	Antisense	47	

Scoring of DNA bands from each CCD4 primers were recorded manually and exclusively the strong and clear bands were scored. The presence of CCD4 primers band at a certain locus was scored as "1" and absence of a band was scored as "0" in order to build binary matrices.

Linkage mapping

The genetic linkage map of LR39 RIL population was constructed with MultiPoint software (Mester et al., 2003) utilizing 96 individuals, genotyped with SNPs based on DArT and CCD4 markers. Linkage analysis was carried out utilizing maximum likelihood mapping algorithm with RIL population type, utilizing function of Kosambi, RIL selfing, a recombination fraction of 0.35 and the odds (LOD) logarithm of 3, as parameters of linkage mapping.

RESULTS and DISCUSSION

Genomic DNA was isolated from 96 RIL individuals and three CCD4 primers (CCD4-P-r1, CsCCD4af, and CsCCD4a/b-r) were surveyed for polymorphisms between the parents of LR39 population in current study (Table 1). Results of PCR analysis were indicated that all primers were amplified. While CCD4-P-r1 and CsCCD4af were produced reproducible polymorphic DNA bands between the two parents, CsCCD4a/b-r was monomorphic. The number of polymorphic DNA band for CCD4-P-r1 was three and detected at 100 bp, 150 bp and 160 bp. On the other hand, CsCCD4af was produced only one reproducible polymorphic DNA bands between the two parents at 550 bp. Finally, a total of four DNA bands were scored and number of each individual bands were equally distributed according to the parents. These results indicated that, lentil includes CDD4 genes, which have play a significant nutritional role as vitamin A precursors and high antioxidant features (Thomas, 2016). Support to our results, presence of carotenoids in lentil was detected in previous studies (EL-Qudah, 2014; Zhang et al., 2014; Thomas, 2016; Lee et al., 2017).

Lentil linkage map was constructed using 1,940 SNPs based on DArT and 2 CDD4 primers. The genomic location of CsCCD4af and CCD4-P-r1 were mapped in current study (Figure 1). On the other hand, CsCCD4a/b-r was amplified but could not mapped due to its monomorphic band profile between parents. This situation showed that these genes actually localized in lentil genome but could not mapped in current study. The two CDD4 (CsCCD4af and CCD4-P-r1) detected four genetic loci on three linkage groups (LGs) (Figure 1). Out of these, CsCCD4af was located at 102.3 cM on LG3 (Figure 1). On the other hand, while CCD4-P-r1-1 and CCD4-P-r1-2 were located at 75.5 cM and 82.9 cM on LG3, respectively, CCD4-P-r1-3 was located at 151.6 cM on LG5 (Figure 1). These genes were mapped by linkage mapping approaches in lentil genome for the first time in current study. In previous study, 143 lentil genotypes were utilized in order to detect SNP markers associated with carotenoid concentration components by association mapping approaches (Thomas, 2016). They reported that 168 SNPs were significantly related with carotenoid concentration components of lentil utilizing the generalized linear model (Thomas, 2016). On the other hand, in previous studies, CDD4 genes also mapped on peach genome utilizing *Y* locus mapping methods (Adami et al., 2013), genome wide association mapping (GWAS) approaches (Gonzalez-Jorge et al., 2013) and fine mapping of the *Y* locus approaches (Ma et al., 2014). CCD4 gene was co-mapped with the *Y* locus and it was localized between markers pchgms3 and PacA18 in the map of peach (Adami et al., 2013) and SSRy was associated with CDD4 gene and co-segregated with the *Y* locus of peach genome (Ma et al., 2014). In other peach studies, GWAS association with β-carotene was detected on chromosome 4 in the map of peach and associated marker was identified as SNP147077 that within the CDD4 coding region (Gonzalez-Jorge et al., 2013).

SNP3635501 and CsCCD4af were located together at the same position of linkage map (on LG3 at 102.3 cM) in current study (Figure 1). Similarly, SNP363448, SNP3634337 and CCD4-P-r1 were located at 75.5 cM and, SNP3635407 and CCD4-P-r1-2 were located at 82.9 cM on LG4 (Figure 1). Also, SNP3634766 and CCD4-P-r1-3 were both located on LG5 at 151.6 cM (Figure 1). These SNP markers are thought to be markers derived from the same region of CDD4 markers in current study.

Two isoforms of CCD4 genes (CsCCD4af and CCD4-P-r1) were mapped at different genome position in the lentil linkage map in current study (Figure 1). Plants produce two CCD4 isoforms as CCD4a and CCD4b that have distinct chemical and biological functions (Ohmiya et al., 2006; Huang et al., 2009) and also have different genome position in plants (Rubio et al., 2008; Ahrazem et al., 2010). Support to our results, Huang et al. (2009) reported that CCD4a and CCD4b were presented different expression patterns in citrus. Later, these findings were confirmed by Pan et al. (2012) demonstrating that isoforms of CCD4 genes have distinct substrates and consequently distinct biological functions. Appreciating the functions of CCD4 genes isoforms, explaining their specificities of substrate and examining their patterns of expression will shed light on their roles in lentil.

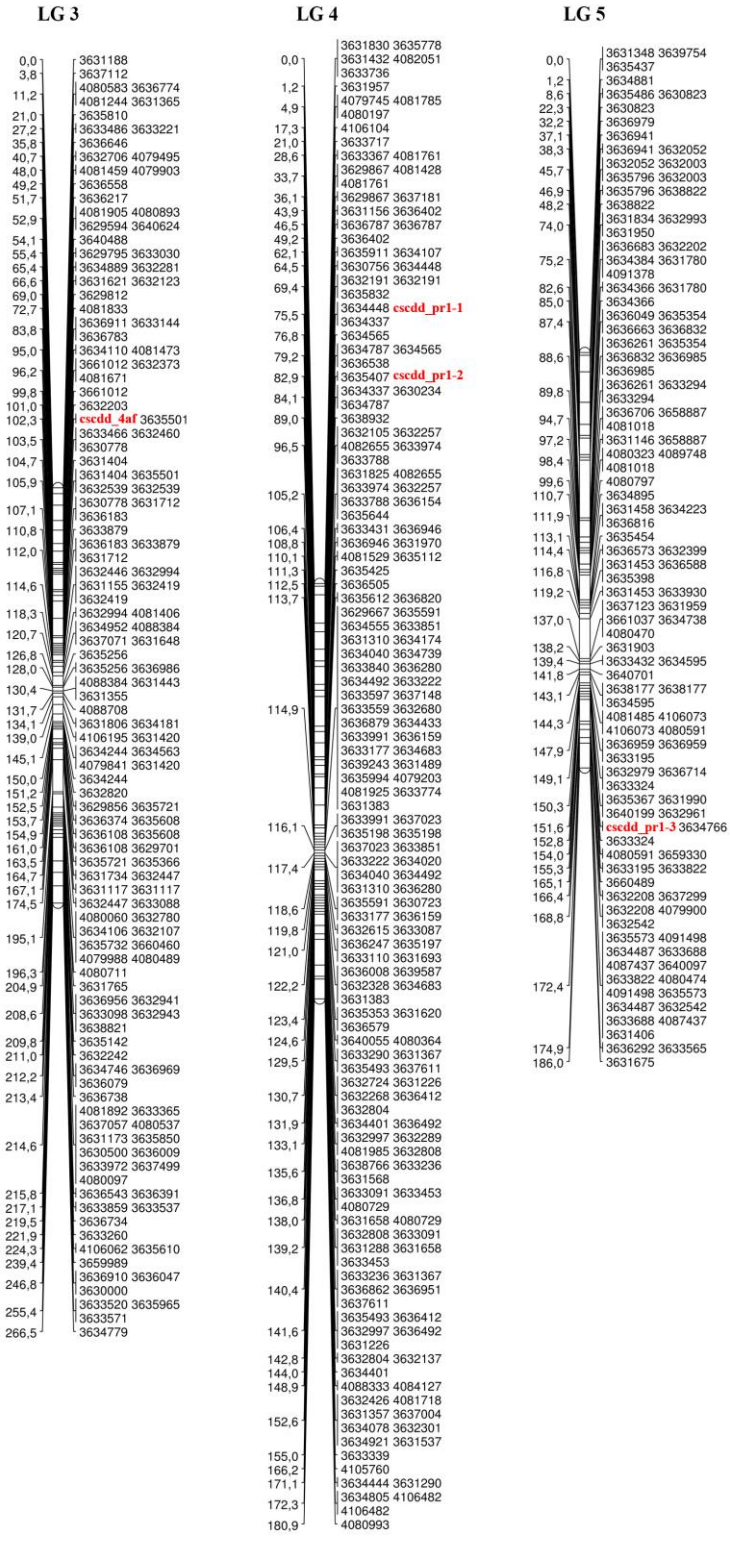

Figure 1. LG 3, 4, and 5 of lentil linkage map derived from a cross between "PI 320937" (P1) × "Eston" (P2). Left bar of the LGs is cM and the right bar is marker names. CCD4 markers were written with red color.

CCD4 genes contain highly conserved motifs within the distinct location of genes (Rokas and Holland, 2000) and these genes were also detected existence in other plants such as *Arabidopsis thaliana* (Vidi et al., 2006; Ytterberg et al., 2006; Gonzalez-Jorge et al., 2013), chrysanthemum (Ohmiya et al., 2006; Huang et al., 2009; Yoshioka et al., 2012), saffron (Rubio et al., 2008; Ahrazem et al., 2010), apple (Huang et al., 2009), rose (Huang et al., 2009), potato (Campbell et al., 2010), rice (Ahrazem et al., 2010), peach (Brandi et al., 2011; Adami et al., 2013; Ma et al., 2014), citrus (Pan et al., 2012), grape (Dockrall, 2012) and Brassica species (Zhang et al.,

2015). In addition, Ahrazem et al. (2010) reported that saffron and rice CCD4 genes promotors grouped together and several conserved motifs were identified, even though changes in spacing were observed. Presence of the same CDD4 gene region in these different plants as well as lentil indicated that this region is well conserved during evolution (Ahrazem et al., 2010).

CONCLUSION

Lentil contains CDD4 genes that have antioxidant features and take a significant nutritional role as vitamin A precursor. Increasing carotenoids concentration in lentils has potential as component of a biofortification program. Location of CDD4 genes that detected in current study on the linkage map of lentil will help breeders improve strategies in order to develop new cultivars with higher carotenoid content.

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