Mapping of Purple Gene in Spears of Asparagus (*Asparagus officinalis* L.)

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Abstract [**Objectives**] This study was conducted to find out regulatory genes related to purple in spears of asparagus (*Asparagus officinalis* L.). [**Methods**] The stable asparagus inbred line JX1513-5 (the base of the spear is purple) and JLV1718-7 (the base of the spear is green) were used as parents to study the genetic law of purple/green traits in their offspring. [**Results**] The results showed that the purple in the basal part of asparagus spear was controlled by a pair of alleles, and purple was dominant over green. The F_2 segregation population was resequenced by the bulk segregation analysis (BSA) method, and the purple trait in the basal part of asparagus spear was located in the interval of 24.51 - 25.08 Mb on Chr07 chromosome, which included 47 genes. According to the annotation information, three candidate genes were screened out: LOC109849403, LOC109849430 and LOC109849442. The candidate genes were verified by real-time fluorescence quantitative PCR (qRT-PCR), and finally LOC109849442 was obtained as the candidate gene for controlling the purple/green trait in the basal part of asparagus spear. [**Conclusions**] This study lays a foundation for the breeding of new asparagus varieties and molecular marker-assisted breeding.

Key words Asparagus; Anthocyanins; Gene mapping; Purple

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Asparagus (*Asparagus officinalis* L.), a perennial herb, originated in the Mediterranean region, has high nutritional and medicinal value and is deeply loved by people. Asparagus has an annual planting area of about 100 000 hm^2 in China, playing an important role in vegetable production[1].

The color of the basal part of asparagus spear is one of the important traits that affect its appearance and quality, and few studies have been conducted on the color formation of asparagus spears at home and abroad. The purple skin of plants is mostly related to the accumulation of anthocyanins. At present, there are many studies on the biosynthesis and metabolism of anthocyanins in plants, and anthocyanin biosynthesis is catalyzed by many enzymes[2-3]. In specific, under the action of phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate-Coa ligase (4CL), phenylalanine is firstly used to synthesize coumaric acid coenzyme A, which is then catalyzed by action of chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H) and dihydroflavonol reductase (DFR) to produce leucoanthocyanidin, which is then transformed into colored anthocyanins under the catalysis of anthocyanidin synthase (ANS), and finally stable anthocyanins are formed through a series of methylation, glycosylation and acylation reactions[4]. There have been some reports about the inheritance law of plant peel and flesh color. Branham *et al.*[5] mapped the major locus related to β-carotene on chromosome 1 in watermelon. Toppino *et al.*[6] mapped two major QTLs for fruit color under the calyx on chromosomes 5 and 10 of eggplant, of which the major QTL on chromosome 10 contributed 82.5%. Qiao[7] used BSA-seq to locate seven genes related to fruit color under the calyx of eggplant.

In recent years, bulked segregation analysis (BSA) has been widely used in watermelon, eggplant, wheat and other species as a technical means of gene mapping research[8]. At present, there has been no report on the genetic law and gene mapping of asparagus epidermis color. In this study, the stable inbred line JX1513-5 with purple basal part of spear and the stable inbred line JLV1718-7 with green basal part of spear as parents to construct a population for studying the genetic mechanism of spear color, and BSA-seq analysis was applied for gene mapping to identify regulatory genes related to purple peel at the base of spear in asparagus, laying a foundation for the breeding of new asparagus varieties and molecular marker-assisted breeding.

Materials and Methods

Experimental materials

The experimental asparagus seeds were obtained from the germplasm resource bank of Institute of Cash Crops, Hebei Academy of Agriculture and Forestry Sciences. In the spring of 2019, JX1513-5 was used as the female parent (P_1, purple at the base of spear) and JLV1718-7 as the male parent (P_2, green at the base of spear) to prepare a hybrid combination and harvest F_1. Next, F_1 was planted in the spring of 2020, and F_2 was obtained through selfing in 2021, while F_1 was backcrossed with parents to obtain BC_1P_1 and BC_1P_2, respectively. In the autumn of 2022, F_2, BC_1P_1 and BC_1P_2 were planted, with 265 plants in the

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F_2 population, 87 plants in the BC_1P_1 population, and 117 plants in the BC_1P_2 population. Conventional cultivation management was adopted.

Construction of extreme population mixed pools and genotyping

From the F_2 population, 30 plants with purple spears and 30 plants with green spears were selected to construct mixed pools for the two extreme traits, and DNA was extracted from the two constructed mixed pools and the two parental materials. The DNA extraction was performed using the CTAB method, and a library was constructed following the Vagyme standard process. Moreover, whole genome resequencing (10 × and 30 × coverage) was performed on the two F_2 mixed pools and two parents using Illumina-HiSeq4000.

Correlation analysis and candidate gene mapping

After quality control and filtering, the resequencing data were mapped to the asparagus reference genome through BWA for comparison (GCA_001876935.1). GATK and SnpEff were used for detecting and annotating the obtained SNPs and InDel, and correlation analysis was then performed. SNP and InDel correlation analysis were conducted using SNP-index and InDel-index to obtain candidate intervals controlling target traits. BLAST was applied for in-depth annotation of the coding genes within the candidate intervals to obtain preliminary candidate genes.

Real-time fluorescence quantitative PCR

According to relevant gene sequences in the NCBI Genome Database (http://cucurbitgenomics.org/organism/21), Primer Premier v5.0 was used to design primers (Table 1). Real-time fluorescence quantitative PCR (qRT PCR) was performed on the Bio Rad CFX96 real-time PCR detection system (Bio Rad, USA) using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). Next, ubiquitin long tail fusion (GenBank: X66875.1) was selected as the internal reference gene, and the relative expression level was calculated by the $2^{-\triangle\triangle ct}$ method. Each experiment was done with three biological replicates and three technical replicates.

Table 1 qRT-PCR primers used in this study

Gene name	Sequence (5′-3′)	Product//bp
LOC109849442	TCCCTGTCGACTACATCCGA	189
	CTCCCCATTCTTTAGCGGCA	
LOC109849403	GCATTTGGAGAGCGCAAGAG	181
	TCTGGGTGCTCCCATCAGTA	
LOC109849430	AGCATTGGAATTGAGCCCGA	176
	GCATCCCCTACATCTGCGAA	

Results and Analysis

Genetic analysis of the purple trait in the basal part of asparagus spear

The color in the basal part of spear in the hybrid offspring of JX1513-5 and JLV1718-7 was investigated, as shown in Table 2, and the results showed that the spears in the F_1 population were all purple at the base. In the F_2 population with 265 plants, color segregation was observed in the basal part of spear, including 62 green plants and 203 purple plants. The results of chi-square testing showed $\chi^2 = 0.363\ 52 < 3.841$ [when $df = 1$, $\chi^2(0.05) = 3.841$], following the 3∶1 segregation law. For the BC_1P_1 population with 87 plants, all plants showed a purple color. Among the 117 plants in the BC_1P_2 population, the ratio of purple to green was 56∶61. The results of chi-square testing showed $\chi^2 = 0.213\ 68 < 3.841$, consistent with the 1∶1 segregation law. It indicated that the color trait in the basal part of asparagus spear was controlled by a pair of genes, with purple being the dominant trait over green.

Table 2 Statistics on the color of the basal part of spear in segregated population

Generation	Total number of plants//plants	Number of purple plants//plants	Number of green plants//plants	Ratio	χ^2 value
P_1	3	3			
P_2	3		3		
F_1	174	174			
F_2	265	203	62	3.27∶1	0.363 52
BC_1P_1	87	87			
BC_1P_2	117	56	61	0.92∶1	0.213 68

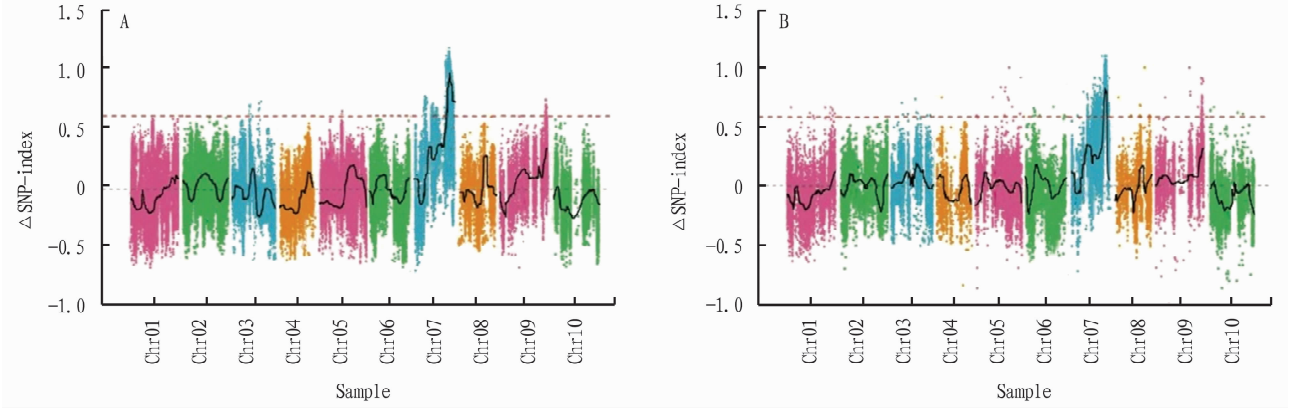
Construction of extreme population mixed pools and analysis of sequencing data

Two mixed pools constructed and two parents were subjected to whole genome re-sequencing, resulting in a total of 70.35G of raw data. The effective sequence data sizes of the four samples after filtering ranged from 9 564.94 to 27 572.64 M, and the total data size was 69.74 G. The sequencing data showed that Q20 > 97.65%, Q30 > 93.17%, and the GC contents were in the range of 35.88% -6.27%. After comparison, 92.5% -96.3% of the sequences were consistent with the reference genome of asparagus. From this, it could be seen that the sequencing quality of all samples was qualified and could be used for subsequent gene mapping.

Gene mapping of purple trait in the basal part of spear

Correlation analysis was performed on BSA seq data from the two parents and two mixed pools using SNP index (Fig. 1). An interval with a length of 2.47 Mb (24.09 -26.56 Mb) was obtained on Chr 07 through SNP correlation analysis, and an interval of 0.57 Mb (24.51 -25.08 Mb) was obtained on Chr 07 through InDel correlation analysis. Intersecting the associated regions of

SNPs and InDel, a candidate region related to purple trait was obtained, and its position on the reference genome was Chr 07 (24.51 – 25.08 Mb). Through comparison of several databases, 47 genes were annotated in this region (Table 3). Therefore, in this study, the gene controlling the purple basal part of asparagus spear was located at chromosome 7, 24.51 – 25.08 Mb, with a total length of 0.57 Mb.



A, SNP correlation analysis; B, index correlation analysis.

Fig. 1 △SNP-index correlation analysis chart based on SNP and InDel of BSA-seq

Table 3 Statistics on annotation of functional genes in candidate intervals

Database	Number of genes
COG	28
GO	35
KEGG	21
KOG	25
Pfam	20
Swiss-Prot	32
eggNOG	31
NR	41
Total	47

Gene functional annotation of candidate regions

In-depth annotation of the 47 genes in the candidate interval was carried out by using KEGG and GO databases, and three genes related to purple peel formation were obtained. LOC109849442 was annotated as ANS-associated in asparagus reference genome. ANS is a key enzyme at the end of plant anthocyanin biosynthesis pathway, which catalyzes the transformation of leucoanthocyanidin into colored anthocyanins. LOC1098494403 was annotated as a chloroplast coding protein, and LOC109849-4430 was a nucleic acid-related protein related to chloroplast development. Both genes were related to chloroplast development. Since chloroplasts can be transformed into plant chromoplasts, these two genes may affect the color of asparagus spears by affecting the formation of chromoplasts.

Functional validation of candidate genes

QRT-PCR was performed on the three candidate genes screened in five purple spear varieties (Pe1-Pe5) and five green spear varieties (Gn1-Gn5), and the relative expression levels are shown in Fig. 2. The expression levels of LOC109849442 in the five purple spear varieties were significantly higher than those in the five green spear varieties, and the expression levels of LOC109849403 and LOC109849430 in the purple spear varieties and green spear varieties showed no significant regularity. Therefore, according to the relative expression levels of the three candidate genes in the 10 materials, the expression levels of LOC109849403 and LOC109849430 had no obvious regularity, while the expression level of LOC109849442 was closely related to the purple basal part of asparagus spear, so LOC109849442 was determined to be the candidate gene for controlling the purple/green trait in the basal part of asparagus spear.

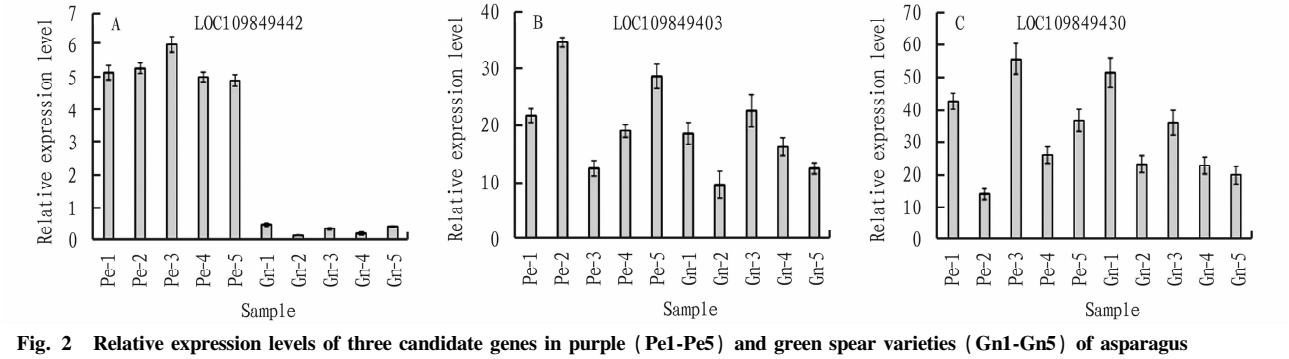


Fig. 2 Relative expression levels of three candidate genes in purple (Pe1-Pe5) and green spear varieties (Gn1-Gn5) of asparagus

Conclusions and Discussion

Anthocyanins are an important class of natural pigments that mainly exist in higher plants in the forms of pelargonidin, cyanidin, delphindin, *etc.* The abundance of anthocyanins leads to the diversification of plant color[9]. The color of the basal part of asparagus spear is one of the important traits that affect its appearance quality. Therefore, conducting research on the localization of color trait genes in the basal part of asparagus spear and screening of candidate genes is of great significance for efficient molecular breeding.

This study investigated the heredity laws of purple/green in spears of JX1513-5, JLV1718-7 and their hybrid offspring. It was found that the purple basal part of asparagus spear was controlled by a pair of alleles, and purple was a dominant trait over green. Meanwhile, the BSA method was used for resequencing and gene mapping of the F_2 segregation population. The purple regulatory gene was located in the 0.57 Mb interval on the Chr 07 chromosome of asparagus. There were 47 genes in the target region, and three genes related to the formation of purple peel were identified through annotation and comparison. After qRT PCR validation, LOC109849442 was ultimately identified as a candidate gene for controlling the purple/green trait in the basal part of asparagus spear. LOC109849442 was annotated as ANS in the reference genome of asparagus, which is a key enzyme at the end of the plant anthocyanin biosynthesis pathway that catalyzes the transformation of leucoanthocyanidin into colored anthocyanins[10]. At present, the isolation of multiple ANS genes from plants has important value in studying the mechanism of plant color formation and abiotic stress physiology.

In this study, the BSA method and asparagus genome information were used to quickly map the purple/green genes in the basal part of asparagus spear, providing a reference for gene mapping of other traits in the future and laying a foundation for the breeding of new asparagus varieties and molecular marker-assisted breeding.

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