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Genetic Mapping of a Soybean Glabrous Gene Using Specific-Locus Amplified Fragment Sequencing Method

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ABSTRACT

Trichome is a natural trait related with plant defense and seed yield, however, the genetic control of this trait has not been extensively studied in soybean. In this study, a cross was made between a soybean landrace “Aijiaomaodou” with normal pubescence and a glabrous soybean line “39002”. The segregation ratio of the F_2 population was fit to the expected value and the glabrous phenotype was controlled by a single dominant gene. Association analysis was conducted to identify candidate genes for the glabrous trait using a high-throughput sequencing technology, specific-locus amplified fragment sequencing. A total of 6,961 polymorphic specific-locus amplified fragments were analyzed and a region of 2.76 Mb at nucleotides 47,315,610-50,072,564 on chromosome 9 containing 551 candidate genes was identified. Four specific genes (*Glyma09G280500*, *Glyma09G282600*, *Glyma09G259400* and *Glyma09G283400*) in the region appeared to be most important candidate genes that are highly related to the glabrous trait. Further studies of these genes may lead to isolation and cloning of the glabrous gene and facilitate the selection procedures in soybean breeding.

Key words: Association analysis, Glabrous trait, Pubescence, SLAF, Soybean.

INTRODUCTION

Plant trichomes are specialized and typically unicellular structures that develop on the surface of stems, leaves and roots. Trichomes are widely distributed in terrestrial plants and are present in a variety of forms. Trichomes perform various functions, including protection against insect and pathogen attack, reducing water loss and increasing tolerance to abiotic stress (An *et al.*, 2011; Gao *et al.*, 2011; Pu *et al.*, 2003; Jain *et al.*, 2018; Rehal *et al.*, 2019). However, some previous studies have showed that some insect-resistant soybean varieties had smooth leaf surface and thin trichomes whereas some insect-susceptible soybean varieties had rough leaf surface and dense trichomes (Yang and Ye, 2013; Hunt *et al.*, 2011).

The genetic control of trichome initiation and development has been extensively studied in *Arabidopsis*. More than 30 genes involved in different stages of trichome development have been identified, including 11 genes that affect trichome occurrence and arrangement (Esch *et al.*, 2003; Gao *et al.*, 2008; Ishida *et al.*, 2008). The *GLABRA1* (*GL1*), the first cloned gene controlling trichome initiation, encodes a R2R3 MYB transcription factor. The GL1 could bind either GLABRA3 (GL3) or ENHANCER of GLABRA3 (EGL3), basic helix-loop-helix type transcription factors. Either GL1-GL3 or GL1-EGL3 could in turn bind TRANSPARENT TESTA GLABRA1 (TTG1), a WD40 type transcription factor to form a ternary complex, GL1-GL3/EGL3-TTG1. This complex could bind *GLABRA2* (*GL2*), a homodomain/leucine zipper transcription factor and then initiate the progression of an epidermal cell development into a trichome.

Unlike *Arabidopsis*, research information on trichome initiation and development has rarely been reported in soybean (*Glycine max*). Compared to soybean with normal pubescence, there are five aberrant pubescence types, including P1 (glabrous), pc (curly pubescence), Pd (dense pubescence), Ps (sparse pubescence) and p2 (puberulent) (Bernard and Singh, 1969). The research showed that each of the five types differed from the normal by a single gene pair and that the five loci segregated independently. These five distinct types of pubescence genotypes provide excellent genetic materials for exploring specific genes controlling the biotypes and studying physiological functions. To date, relevant molecular research is relatively limited and study on genetic mechanism and regulation is rarely reported.

The glabrous and pubescent characters of soybean were controlled by a pair of alleles and the glabrous trait was completely dominant (Sun, 2007). Using bulked segregant analysis (BSA) and genetic mapping, the *P1* gene was mapped on molecular linkage group K (chromosome 9), between the simple sequence repeat (SSR) makers Sat_126 and Sat_020 with the distances of 3.6 and 10.8 cM, respectively. The transcriptional profiles in wild-type (cv. Clark standard, CS) and a mutant (cv. Clark glabrous, CG) soybean isoline that carried the dominant *P1* allele were

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compared using Digital Gene Expression Tag profiling and RNA Sequencing (Hunt *et al.*, 2011). The genome-wide comparative analysis revealed a number of differentially expressed genes. One gene, *Glyma04g35130* (a BURP family member) was abundantly expressed in the CS line and very weakly expressed in the CG line. However, this gene was not a candidate for the *P1* locus.

Soybean stem and leaf trichomes are closely related with seed yield and stress tolerance (Hunt *et al.*, 2011; Lam and Pedigo, 2001; Ren *et al.*, 2018). Pod trichomes also have a direct impact on the picking and commodity of vegetable soybean. Therefore, it is important to explore genes and proteins that regulate trichome formation in soybean. To make use of glabrous character and breed glabrous soybean variety, we reported on the hereditary analysis and genetic mapping of a *P1* (glabrous) gene in soybean using specific-locus amplified fragment sequencing (SLAF-seq) method. This may lead to isolation and cloning of this *P1* specific gene. This work will greatly facilitate the selection procedures in soybean breeding programs and it lays a foundation for further cloning of the *P1* gene.

MATERIALS AND METHODS

Plant materials

The pubescent soybean landrace "Aijiaomaodou" was crossed with the glabrous soybean line "39002" (the progeny from the cross of "4074" and "Ya99009") to obtain the F_1 hybrid seed in 2015. Then the F_1 seeds were grown to produce the segregating F_2 population in 2016. The phenotypic classification of the 229 F_2 individuals was verified by the $F_{2:3}$ lines derived from the F_2 plants in 2017 (Singh and Sharma, 2016). The plant populations were grown in the research fields of Zhejiang Academy of Agricultural Sciences, Hangzhou, China.

DNA extraction

Ten respective individuals each of the glabrous and pubescent phenotype were selected at the F_2 population. Genomic DNAs were isolated from young leaves of both parents and the F_2 individuals using CTAB method (Murray and Thompson, 1980). The DNA was diluted to 50 ng/µl with OD260/280 of 1.8~2.2 for SLAF sequencing.

SLAF library construction

The specific-locus amplified fragment (SLAF) library was constructed based upon the previous research (Abe *et al.*, 2012; Xia *et al.*, 2015; Geng *et al.*, 2016). The reference genome of soybean was used with a size of 978.52 Mb (*http://phytozome.jgi.doe.gov/pz/portal.html#!bulk?org=Org_Gmax*). The enzyme digestion was predicted electronically on the reference genome and the restriction enzyme was determined as HaeIII. Purified DNAs were digested into DNA fragments with 264-314 bp in size. These fragments were defined as SLAF labels. Then fragment ends were repaired, index paired-end adapters were ligated and adapter-modified ends were obtained. Size on gel was selected and the fragments were amplified in PCR. The sequencing was executed using IlluminaHiSeq™ 2500 (Illumina, USA).

Association analysis

SNP-index association analysis was used to calculate genotype frequency differences between the two bulks that are satisfied by DSNP-index. DSNP-index was calculated using the procedures described by the previous studies (Abe *et al.*, 2012; Geng *et al.*, 2016). In this study, P stands for the male parent (glabrous phenotype) and M stands for the female parent (pubescent phenotype); aa represents the pubescent-phenotype pool and ab represents the glabrous-phenotype pool. Maa stands for the depth of the aa group derived from M while Paa means the depth of the aa group derived from P. Mab indicates the depth of the ab group derived from M while Pab means the depth of the ab group derived from P.

SNP_index (ab) = Mab / (Pab + Mab);
SNP_index (aa) = Maa / (Paa + Maa);
ΔSNP_index = SNP_index (aa) – SNP_index (ab).

Quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) analysis was performed according to the previous reports (Yuan *et al.*, 2017; Jin *et al.*, 2016). Total RNA was isolated from young leaves of two parent lines using a MiniBEST Plant RNA Extraction Kit (TaKaRa, Beijing, China). The quality and quantity of the total RNA were assessed using a UVS-99 spectrophotometer (ACTGene, USA). The housekeeping gene *β-actin* was used as an internal reference gene. First-

Table 1: Primers for the qRT-PCR of the tested genes.

Gene	Primer specificity	Primer sequence (5′-3′)
Glyma09G280500	Forward	CAGCTCGAAGAATGGGAAAG
	Reverse	CCCCTTGGTGCTCTGATAAA
Glyma09G282600	Forward	GCGAGCAAGGTAGAGGACAC
	Reverse	ACTAGCACGGACATCCCAAC
Glyma09G259400	Forward	ACCTGGTTTCATCGTCAAGG
	Reverse	ATTAACACGGTCACCGCTTC
Glyma09G283400	Forward	TTCACCCTTCAAACCTCCAC
	Reverse	AGCGATACGCGACTTTGACT
β-actin	Forward	GAGCTATGAATTGCCTGATGG
	Reverse	CGTTTCATGAATTCCAGTAGC

strand cDNA was synthesized using oligo (dT) primers and the genes were subjected to qRT-PCR with specific primers (Table 1). The qRT-PCR assay was performed in triplicate with SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) on a LightCycler® 480 II instrument (Roche, Germany).

RESULTS AND DISCUSSION

Genetic analysis

To study the genetic characteristics, the pubescent landrace "Aijiaomaodou" was crossed with the glabrous line "39002" to obtain F_1 seeds. All F_1 plants from the cross of "Aijiaomaodou" and "39002" were glabrous indicating dominant nature of glabrous type. The 229 F_2 individuals showed two phenotypes, 163 glabrous and 66 puberulent plants. The segregation ratio fits to the expected 3:1, with χ^2 = 0.8499 and P = 0.357. The glabrous F_2 individuals segregated in the $F_{2:3}$ generation while all puberulent plants remained pubescent. The segregation of glabrous and puberulent individuals in the $F_{2:3}$ population perfectly fit in 5:1 ratio. These findings indicated that the glabrous trait was controlled by a single dominant gene.

Association analysis

After SLAF library construction, the SLAF tags were well-distributed on each chromosome (Fig 1). A total of 161,367 SLAF tags were developed as the basis for sequencing and 82,533 SNP markers were identified (Table 2). A total of 6,961 SNP markers were finally selected for further association analysis. The association value was calculated using the SNP-index method and ΔSNP-index was fitted using LOESS method (Abe *et al.*, 2012; Geng *et al.*, 2016). The threshold value was 0.667 according to the expected segregation ratio (Fig 2). The association region was on chromosome 9 and had a size of 2.76 Mb at nucleotides 47,315,610-50,072,564. Bernard and Singh (1969) reported a single gene difference between the glabrous (*P1*) and pubescent (*p1*) plants with glabrous being dominant in

Table 2: The distribution of Specific-Locus Amplified Fragment (SLAF) and Single Nucleotide Polymorphism (SNP) on soybean chromosomes.

Chromosome number	SLAF number	SNP number
01	10,641	4,064
02	7,518	2,990
03	6,947	5,082
04	9,947	3,643
05	7,177	1,882
06	7,727	4,673
07	7,187	2,264
08	7,484	2,085
09	8,230	2,913
10	8,887	4,177
11	5,386	2,290
12	6,506	1,722
13	7,095	5,098
14	8,968	3,651
15	8,213	8,708
16	5,824	4,461
17	6,879	3,380
18	9,126	8,058
19	8,177	6,349
20	8,350	2,504
Other	5,098	2,539
Total	161,367	82,533

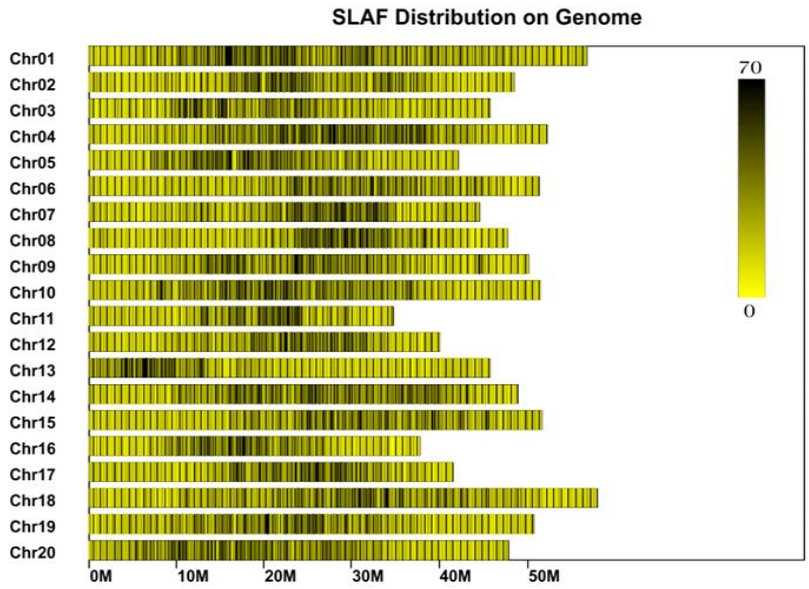


Fig 1: SLAF distribution on soybean genome.
The x-axis is the length of chromosome. Each yellow band represents a chromosome. The more the SLAF number, the darker the color.

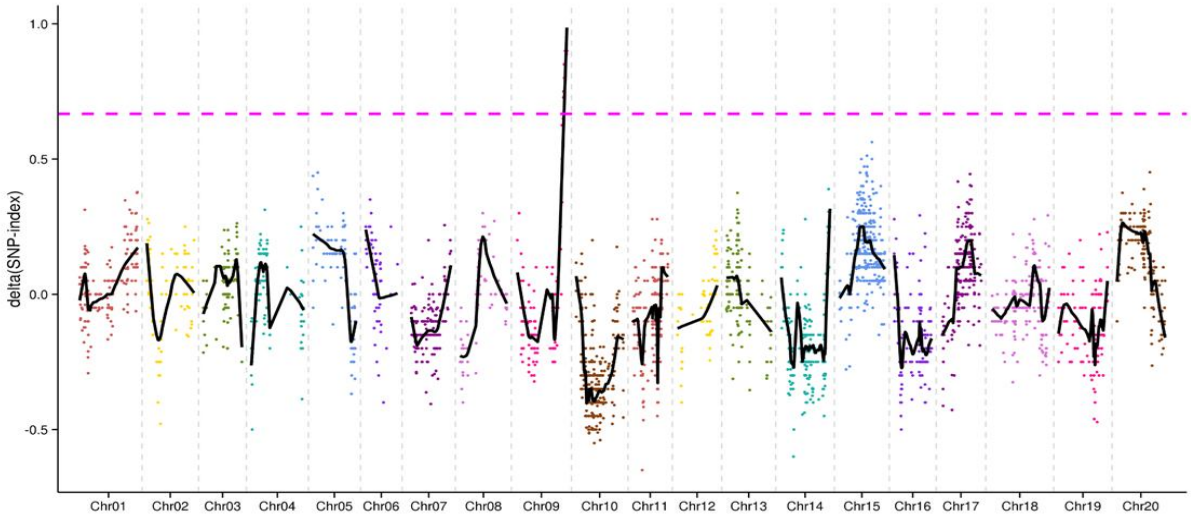


Fig 2: The distribution of ΔSNP-index on soybean chromosomes

The x-axis is the chromosome number. The colorful dots represent the calculated values of DSNP-index. The black lines are the fitting values of ΔSNP-index. The purple dashed line represents the threshold value.

Table 3: SNP annotation of the associated region for the glabrous trait in soybean.

Type	Different SNP
INTRON	9
UPSTREAM	14
DOWNSTREAM	13
UTR_5_PRIME	2
SYNONYMOUS_CODING	3
NON_SYNONYMOUS_CODING	7
Other	0

soybean. A linkage genetic map was constructed using a F_2 population with 400 SSR markers and detected a QTL for glabrous phenotype (Sun, 2007). This QTL was located on molecular linkage group K (chromosome 9) in soybean. These findings were consistent with our results.

Sun (2007) reported that the genetic distances between the P1 gene and the two SSR markers (Sat_126 and Sat_020) were 3.6 and 10.8 cM, respectively. SLAF-seq is a large-scale genotyping method with high efficiency and resolution (Sun, 2007; Sun *et al.*, 2013). Compared with traditional methods, the SLAF-seq method is more effective with a low cost for constructing high-density linkage maps (Jia *et al.*, 2016). In this study, the F_2 population was divided into glabrous and puberulent pool, with 10 individuals in each pool. With the application of SLAF-seq method, the *P1* gene was further narrowed downed to a region at nucleotides 47,315,610-50,072,564 of chromosome 9 in this study. There were approximately 551 genes in the association region. The variation of the SNP was annotated based on SNP difference on the exon region between the two parents, “Aijiaomaodou” and “39002” (Table 3). This internal identified by SLAF-seq provided a clue to further isolate the target gene.

Gene annotation

To explore putative functions of the genes in the association region, the candidate genes were blasted with *Non-redundant protein* (NR), *Swissprot*, *Gene ontology* (GO), *Kyoto Encyclopedia of Genes and Genomes* (KEGG) and *Cluster of Orthologous Groups of proteins* (COG) databases, respectively. This region on chromosome 9 containing 551 candidate genes was identified as potentially associated with the glabrous trait. Finally, 538 of the 551 genes were annotated by the blasting. Among these annotated genes, 178 were found to be involved in NR database, 128 annotated in Swissprot database, 138 included in GO database, 39 involved in KEGG pathway and 58 annotated in COG database.

Since nonsynonymous substitutions alter amino acid sequences and thus result in a biological change, we primarily considered this type of genes and then four genes were identified from annotation information. In this region, there were seven nonsynonymous mutations associated with four soybean genes, including *Glyma09G280500*, *Glyma09G282600*, *Glyma09G259400* and *Glyma09G283400*. Therefore, one of these four genes might be a functional gene directly related to the glabrous trait in soybean. However, the other genes might be interesting genes highly related to the trait.

Confirmation of genes by qRT-PCR

To certify the reliability of the SLAF-seq method, the expression of the four genes (*Glyma09G280500*, *Glyma09G282600*, *Glyma09G259400* and *Glyma09G283400*) was examined by qRT-PCR. The soybean *β-actin* gene was used as an internal control. The expression data showed significant differences between the two parents (Fig 3). The qRT-PCR expression of the gene *Glyma09G282600* was higher in “39002”, whereas the expressions of the other three genes were higher in “Aijiaomaodou”.

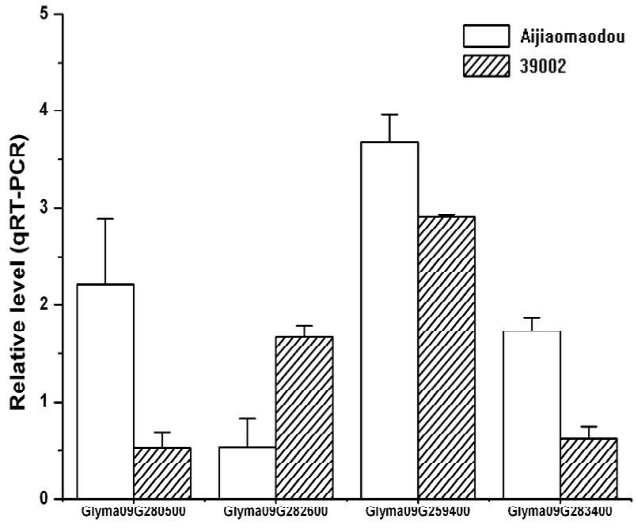


Fig 3: Verification of SLAF-seq results by quantitative real-time PCR (qRT-PCR).
The relative expression level was determined by qRT-PCR using the 2-ΔΔCT method for three biological replicates.

The genes involved in trichome initiation and development have been well characterized in Arabidopsis (Hunt *et al.*, 2011; Esch *et al.*, 2003; Gao *et al.*, 2008; Ishida *et al.*, 2008). However, soybean orthologs potentially regulating trichome development have not been investigated in soybean. In this study, four candidate genes were found to be possibly associated with the glabrous phenotype in soybean. Three of these four genes were annotated in GO database, in which the *Glyma09g280500* was glucosyl transferase, the *Glyma09g259400* was RNA polymerase II transcription mediator and the *Glyma09g283400* was molecular chaperone. Further comparative sequencing and expression analysis are under way in our lab to identify the potential *P1* gene for glabrous phenotype in soybean.

CONCLUSION

Trichome is a direct trait for plant defense and seed yield. The glabrous (*P1*) gene was mapped to the chromosome 9 with an interval of 2.76 Mb. Among the 551 genes in this region, four specific genes (*Glyma09G280500*, *Glyma09G282600*, *Glyma09G259400* and *Glyma09G283400*) appeared to be the most important candidate genes that are highly related to the glabrous trait. The application of SLAF-seq method could help identify the target region of the *P1* gene and further unravel the molecular mechanism of the glabrous phenotype.

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Conflict of interest

The authors declare that they have no conflict of interest.

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