Fine Mapping and Identification of the Lobed-Leaf Gene in Radish (*Raphanus Sativus* L.)

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Research Article

Keywords: R. sativus, the lobed-leaf trait, BSA

Posted Date: June 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-636204/v1

Abstract

Leaf shape is one of the important factors affecting the yield and quality of radish (*Raphanus sativus* L.). In this study, an F_2 population obtained from the cross of the lobed-leaf cultivar J4 with the serrated-leaf cultivar WA was used for genetic analysis and gene mapping of the lobed-leaf trait. The lobed-leaf trait is controlled by a single dominant nuclear gene, and dominant over the serrated-leaf trait. Through bulked segregant analysis (BSA) and genotyping by sequencing (GBS), the lobed-leaf gene was initially mapped to the genomic region from 0.66 Mb to 8.19 Mb on chromosome R7. By using nine insertion/deletion (INDEL) markers, the gene was further narrowed down to a 1.53 Mb region. The comparative analysis of the collinear region between *Raphanus sativus* and *Brassica napus* identified *Rs390250*, an *HD-Zip I* transcription factor as the most probable candidate gene. Phylogenetic analysis supported that *Rs390250* is an *RCO* (*REDUCED COMPLEXITY*) orthologous gene, and the single nucleotide variation (C425T) of *Rs390250* which caused an amino acid substitution from serine (S) to lysine (L) in conserved leucine zipper domain, may destroy its DNA-binding function and be supposed to response for the morphological variation.

Introduction

Leaf morphology is an important agronomical trait which affects sunlight penetration, air exchange, disease occurrence, and ultimately influences the crop quality and yield (Vogel 2009; Zhu et al. 2010). Leaves of eudicots can be simple or dissected as the blade are entire or divided into leaflets, and have margins that are entire (smooth), serrated, or lobed (Hay and Tsiantis 2006; Sicard et al. 2014). Among the evolutionary history, leaf shape of many species have experienced artificial selection during domestication and breeding (Hafeez-ur-Rahman 2005). Radish (*Raphanus sativus* L.), an annual or biennial herb of tribe *Brassicaceae*, is one of the most popular vegetables all over the world. The leaves of radish cluster on a shortened stem and have serrated even lobed margins varying in sinus depth and the number of lobes. Lobed-leaf trait is important for radish growth, which can beneficial to photosynthesis enhancement and disease reduction, but there is little know about how it developed in radish. Thus, it is necessary to analyse the genetic mechanism of radish lobed-leaf trait.

Previous studies shown that lobed-leaf results from the interaction of plant hormones, transcription factors and microRNAs. Auxin is essential to the leaf margin development. It is necessary to yield auxin maxima by accumulating the auxin efflux carrier *PIN1* (*PIN-FORMED*1) at the leaf margin to form the early tooth tips (Vernoux et al. 2000; Aloni et al. 2003). *CUC2* (*CUP-SHAPED COTYLEDON2*) promotes the transportation of *PIN1* but is repressed by auxin, thus it will accumulates at the lower-auxin-concentration locations of leaf margin (Kawamura et al. 2010). The relative distribution of CUC2 and PIN1 helps to maintain the high auxin concentration, and eventually leads to the formation of serrations, the small outgrowth at leaf margin (Furutani et al. 2004; Bilsborough et al. 2011). In addition, *CUC2* is regulated by the *miR319-TCPs-miR164* pathway, over-express *miR319* or down-regulate *TCPs* (*TEOSINTE BRANCHED*1, *CYCLOIDEA* and *PCF*) and *miR164* would increase the expression of *CUC2*, the leaf margin accordingly be more lobed (Nikovics et al. 2006; Koyama et al. 2010; Hasson et al. 2011). Class

I *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) are required for shoot apical meristem (SAM) maintenance and dissected leaf formation (Hay and Tsiantis 2006; Shani et al. 2009). There are four *KNOX* genes in *Arabidopsis thaliana* including *STM* (*SHOOT MERISTEMIESS*), *BP* (*BREVIPEDICELLUS*), *KNAT2* (*KNOTTED*-like from *Arabidopsis thaliana* 2), and *KNAT6*. It has been reported that overexpression of any the four *KNOX* genes would lead to deep lobes in *A. thaliana* (piazza et al. 2010). Meanwhile, *KNOX* genes were regulated by many plant hormones, high concentration of auxin inhibits the expression of *KNOX1* and subsequently affects the content of gibberellic acid (GA) and cytokinin (CK), which leads to lobed margins in different degrees (Scanlon et al. 2003; Jasinski et al. 2005). It is known that *LATE MERISTEM IDENTITY* 1 (*LMI1*)-like genes play an important role in leaf shape development (Saddic et al. 2006). *A. thaliana* has one *LMI1*-like gene (*LMI1*), while *Cardamine hirsute* has three as a tandem gene triplication, *LMI1*, *RCO* (*LMI1-like2, REDUCED COMPLEXITY*), and *LMI1-like3* (Vlad et al. 2014). Previous study showed that *LMI1* is required for the formation of simple serrated leaves, *lmi1* plants became lobed and divided at the base of the leaf (Saddic et al. 2006). *RCO* is able to affect the complexity of leaf shape in *Brassicaceae*, introducing *RCO* into *A.thaliana* would convert the simple entire leaf into lobed (Vlad et al. 2014; Sicard et al. 2014). Nowadays, *LMI1*-like genes have been cloned in different species, and also confirmed to regulate the leaf margin development (Chang et al. 2016; Ni et al. 2017; Andres et al. 2017; Wei et al. 2017; Hu et al. 2018; He et al. 2018; Ren et al. 2019), but the molecular mechanism is still uncertain and needs further study.

In this study, we conducted an F_2 population segregating in leaf shape of radish for inheritance analysis and gene mapping. A *RCO* homologous gene (*Rs390250*) was supposed to determine the lobed leaf margin. Our study can help to analyse the molecular mechanism of *RCO*-like genes and lay a foundation for molecular marker-assisted selection of leaf shape trait in radish.

Materials And Methods

Plant materials

WA is a cytoplasmic male sterile (CMS) line with serrated leaves. J4 is a restorer line with lobed leaves which have deep sinuses and paired lobes. F_1 hybrids were obtained by crossing WA with J4 at the experimental field at the Institute of Vegetables, Wuhan Academy of Agricultural Science. F_2 population was grown at the experimental farm of Huazhong Agricultural University, Ezhou, China.

Eleven more radish varieties with various leaf shape (Table 1) were provided by the Institute of Vegetables, Wuhan Academy of Agricultural Science for single nucleotide polymorphism (SNP) analysis.

Trait measurement

The lobed and serrated leaves are obviously distinguishable when plants are at 5-6 leaf stage. The leaf shape was investigated when the two parents and the F_2 plants had 5 leaves. All the 287 F_2 plants were labeled with plastic tags and their leaf shape was scored. Chi square test was used to analyze the inheritance of leaf shape.

DNA extraction and sequencing library construction

Genomic DNA of parents and F_2 plants were extracted from about 0.25 g fresh leaf using the cetyltrimethyl ammonium bromide (CTAB) method. For bulked segregant sequencing (BSA-seq) and initial mapping, equal amount of genomic DNA from 20 lobed- and 20 serrated-leaf plants ware pooled to build the lobed-leaf bulk (Bk1) and the serrated-leaf bulk (BK2), respectively. Two parents and two bulks were next subjected to genotyping by sequencing (GBS) as described previously (Chen et al. 2013). Briefly, 100 ng DNA was digested with restriction enzymes SacI and MseI. The restriction fragments were ligated to SacI and MseI adaptors with unique barcode combinations to differentiate the samples. The ligated products of all samples were pooled and were separated on 2 % agarose gel. Fragments ranging from 220 bp to 500 bp were recovered from agarose gel and purified with the gel purification kit (Sangon, Shanghai, China). The mixed libraries were amplified in a 50 µl of volume with 50–100 ng of adaptor-ligated DNA fragments as template, 1× HF buffer, 3.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U iProof polymerase (Bio-Rad), 5 pmol of two primers (PF: AATGATACGGCGACCACCGAGATCTACACTCTTTC CCTACACGACGCTCTTCCGATCT and PR: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT). PCR amplification was performed as follows: 98 °C for 2 min, followed by 11 cycles at 98 °C for 30 s, 65 °C for 30 s and 72 °C for 15 s, and a final extension at 72 °C for 5 min. PCR products were separated on 2 % agarose gel and fragments in the size range of 270–550 bp were purified with the Sangon gel purification kit (Sangon, Shanghai, China) and sequenced on HiSeq 4000.

Data analysis of BSA-seq

To obtain the genome-wide variations between parents and between the two bulks, the sequencing reads were aligned to the reference genome of *R. sativus* (Jeong et al. 2016; http://radish-genome.org/) with BWA software. Genome-wide variations between the lobed- and the serrated-leaf bulks were obtained with GATK (McKenna et al. 2010). For each identified SNP, QTLseqr (Mansfeld and Grumet 2018) was used to calculate the SNP-index values at each quality-controlled locus. The raw sequence date were checked with the FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the quality of reads was controlled with Trimmomatic (Bolger 2014).The filtration parameters of GATK was “QUAL < 30.0 || QD < 4.0 || FS > 60.0 || MQ < 40.0”. The SNP index plots and corresponding Δ (SNP index) plot of two bulks were drawn with a moving window of 2 Mb along the physical map of the radish reference genome.

Molecular marker development and genotyping

Based on the BSA-seq data, 16 INDEL markers were designed according to 16 insertion/deletion variations of parents in the initial candidate interval. All primers used in this study are listed in Table 2. PCR amplifications were performed in a volume of 10 ml containing 50 ng genomic DNA, 1´ Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each primer and 0.25 U Taq DNA polymerase (Vazyme, Nanjing, China). The reaction mixture was initially denatured at 94 °C for 5 min, followed by 40 cycles of amplification at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min.

The PCR products were separated on 6 % denaturing polyacrylamide gels and stained with silver nitrate solution.

Collinearity analysis

All the predicted proteins of *R. sativus* were aligned to the *B. napus* reference genome of Darmor-*bzh* by BLASTP. The alignment were used as the input file of MCScanX (Wang et al. 2012) to construct the collinear segments between the radish and rapeseed genome. The collinear segment containing the candidate interval for lobed-leaf gene on *R. sativus* chromosome R7 was selected and gene-annotated.

Comparative sequencing

Based on the reference genome sequence of *R. sativus* (Jeong et al. 2016), three pairs of primers (Rs-1F/Rs-1R; Rs-2F/Rs-2R; Rs-3F/Rs-3R) were designed to amplify the gene *Rs390250* including 1 kb regulatory sequence, the 1.8 kb coding region and 200 bp downstream sequence. PCR products amplified from both WA and J4 were separated on agarose gel and the target fragments were recovered from the gel with the Sangon gel purification kit (Sangon, Shanghai, China) and sequenced. All sequences were assembled together using Sequencher (GeneCodes, USA) to identify variations between parents.

Semi-quantitative reverse-transcriptase PCR (RT-PCR)

Total RNA of parents were extracted from fresh leaves using the TRIpure Reagent kit (Aidlab, Wuhan, China). RNA quality was check by electrophoresis on 2 % agarose gel, purity and concentration were measured with Nanodrop (Thermo Scientific, USA). 2 mg RNA was reverse transcribed into cDNA using the RevertAid First strand cDNA synthesis kit (Thermo Scientific, USA). RT-PCR was performed with the cDNA to measure the relative expression level of *Rs390250* in WA and J4. Gene-specific primers were obtained from a qPCR primer database at https://biodb.swu.edu.cn/qprimerdb/ (Lu et al. 2018). *Actin* was used as the reference gene to control the amount of input RNA.

Results

Inheritance analysis

To analyze the inheritance of leaf shape in *R. sativus*, a cross between WA, a CMS line with serrated leaf, and J4, a restorer line with deeply lobed leaf was conducted (Fig. 1). All F_1 plants had lobed-leaves, while in F_2 population (WA×J4), there were 68 plants with serrated leaves and 219 plants with lobed leaves, which fit an expected Mendelian segregation ratio of 1:3 ($\chi^2 = 0.227$, $p > 0.05$). These results indicated that the lobed-leaf is controlled by a dominant nuclear gene and dominant over the serrated-leaf.

Bulked segregant sequencing of the lobed leaf gene

To quick map the leaf shape gene, we did BSA-seq by incorporating BSA and GBS. After sequenced on HiSeq 4000, there were 2,500,383 reads and 3,453,168 reads obtained form J4 and WA, 2,851,995 reads

and 4,844,581 reads obtained form Bk1 and BK2, respectively. All reads were aligned to the radish reference genome (Jeong et al. 2016) to identify single nucleotide polymorphisms (SNPs) and small INDELs between parents and between two Bulks. Totally, 4,544 high-quality polymorphic loci including 3,852 SNPs and 692 INDELs were identified. For each identified SNP, the SNP index was calculated, and a corresponding Δ (SNP index) plot of two bulks was drawn along each chromosome (Fig. 2). It was found that the Δ (SNP index) in a genomic region from 0.66 Mb to 8.19 Mb on chromosome R7 exceeded the threshold of 0.5, indicating that the leaf-shape gene may be encompassed.

Rs390250 is the lobed leaf candidate gene of radish

For further mapping, 16 INDEL markers among the initial candidate interval on chromosome R7 were designed based on the INDELs between parents. These markers were used to amplify the genomic DNA of WA and J4, 9 (RIL_1, RIL_2, RIL_3, RIL_7, RIL_11, RIL_12, RIL_14, RIL_15 and RIL_16) out of the 16 markers detected distinguishable polymorphisms (Fig. 3a). Then, these polymorphic INDEL markers were used for recombinant screening of 287 F_2 individuals. Finally, the genomic region containing lobed-leaf gene was narrowed down to a 1.53 Mb region between RIL_3 (0.87 Mb) and RIL_11 (2.40 Mb) on chromosome R7 (Fig. 3).

Radish belongs to the tribe *Brassicaceae* and closely related to *B. rapa* and *B. napus*, two important commercial crops as vegetables and vegetable oil. Comparative mapping using molecular markers and whole-genome alignment revealed extensive syntenic blocks all over the genomes of *R. sativus* and *B. rapa* (Li et al. 2011; Jeong 2016). To identify the candidate gene, we did whole-genome collinearity analysis between *R. sativus* and *B. napus.* The region from 0.87 Mb to1.3 Mb of chromosome R7 on radish which within the fine mapping region showed a good collinearity with the region between 16.3-16.8 Mb of chromosome A10 on *B.napus* (Fig. 3b). In the collinear region of *B. napus,* there were two *LMI1-like* genes, *BnaA10g26320D* (*BnLL1.LMI1*) and *BnaA10g26330D* (*BnA10.LMI1*) which were considered as the lobed leaf genes (Ni et al. 2017; Hu et al. 2018). Both *BnLL1. LMI1* and *BnA10. LMI1* are orthologous to *Rs390250* (899,863-901,651 bp), one of the 53 gene models in the mapping region of radish. All these genes encode homeodomain leucine zipper class I (*HD-Zip I*) transcription factors. Previous results indicated that several class I HD-Zip regulators control leaf shape and size (Saddic et al. 2006). We thus listed *Rs390250* as the lobed leaf candidate gene of radish.

Sequence comparison analysis of Rs390250

According to the annotation of the radish genome, *Rs390250* consists of three exons and two introns, and encodes a protein with 220 amino acids. To compare the nucleotide sequences of *Rs390250* between J4 and WA, a 3.0 kb genomic region including the 1.0 kb upstream regulatory sequence, the 1.8 kb coding region and the 0.2 kb downstream untranslated region of *Rs390250* was amplified. Comparative sequencing identified three SNPs (C84G□T177A and C425T) in the coding sequence (CDS), one 149 bp insertion in the regulatory region and one 666 bp insertion in the first intron of *Rs390250* from J4 (Fig. 4a). The 149 bp insertion in the regulatory region is a duplicate copy of a neighbouring 130 bp

sequence plus a 19 bp spacer with unknown function. By searching against the transposable element (TE) database (http://pmite.hzau.edu.cn) (Chen et al. 2013), we found that the 666 bp insertion is a Mutator-like TE, which has a 9 bp target site duplication (GTTTTTAAA) and 46 bp terminal inverted repeate (GAGTTATTCTTGGGTTCACCCCCTAGGGTGAACCTTTAGGTTCACC). To detect whether the TE is co-segregate with leaf shape variations, we designed a pair of primers (Rs-3F/Rs-2R) to specifically amplify the 666 bp insertion in the 68 serrated-leaf plants selected from the F_2 population, and found that all the 68 serrated-leaf plants did not have the 666 bp insertion while the lobed-leaf plants had (Fig. 4c), suggesting that the TE insertion in the first intron of *Rs390250* co-segregated with the lobed-leaf. To reveal if the 149 bp duplication in the regulatory region affect the expression level of *Rs390250*, we performed RT-PCR to measure the relative expression level of *Rs390250* in J4 and WA, and found that there is no significant difference between parents (Fig. 4d), indicating that the insertion is unrelated to the difference of leaf shape between J4 and WA .

Rs390250 encodes an *HD-Zip I* transcription factor. HD-Zip proteins contain a homeodomain (HD) domain and a leucine zipper (LZ) domain. The HD domain binds to double helix of target DNA, and the LZ domain helps the HD to recognize the downstream genes by forming a homo- or heterodimer structure (Tron et al. 2004). Both the HD and the LZ domains are essential for the HD-Zip I proteins to regulate the transcription of target genes. Of the three SNPs in the CDS of *Rs390250*, T177A is a synonymous mutation while C84G and C425T are non-synonymous mutations. The C425T mutation caused an amino acid substitution from serine (S) to leucine (L), which exactly located in the LZ domain (Fig. 4b). we aligned few of amino acid sequences of the LZ domain in *LMI1-like* genes from different species with various lobed-leaves, the result showed that the serine (S) residue is absolutely conserved in these species, except for the serrated-leaf WA which has a leucine (L) residue (Fig. 5a). Besides, Phylogenetic analysis of the LZ domain indicated that Rs390250 belongs to RCO orthologous gene (Fig. 5b). Analysis of conserved domains using the NCBI Conserved Domains tool showed that both RCO and Rs390250-J4 have the HD domain and the LZ domain, while Rs390250-WA only has the HD domain but does not have the LZ domain, suggesting that the substitution of the conserved serine by leucine may destroys the structure of DNA-binding LZ domain in WA. We thus supposed that the C425T may cause the different morphological variation between J4 and WA. To verify our speculation, we amplified and analyzed the *Rs390250* sequence from 11 more radish varieties with various leaf shape. All the serrated-leaf varieties are T/T homozygous as WA, while the lobed-leaf varieties with deep lobes are C/C homozygous as J4 or heterozygous (C/T) (Table 1). It is known that the *rco* mutant in *Cardamine hirsuta* resulted in a lighter leaf margin dissection (Vlad et al. 2014; Sicard et al. 2014). Thus, we speculated that the C425T transition is the causal variation resulting the serrated leaf of radish.

Discussion

Lobed leaf is a very common type of leaf shape in eudicots such as lettuce, watermelon, rapeseed etc. *LMI1-like* genes regulate the leaf shape formation. In previous studies, *LMI1* was responsible for the

margin development of simple leaf, while *RCO* was required for the leaf complexity which played an essential role in leaflet formation of dissected leaf (Sicard et al. 2014). Most lobed leaf genes were homologous to *LMI1*, while in recent years, *RCO* was considered as a candidate gene for the lobed margin development of simple leaf, but the molecular mechanism is unclear (Ni et al. 2017; Hu et al. 2018). *RCO* was first identified in *Cardamine hirsuta.* It was confirmed that *RCO* has been lost in the evolution of *A. thaliana*, and contributing to a simple leaf form (Vlad et al. 2014). *RCO* affected the leaflets developing of *Cardamine hirsuta* mainly through local growth repression of their flanks, introducing *RCO* into *A.thaliana*, the simple serrated leaf became lobed (Vlad et al. 2014). As we know, the more lobes per leaf and the deeper sinuses between the lobes, the leaf margin dissection would be more severe. Thus, it is believed that *RCO* also plays an important role in lobes' development of simple leaf, and the lose of *RCO* would result in less lobed. Our results coincided with it. In this study, we identified *Rs390250*, an *HD-Zip I* transcription factor, as the lobed-leaf candidate gene, which belongs to *RCO*-like gene (Fig. 5). Comparative sequencing of *Rs390250* in different varieties showed that all the serrated-leaf varieties have the mutant genotype as *Rs390250-WA* (Table. 1), which indicated that when *Rs390250* mutanted, the leaf margin would be less lobed. Further more, the different allelic variation (C425T) in the conserved zipper domain which caused the amino acid substitution of *Rs390250* may destroy the structure of DNA-binding leucine zipper domain in serrated-leaf varieties, thus the HD-Zip I protein in WA may fail to regulate the lobed leaf margin formation.

Declarations

Funding

Not applicable

Conflicts of interest/Competing interests

All the work described in this manuscript was original and has not been published or under consideration by any other journal. And I confirm that all authors are familiar with and agree with submission of the contents of the manuscript.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Code availability

Not applicable

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Tables

Table 1 The genotype of the 425th nucleotide in the coding sequence of *Rs390250* in 11 radish varieties

Variety	Genotype at the 425th nucleotide	Phenotype
1	T/T	serrated
2	C/C	lobed
3	T/T	serrated
4	C/T	lobed
5	C/T	lobed
6	C/T	lobed
7	T/T	serrated
8	C/C	lobed
9	C/T	lobed
10	T/T	serrated
11	C/C	lobed
J4	C/C	lobed
WA	T/T	serrated

Table 2 Primers used in this study

Marker name	Forward primer (5'-3')	Reverse primer (5'-3')	Primers for
RIL_1	cgcctcatacatttgtttgg	tgctaatgttttcgttaacct	gene mapping
RIL_2	accaattacaaaacaacggt	taaacgaccagtacccaaag	
RIL_3	aagtaatggagagacctcga	gagttaaacgttctgttgca	
RIL_7	gacataagtctgtctggaca	tctgttgatacaaagtctctct	
RIL_11	atcttggtacagaactctcg	atcatgtactctcactctgc	
RIL_12	gtagaagatgacgccgac	gaaaccctaatcgtggctaa	
RIL_14	tggaagtaaaagacagacga	ggattcttgaacttttgtgct	
RIL_15	acctctttaactgcgtttga	agaaggatcggagaagaaga	
RIL_16	tcactcaaacccattatgtct	cgcaaatcacctaccaaaat	
Rs_1	tagagagggaagccatgttgtc	aacgttgctcttcgtagaccat	gene clone
Rs_2	ctttctcaccgcatgctctc	gtgctctatatcccacttctcat	
Rs_3	ccctatcgagtttcctgtgttc	agtctgccttcgtctaacatt	
Rsa	cgcagtttcaagacagaatcaa	caacaagtagaagcaacacaca	RT-PCR
Actin	atcaggaaggacttgtacggtaac	gctgagggaagcaagaatggaacc	internal control

Figures

Figure 1

Morphological performance of the lobed-leaf trait at seedling stage of Raphanus sativus (a) The J4 plant at seedling stage. (b) The WA plant at seedling stage. (c) The leaves of J4, WA and some F2 plants of WA×J4 at seedling stage (from left to right).

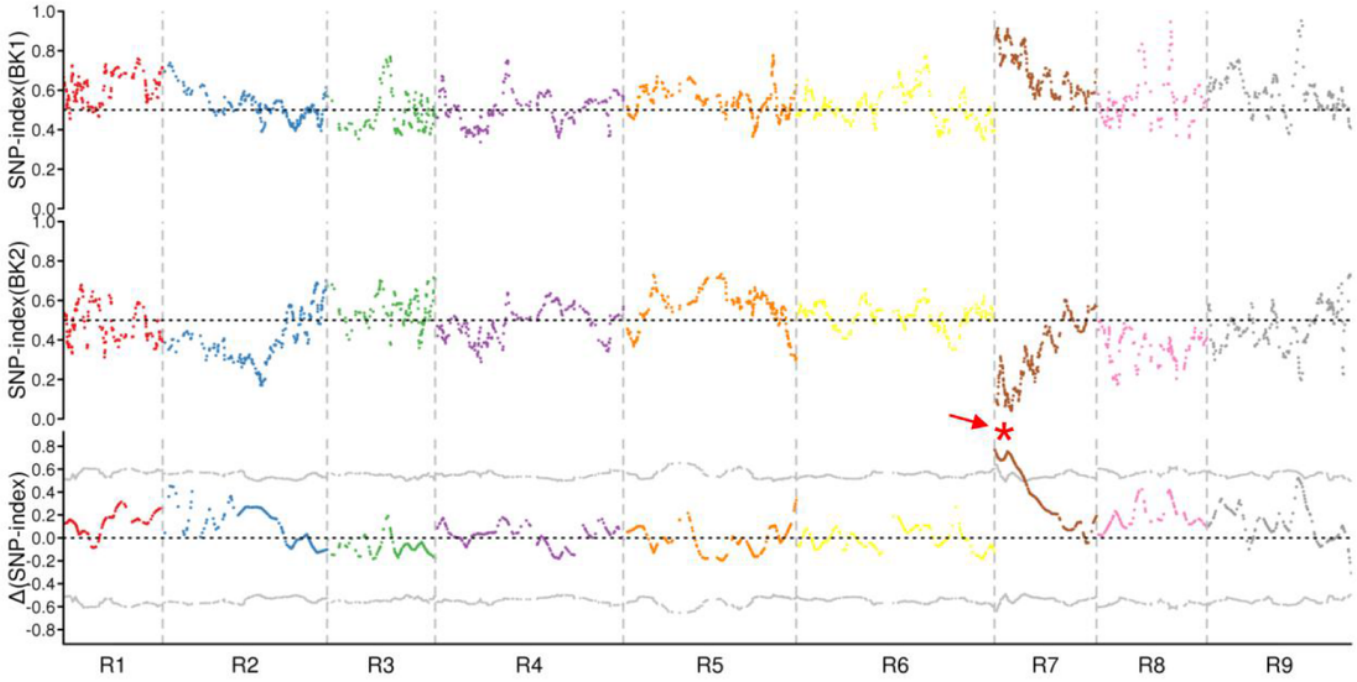

Figure 2

Genetic mapping of leaf shape gene by BSA-seq The SNP-Index attribution of the lobed-leaf DNA bulk (Bk1) and serrated-leaf DNA bulk (BK2). X-coordinate represents the location in chromosomes, Y-coordinate represents the SNP-index value and the delta SNP-index value of Bk1 and BK2 (from top to bottom). '*'indicates the region where the delta SNP-index exceeds the threshold of 0.5.

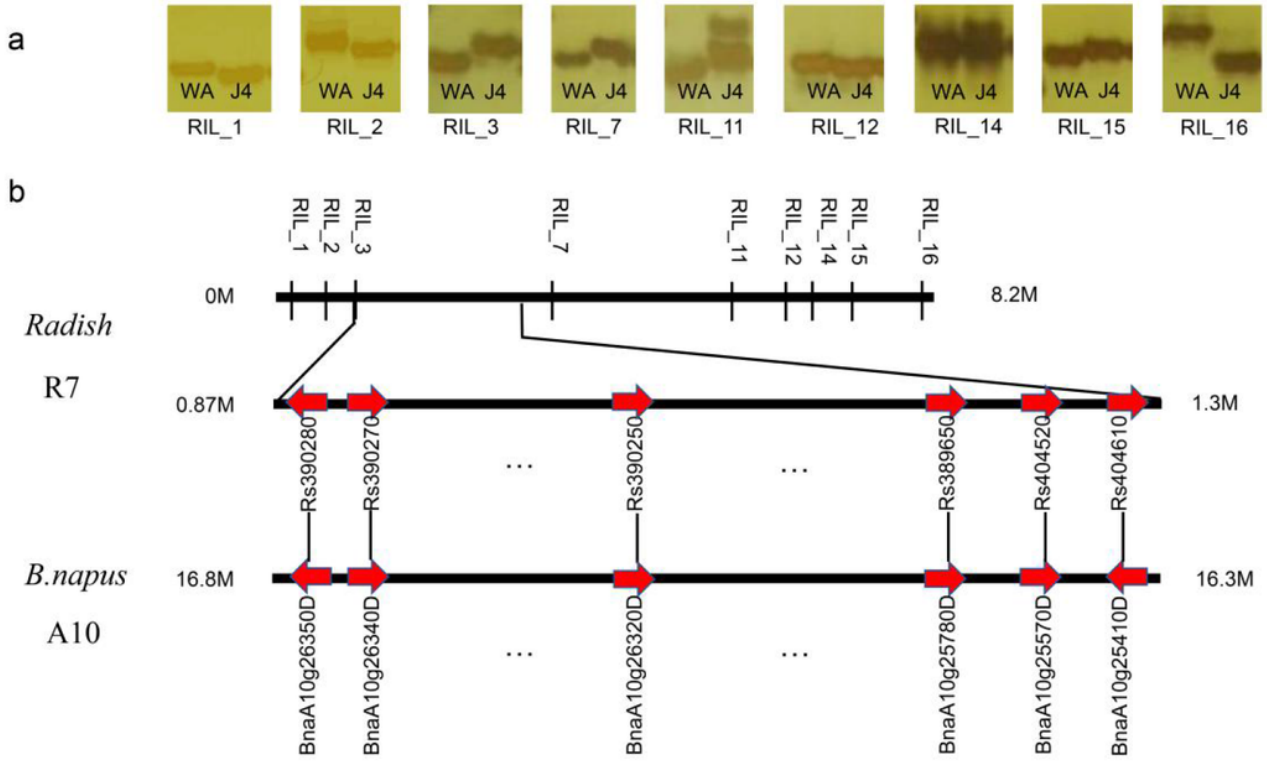

Figure 3

The lobed-leaf gene mapping in R. sativus and the collinearity analysis of candidate interval (a) Polymorphisms between WA and J4 detected by INDEL markers. (b) Collinearity between the target region of lobed-leaf gene on chromosome R7 of R. sativus and chromosome A10 of B. napus.

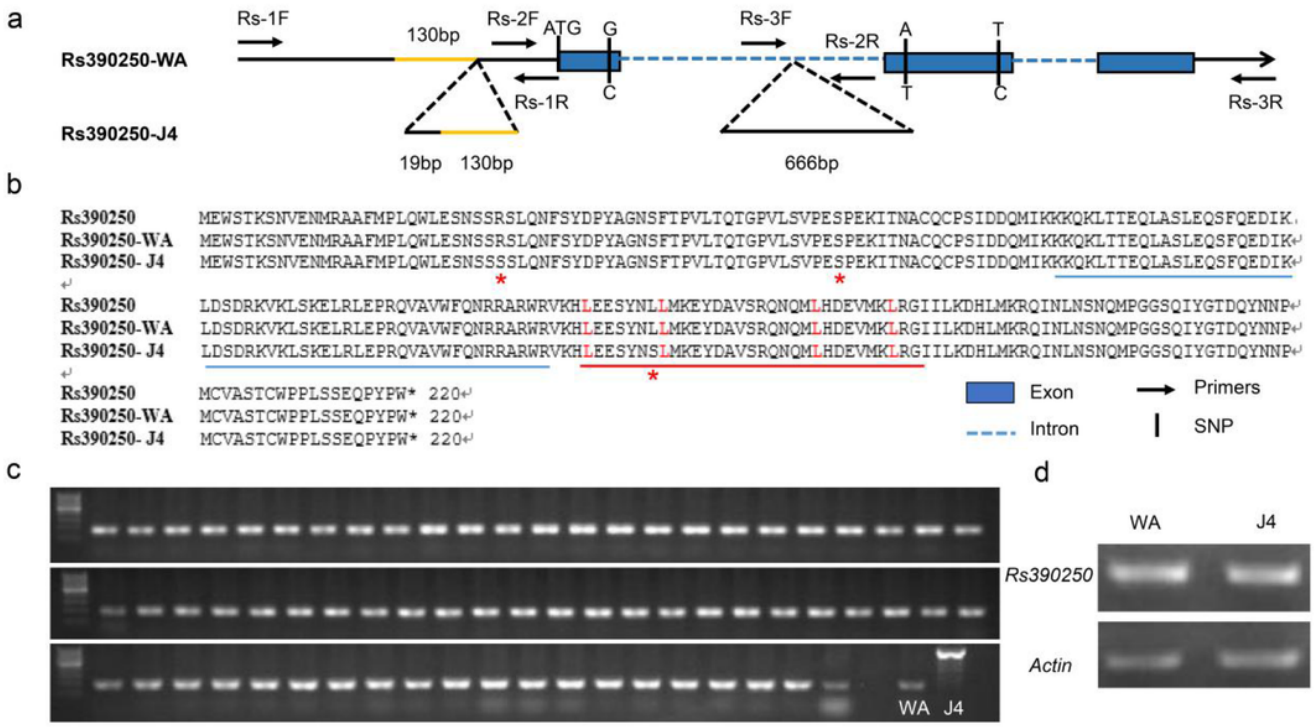

Figure 4

Gene structure and expression level of candidate gene Rs390250 (a) The gene structure of Rs390250; (b) Alignments of Rs390250 amino acid sequences of WA and J4. '*' indicates amino acid change; Blue lines indicate the homeobox and red lines indicate the homeobox-associated leucine domains. (c) Primer Rs-3F/Rs-2R was used to detect the co-segregation of Rs390250 with lobed-leaf trait. All 68 non-lobed leaves do not contain a 666 bp transposon sequence. (d) The expression level of Rs390250 in WA and J4. Primer Rsa is used for RT-PCR.

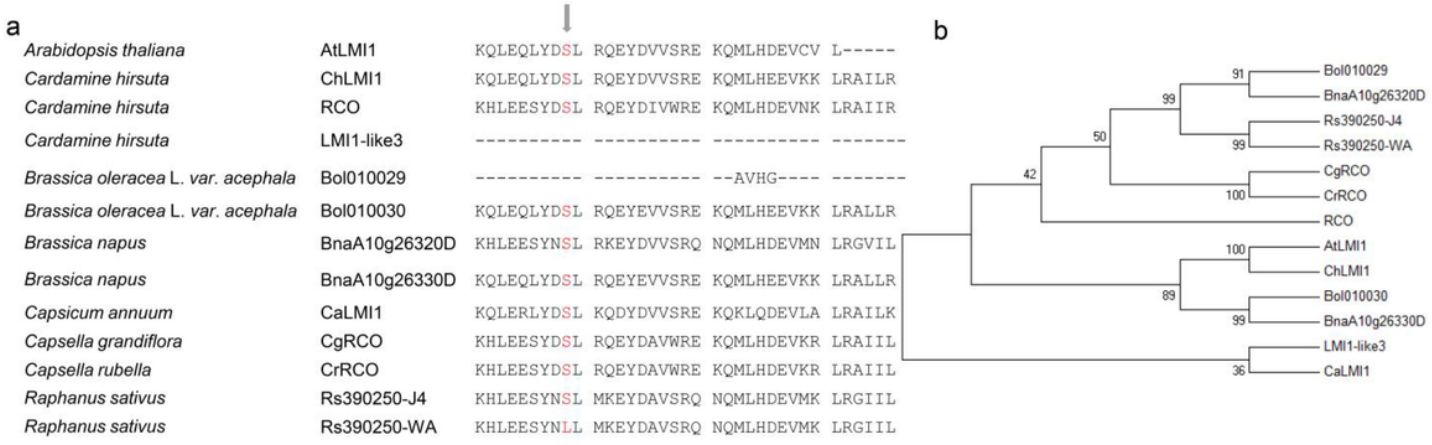

Figure 5

Comparison and phylogenetic analysis of the LMI1-like genes based on amino acid sequence (a) Alignment of amino acid sequences in the LZ domain of LMI1-like genes from different plant species with different leaf shape. The sequences from A. thaliana, B. napus and B. oleracea were download on the Brassica database (http://brassicadb.org/brad/). The others were download on the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/) by searching their genbank accession numbers: ChLMI1 (KF939590), RCO (KF939591), LMI1-like3 (KF939592), CaLMI1 (PHT71668), CgRCO (KM214233) and CrRCO (KM214232). The arrow indicates the position of the absolutely conserved amino acid. (b) Phylogenetic analysis of the LMI1-like genes based on amino acid sequence from different plant species.