Detection of Candidate Gene *LsACOS5* and Development of InDel Marker for Male Sterility by DdRAD-Seq and Whole-Genome Sequencing in Lettuce (*Lactuca Sativa* L.)

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Research Article

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Abstract

A new breeding method of F_1 hybrid using male sterility would open an exciting frontier in lettuce breeding, a self-pollinating crop. Male sterility is a crucial trait in F_1 hybrid breeding. It is essential to map the causative gene for using male sterility. The *ms-S*, male-sterile gene of 'CGN17397', was mapped to LG8 by double-digest restriction site-associated DNA sequencing (ddRAD-seq) and narrowed down between two markers using two F_2 populations. This region spans approximately 10.16 Mb, where 94 genes were annotated according to the lettuce reference genome sequence (version8 from crisphead cultivar 'Salinas'). The whole-genome sequencing of the male-sterile and fertile lines of 'CGN17397' revealed that only one gene differed in the area of *Lsat_1_v5_gn_8_148221.1*, a homolog of *Arabidopsis acyl-CoA synthetase5* (*AtACOS5*), and was deleted in the male-sterile lines. It was reported that *AtACOS5* was needed for pollen wall formation and that the null mutants of *AtACOS5* were entirely male sterility. Thus, I concluded that *Lsat_1_v5_gn_8_148221.1* designated as *LsACOS5* was a biologically plausible candidate gene for the *ms-S* locus. By using the structural polymorphism of *LsACOS5*, an insertion/deletion (InDel) marker was developed to select the male-sterile trait. The results obtained here provide valuable information for the genic male-sterility in lettuce.

Introduction

Lettuce (*Lactuca sativa* L.), a cool-season vegetable crop, is stressed in high-temperature environments^{1,2}. Increasing temperatures associated with climatic change have been shown to affect negatively the growth of lettuce, a major leafy vegetable, and necessitate the development of new cultivars with enhanced stress tolerance. Hybrids usually have better stress tolerance due to hybrid vigor than pure lines and have also been extensively used in leafy vegetable crops such as cabbage and Chinese cabbage to enhance crop production^{3,4}. Harnessing hybrids are considered as one of the effective approaches for many leafy vegetable crops⁵, and the cultivation of F_1 hybrids allows quantum jump in their productivity. Since a cultivation test has already confirmed that lettuce yield of F_1 hybrids increased over the parent, and exploitation of hybrid vigor allowed to promise in improving the yield and other quality parameters⁶. Precise control over pollen fertility is a key factor in the production of F_1 hybrids in self-pollinating crops⁷. Although the F_1 hybrid breeding of the self-pollinating crops such as rice, soybean, wheat, and lettuce would challenge many common-sense assumptions in plant breeding, developments of hybrid rice using genic male sterility (GMS) and cytoplasmic male sterility (CMS) are already underway with great success in China^{8,9}. In addition, numerous studies have been also performed for male sterility in soybean and wheat^{7,10-14}.

The present study began from the finding of a GSM plant in the inbred lines of 'CGN17397' (Fig. 1)¹⁵. Because lettuce has a compound autogamous floral structure, it is impossible to completely remove pollen from the flower¹. Male sterility which can avoid unnecessary maternal self-pollination is not only an essential trait for the hybrid breeding approach in lettuce, and is also useful in the study of genetic

investigations such as disease resistance. In contrast to CMS, the phenotype of GMS is recognized after flowering. Hence, genetic markers linked to the male-sterile locus are needed to select male-sterile plants at the pre-planting stage¹⁶. The markers for the *ms-S* gene have been developed by an amplified fragment length polymorphism (AFLP) technique so far, but all markers were located on the same side of the gene¹⁵. In this study, genetic mapping of the *ms-S* gene was conducted in two F₂ populations obtained from a cross between male-sterile and fertile plants. Additionally, by employing the whole-genome sequencing of male-sterile and fertile lines of 'CGN17397', the causal gene for male sterility was identified to develop a reliable PCR-based marker for MAS (Marker Assisted Selection).

Results

Inheritance of male sterility

Male-sterile phenotypes of the F₂ individuals from a cross between '2008-83 (MS)' and 'UenoyamaMaruba', and a cross between 'MS1024' and 'Salinas' were visually determined. The male-sterile trait derived from 'MS1024' was proposed to be controlled by a single recessive gene, according to the segregation of putative genotype of the male-sterile gene showing a 1:3 ratio in the two F₂ populations (Table 1). These results are consistent with the previous study¹⁵.

Linkage analysis for male sterility trait by ddRAD-seq analysis

For genetic mapping of the locus for the male sterility, ddRAD-seq analysis was conducted for constructing a linkage map using the F₂ population from a cross between '2008-83(MS)' and 'UenoyamaMaruba'. For the setting of RAD-R scripts¹⁷, BWA mode, construction method, and correction approach were "mem_60", "ABH", and "6US" respectively. Then, the 1241 pairs of RAD tags in two parents were employed as codominant markers for genetic mapping of male sterility and used for linkage map construction (Fig. S1). By summarizing the linkage map, the total length of the linkage map was 1815.6 cM. Marker density ranged from 1.2 cM (LG2) to 2.0 cM (LG1) per marker. The number of markers in the linkage groups ranged from 93 (LG1) to 194 (LG5). Summary statistics of the linkage map are shown in Table 2. The segregation data of the genotype of the F₂ population and the phenotype of male-sterile traits showed that the *ms-S* gene was located at the position between 238.429 Mbp and 257.031 Mbp with the interval of 4.6 cM on LG8 (Fig. 2a). Genotyping using three PCR-based markers designed in this region was conducted for fine mapping (Table 3). However, the area could not be further narrowed in this population because these three markers showed complete cosegregation with male sterility (Fig. 2a). Then, the F₂ population derived from a cross between 'MS1024' and 'Salinas' was employed to further mapping of the target locus using PCR-based markers. The gene of the male sterility was located at the position between 246.869 Mbp and 263.743 Mbp with the interval of 6.6 cM on LG8 (Fig. 2b), and *LG8_v8_250.793Mbp* indicated complete cosegregation with the male sterility based on the two F₂ populations (Fig. 2). The results of mapping using the two F₂ populations demonstrated that the *ms-S* gene is located at the position between 246.869 Mbp and 257.031 Mbp on LG8 (Fig. 2).

Identification of candidate genes in *ms-S* locus by whole-genome sequencing

The *ms-S* locus was found to include 94 genes annotated according to the lettuce reference genome sequence (version8 from crisphead cultivar ‘Salinas’) (Table S1). Whole-genome sequencing data of the male-sterile and fertile lines revealed that a genomic region of about 4 kb containing the *Lsat_1_v5_gn_8_148221.1* was completely deleted in only the male-sterile lines (Fig. S2). According to the reference genome sequence, *Lsat_1_v5_gn_8_148221.1* encodes an *acyl-CoA synthetase5* (*ACOS5*), which might be orthologous to Arabidopsis male-sterile gene *AtACOS5*^{18,19}. To further elucidate the relationship between *Lsat_1_v5_gn_8_148221.1* and *AtACOS5*, these two genes were examined for amino acid alignment by employing Clustal W. The results showed that there was a 68.02% identity between the two and significant conservation within the AMP-binding domain and the fatty acid-binding domain of *ACOS5* (Shockey and Browse 2011) (Fig. 3a). The phylogenetic analysis showed that *Lsat_1_v5_gn_8_148221.1* was categorized into the *ACOS5* group, which is related to male sterility in some plant species (Zou et al. 2017; de Azevedo Souza et al. 2009) (Fig. 3b). Based on the results, the gene might be the candidate gene for *ms-S* because of its homology with the known recessive male-sterile gene and was designated as *LsACOS5*. For the other 93 genes, there were no differences in the coding region between the two lines (Table S1). The *LG8_v8_250.793Mbp* designed using the genomic regions of the candidate gene (Fig. S2, Table S1) had polymorphism between the male-sterile and fertile lines of ‘CGN17397’ and was completely cosegregated with the male-sterile trait in the two F₂ populations (Fig. 2). These results suggest that *LsACOS5* is a biologically plausible candidate gene for *ms-S*.

Discussion

Because an F₁ hybrid has a potential character that grows faster and has a shorter cultivation period in a field, the risk against bacterial disease accelerated by rain would be below. Thus, F₁ hybrids are commonly anticipated to display high productivity under stressful conditions. In lettuce, the exploitation of the F₁ hybrid could be one of the effective approaches to maintain a stable yield, particularly in tropical and subtropical regions. A new crisphead cultivar ‘Fine green’ was indeed the first F₁ hybrid bred by Kaneko seeds CO., LTD. in Japan, but unfortunately, the technical detail of the breeding method was not announced publicly. In general, the male-sterile plant is worth exploring as the key factor of F₁ hybrid breeding, and several GMS mutants were also reported in lettuce so far²². The genetic mechanism is not understood, and this is the first report of the identification of the male-sterile gene in lettuce. It is valuable to ascertain the genetic mechanism of male-sterile plants to select a future breeding strategy.

In this study, the two F₂ populations were used to locate the male-sterile gene to the region between the two PCR-based markers, *LG8_v8_246.869Mbp* and *LG8_v8_257.031Mbp*. Although the genomic region of the *ms-S* locus was relatively large, the whole-genome sequencing for male-sterile and fertile lines of ‘CGN17397’ revealed only 1 different gene, *Lsat_1_v5_gn_8_148221.1*, between 2 lines in these 94 annotated genes in the *ms-S* locus (Table S1, Fig. S2). The gene encoded an *acyl-CoA synthetase 5* (*ACOS5*) and was a potential ortholog of the key male-sterile gene *AtACOS5* in *Arabidopsis*¹⁸ (Fig. 3a).

The *AtACOS5* acted as acyl-CoA synthase to regulate the biosynthesis of sporopollenin to affect male fertility, and a null mutant was entirely male-sterility¹⁸. The male-sterile line of 'CGN17397' displayed normal vegetative growth and complete male-sterility insensitive to environmental conditions. There were no other obvious morphological differences between the male-sterile and fertile lines. Lettuce was generally only flowering for about two hours in the morning, but the male-sterile lines could continue to flower through the afternoon. Thus, the male-sterile mutants of lettuce and *Arabidopsis* showed phenotypic similarities¹⁸. I concluded that *LsACOS5* was a biologically plausible candidate gene for the *ms-S* locus (Figs. 2, 3, Table S1, Fig. S2).

In addition, the InDel marker—*LG8_v8_250.793Mbp*—tightly linking to the *ms-S* gene was developed. By using the InDel marker, it was possible to select male-sterile plants for a conventional-breeding program (Fig. 1c, Fig. 2). Due to the structure of the lettuce flower, it was challenging to examine the inheritable characteristics of valuable traits¹, such as disease resistance in only the F₁ seeds because crosses produced not only F₁ seeds but also self-pollinated seeds. Because only F₁ hybrid seeds can be produced using GMS plants for crossbreeding, research on valuable traits that could not be analyzed in the past would be facilitated.

The F₁ seed production system was needed to promote the commercial production of F₁ hybrids. To propagate the F₁ hybrid seeds in the case of rice, the maternal and paternal plants were alternately cultivated in a field to cross by the wind and artificial pollination²³. But lettuce pollen was not dispersed by wind, the F₁ seed production system has been already developed using insect pollination at a greenhouse. The fact that flies and bees were adopted for the system due to an absence of specialist pollinators of lettuce, the self-pollinating crop, could propagate the F₁ hybrid seeds^{24,25}. Moreover, the F₁ hybrids are likely to be suitable for cultivation in not only fields but also plant factories. The trait of rapid growth was economically important for the cultivation in plant factories. The breeding of F₁ hybrids suitable for cultivation in fields and plant factories is an issue for the future.

To date, genome editing technology makes it possible to create knockout mutants of the target gene. GMS plants generally have a problem of seed mixture for the male-sterile and fertile progeny. Still, a novel hybridization platform known as the third-generation breeding technique has been successfully selected for non-transgenic GMS seeds⁹. Combining these two techniques could also be applied for the F₁ hybrid breeding in lettuce, and it converts any elite cultivars into a commercial male-sterile plant and accelerates the development of F₁ hybrid cultivars. The applications of the GMS plant initiative to the rise of considerable potential for lettuce breeding.

Methods

Plant materials

The plant materials were grown at the Nagano Vegetable and Ornamental Crops Experiment Station (Shiojiri City, Nagano prefecture, Japan; 36° 10' N, 137° 93' E). The genic male-sterile plant was discovered as a spontaneous mutation in 'CGN17397' (Fig. 1). The male-sterile and fertile lines of 'CGN17397' were used for whole-genome sequencing. The male-sterile line and its causative gene were designated 'MS1024' and *ms-S*, respectively¹⁵. '2008-83 (MS)' was obtained from a cross between 'MS1024' and a cultivar 'Patriot' at Nagano Vegetable and Ornamental Crops Experimental Station. A total of 90 individuals from the F₂ progeny obtained from a cross between '2008-83(MS)' and 'UenoyamaMaruba' (*L. serrila*) were used for linkage analysis using ddRAD-seq. The male-sterile trait was visually examined. Additionally, 96 individuals of F₂ progeny obtained from a cross between 'MS1024' and 'Salinas' were used for further mapping using PCR-based markers.

Linkage analysis based on ddRAD-seq

Genomic DNA was extracted from leaves using the Nucleo-Spin Plant II Extract Kit (Machery-Nagel, Duren, Germany). The RAD-seq library construction was performed following a previously described method^{2,26}. The ddRAD-seq libraries were sequenced using the HiSeq4000 platform (Illumina, San Diego, CA, USA). Paired-end sequencing reads (100 bp × 2) were analyzed for ddRAD-seq tag extraction, counting, and linkage map construction using RAD-R scripts¹⁷. The read mapping was performed with the RAD tags in each parent against the lettuce reference genome sequence [version8 from crisphead cultivar 'Salinas' (<https://genomevolution.org/coge/GenomeInfo.pl?gid=28333>)]. The linkage map was graphically visualized using Mapchart and R/QTL^{27,28}. Raw sequence data (FASTQ) in this ddRAD-seq were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (http://ddbj.nig.ac.jp/dra/index_e.html) under accession number DRA012711.

Designing PCR-based markers and their amplification

Polymorphisms between parental lines around the *ms-S* locus, including insertion, deletion, and SNP, were surveyed to identify the marker sites using the IGV software²⁹. Primers for amplifying the markers were designed using the Primer3 website (<http://bioinfo.ut.ee/primer3-0.4.0/>), and their IDs (names) were defined as (linkage group) _ (genome version) _ (genome position). PCR was conducted using 0.5 µL of DNA template, 0.4 µL of each primer (50 µM), 2 µL of dNTP (2 mM), 5 µL of 2× PCR Buffer, 0.2 µL of KOD FX (1 U/µL, TOYOBO, Japan), and distilled water (dH₂O) to a final volume of 10 µL. PCR conditions were as follows: at 94°C for 5 minutes, 30 cycles of at 94°C for 30 s, and at 61°C for 30 s followed by 1 cycle at 72°C for 4 minutes. 9 µL of PCR products were employed to electrophoresis on 2.5% agarose gel (Takara-bio, Japan) at 100 V after amplification.

Resequencing analysis

Genomic DNA was extracted from young leaves of the two lines (male-sterile and fertile lines of 'CGN17397') using NucleoSpin Plant II (Machery-Nagel, Duren, Germany) and was used to construct paired-end sequencing libraries (100 bp x2) and subjected to whole-genome sequencing using the HiSeqX

(Illumina) and DNBSEQ-500 (MGI) platform. The resequencing analyses were conducted according to the previously described method ². Raw sequence data (fastq) for this resequencing analysis are available in the DDBJ Sequence Read Archive at accessions DRA012737.

Phylogenetic analysis

The protein sequence of the candidate gene was searched for homologs from the plant species using basic local alignment search tools (BLAST) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments of the full-length protein sequences were conducted using ClustalW and displayed using BOXSHADE (https://embnet.vital-it.ch/software/BOX_form.html). The phylogenetic tree was generated using MEGA X program ³⁰ using the neighbor-joining method with default parameters besides 1000 bootstrap replications.

Declarations

Ethical statement

The author assures that legislation on seed collection has been accomplished. Permission obtained from responsible authority to collect seeds.

Ethical approval

All the experiments carried out on plants in this study were in compliance with relevant institutional, national, and international guidelines and legislation.

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Author contributions statement

KS planned the experiments, performed all experiments and analyses, and wrote the manuscript.

Additional Information

The author declares that there is no conflict of interest.

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Figures

Figure 1

a An inflorescence of *Lactuca sativa*: The inflorescence is composed of 7–15 yellow florets. b Pistil of a male-fertile flower: There are pollen grains on the stigma. c Pistil of a male-sterile flower: There are no pollen grains on the stigma

Figure 2

The mapped location of the ms-S gene on LG8 in two populations. Genetic distances (cM) were shown between the markers. “(RAD)” and “(PCRbased)” in the marker name indicate ddRAD-seq markers and PCR-based markers, respectively. “ms-S” indicates the position of the causal gene for male sterility. a Linkage mapping of the ms-S gene using an F2 population derived from a cross between ‘2008–83(MS)’

and 'UenoyamaMaruba'. b Mapping of the ms-S gene using an F2 population derived from a cross 'MS1024' and 'Salinas'

Figure 3

Sequence alignment of LsACOS5 and its homologs. a. Amino acid sequences alignment of LsACOS5 (Lsat_1_v5_gn_8_148221.1) and AtACOS5 (AT1G62940). The sequences were aligned using ClustalW and displayed using BOXSHADE with MEGA X. Red frames indicate the conserved AMP-binding, fatty acid-binding domains. b A neighbor-joining phylogenetic tree of LsACOS5 and its homologs in some plants. Bootstrap values are the percentage of 1000 replicates

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