Introduction

Genetics of male sterility expression in *Daucus carota* (carrot) is known to be complicated (Kozik et al. 2012). In this species, two different types of cytoplasmic male sterility (CMS) are known. Formation of brownish unfertile anthers, commonly known as the brown-anther sterility phenotype, is one such trait being utilized in F1 hybrid breeding programs. Conversion of stamens into petal-like or carpel-like structures, known as the petaloid or carpeloid, is another type of male-sterility known in this species (Linke et al. 2003). This phenotype is expressed as a result of nuclear-cytoplasmic genotype interaction. The cytoplasmic genotypes of the two-types are known to be distinct. Different genetic models with the involvement of at least two genetic loci in the expression of petaloid in carrots have been proposed by past studies (Banga et al. 1964, Kozik et al. 2012). Furthermore, expression of the CMS phenotype is reported to be influenced by environmental backgrounds such as high temperatures (Wolyn and Chahal 1998). Therefore, molecular markers that enable genetic diagnosis of the male-sterility phenotype are invaluable in carrot breeding.

Recently, gene identification is facilitated by high-throughput sequencing technology. Notably, identification of the quantitative trait loci using bulked-segregant analysis, or QTLseq, is becoming a powerful strategy for the discovery of causal genetic loci of a phenotype (Takagi et al. 2013). Here we employed the QTLseq approach to identify the causal gene locus of the petaloid phenotype in carrots.

Materials and Methods

Plant materials

We used a segregated population obtained from a cross between a petaloid strain (YSN6MS) and a fertile individual spontaneously found in the green house of Yamato Noen Co. Ltd. (YSN6spF). Carrots were grown in a field at Tenri, Japan during 2015. Leaves from fifteen individuals from each of the fertile (F) and male-sterile (MS) populations were sampled.

Sequence analysis

DNA from the bulked leaves was extracted by using the DNeasy Plant mini kit (Qiagen) following the manufacturer’s protocol. The library preparation and sequence run were conducted by Macrogen (Seoul, Korea). The TruSeq DNA PCR-Free kit (Illumina) was used to prepare libraries. Paired end reads were prepared and a sequence run performed using the HiSeq X Ten platform. Raw sequence data files were deposited to the National Center for Biotechnology...
PCR Restriction Fragment Length Polymorphism (PCR-RFLP) analysis

A pair of PCR primers (5′-GGGATATTACAGGCTCTCAGCAATG-3′/5′-TTAGGCTGCAAGCGGAAGGCAAAG-3′) were designed for the PCR amplification of the region surrounding the DraI site on position 22,416,901 of chromosome 4. Tks Gflex DNA Polymerase (TaKaRa) with the following PCR routine: 94°C 1 min; (98°C 10 sec, 55°C 15 sec, 68°C 30 sec) × 35 cycles; 68°C 5 min, was used to amplify the PCR fragment. Five μl of the PCR product was digested by 0.2 μl of DraI (15 U/μl, TaKaRa) with incubation at 37°C for two hours. PCR products were electrophoresed on a 1% agarose gel.

Results and Discussion

We obtained ca. 417 million and ca. 536 million reads from the F and MS bulks via the deep-sequencing runs, respectively. Mean coverage depths of the carrot genome were 132.90 and 169.05 for the F and MS bulks, respectively. Detailed statistics for the sequencing results are presented in Table 1. The total number of SNPs called were 6,836,994 for the F bulk, and 6,629,607 for the MS bulk (Table 1).

From the QTLseq analysis, we detected the strongest contributing peak on the long arm of chromosome 4, at approximately the 17.50 to 27.75 mega-base-pair (mbp) position on the pseudomolecule (Fig. 1B, 1C). The window in the center of the peak harbored SNPs between the 22.25 and 22.75 mbp position on the pseudomolecule of chromosome 4 (Fig. 1C).

We hypothesized that a causal gene for the petaloid phenotype is related to the B-class floral homeotic gene. To identify such a candidate, we used the protein sequences of 1,296 gene models within this 17.50 to 27.75 mbp region for BLAST searches against the Arabidopsis thaliana proteins. As a result, we found a gene (LNRQ01000004.1_prot_KZM98268.1_1702 in the Daucus carota genome assembly PRJNA268187) with a homology against A. thaliana AT5G20240, or the PISTILATA gene (Piwarzyk et al. 2007).

The genomic region of this gene spanned from 22,416,479 to 22,417,257 bp region of the pseudochromosome, which is approximately at around the center of this QTL region, meeting the criteria of a suitable candidate. This gene was named as DcMADS2 by a previous study, and was found to be down-regulated in the carpeloid CMS flower (Linke et al. 2003). It is possible that DcMADS2 is the causal gene for the petaloid phenotype investigated in this study.

We searched manually for a SNP by assessing changes in the restriction enzyme digestion patterns within the DcMADS2 gene.

Table 1. Summary of sequence runs

| Sample | Total reads | Mapped reads | Mean depth | Number of SNPs |
|--------|-------------|--------------|------------|----------------|
| F      | 417,577,082 | 368,311,807  | 132.9      | 6,836,994      |
| MS     | 536,062,242 | 468,086,107  | 169.05     | 6,629,607      |
QTLseq analysis of the petaloid phenotype in carrots

Acknowledgments

We thank M. Okamura, M. Niidome and M. Tsuda for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (16H01467, 18H04776 to S.F.), Grants-in-Aid for Scientific Research (18H02456 to S.F.), Grant-in-Aid for Challenging Exploratory Research (15K14626 to S.F.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and Japan Science and Technology Agency (JST) PRESTO program (JPMJPR16Q8) to S.F..

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Fig. 2. The PCR-RFLP analysis of DcMADS2. M indicates a 100-bp-sized DNA ladder marker. F: fertile individual. MS: male-sterile individual. Restriction enzyme Dral was used to digest the PCR product.

22,416,901 bp of chromosome 4 in the MS bulk, whereas a series of SNPs were found on this site in the F bulk. To enable detection of PCR-RFLP, we designed PCR primers that could amplify the DNA fragment surrounding this site. As expected, Dral-treatment yielded complete digestion of the PCR fragments from an MS individual, whereas a mixture of digested and undigested bands from an F individual was detected (Fig. 2).

In this study we found the QTL responsible for the carrot petaloid phenotype analyzed in this study. It is possible that this QTL is related to the petaloid previously reported as the phenotype of CMS. However, the locus we identified is distinct from the fertility restorer locus previously reported on chromosome 9 (Alessandro et al. 2013). Breeding programs should benefit from the DNA marker established in this study. Future studies may aim to clarify the role of DcMADS2 in the expression of the petaloid phenotype.