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RESEARCH ARTICLE

Genome-wide association study of appearance quality traits and development of KASP markers in vegetable soybean

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Highlights

- Genome-wide association study (GWAS) analysis of 264 soybean accessions identified 525 SNPs significantly associated with pod appearance traits (length, width, and color) across multiple environments.
- Five candidate genes were pinpointed as potentially regulating these key pod quality traits.
- Functional KASP markers were developed for three major SNPs, providing practical tools for molecular breeding of vegetable soybean with improved visual appeal.

Abstract

Vegetable soybean ((*Glycine max* (L.) Merr.), commonly referred to as edamame, holds significant agricultural importance in China as a legume vegetable harvested at the pod-filling stage (R6). The visual appeal of vegetable soybeans is crucial for consumer preference and marketability, and it depends on factors such as pod length, pod width, and pod color. This study cultivated 264 vegetable soybeans in Nanjing, Huai'an, and Nantong, Jiangsu Province, China to assess pod traits using PlantPhenoM, a system for pod phenotypic identification and analysis. The results revealed a variability range of 8.64 to 30.00% in appearance quality traits among the vegetable soybeans. Leveraging phenotypic data and employing a genome-wide association study (GWAS) identified 525 SNPs significantly linked to the appearance quality traits in different regions. In addition, five candidate genes (*Glyma.04G004700*, *Glyma.15G051600*, *Glyma.18G225700*, *Glyma.18G225900*, and *Glyma.18G272300*) associated with target traits were identified, and KASP markers for S04_372771 (pod length), S18_51477324 (pod width), and S18_55553200 (pod color) were developed. This study offers valuable insights for breeding superior vegetable soybean varieties and lays the groundwork for exploring candidate genes and molecular markers related to appearance and quality traits in vegetable soybeans.

Keywords: appearance quality, genome-wide association study, KASP marker, vegetable soybean

1. Introduction

Vegetable soybeans are gaining increasing recognition for their rich nutritional profile and delightful, tender texture. Their abundant supply of protein, fiber, and essential nutrients has made them a popular choice among health-conscious consumers. China boasts a rich tradition of cultivating vegetable soybeans, and it has emerged as a primary global exporter of this crop over the past decade (Williams 2015; He *et al.* 2023; Zhang L *et al.* 2023). Vegetable soybeans are evaluated based on four essential criteria based on their appearance, health, nutritional, and edible qualities (Wang *et al.* 2002; Zhang L *et al.* 2023). Among them, appearance quality plays a pivotal role in determining the grade of vegetable soybean products (Zhang *et al.* 2013). The quality of appearance, including factors such as pod size, pod color, and grain count, holds particular significance as it directly

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influences consumer preferences and bolsters the farmers' bargaining power in trade negotiations (He *et al.* 2023). Green and sizable pods are the defining features of vegetable soybeans (Dong *et al.* 2014). Amidst the challenges facing the soybean industry and the surging demand for vegetable soybeans at home and abroad, developing premium-quality vegetable soybeans is necessary. Consequently, the selection and breeding of germplasm resources to enhance appearance quality has emerged as a crucial focus in molecular breeding efforts for vegetable soybeans.

In recent years, significant progress has been made in our understanding of the genetic mechanisms underlying the quality traits of vegetable soybeans. Internationally, the increasing demand for vegetable soybeans has imposed a high yield requirement. Totals of 27, 18, 24, 46, and 20 SNPs and 15 potential candidate genes were found to be associated with the 100-grain fresh weight, 100-grain dry weight, yield rate, grain number per pod, and water content of fresh grains in vegetable soybean, respectively. Among which two new SNPs were found in this study, while the others were previously identified (Li X *et al.* 2021). Metabolome and transcriptome analyses have shown that compared to Williams82, vegetable soybean Jiaoda 133 exhibits higher levels of free amino acids, carbohydrates, sterols, and flavonoids, while the levels of fatty acids are lower (Chen *et al.* 2022). Protein, starch and water content are the main factors significantly associated with vegetable soybean seed hardness (Bu 2019). That study identified 11 SNPs significantly associated with seed hardness through genome-wide association analysis of a mini-core collection containing 224 pod-filling (R6)-stage soybean seeds, with Q-05-0194474 detected seven times (Bu 2019). Soluble sugar has an essential influence on the sweet taste of vegetable soybeans. Thirteen QTNs associated with total soluble sugar content were identified by genome-wide association study (GWAS) (Lu *et al.* 2022). Coincidentally, a total of 27 SNPs significantly associated with total soluble sugar were identified *via* GWAS in 264 soybean accessions (Xu W *et al.* 2022). Free amino acids play a key role in sweetness and umami (Song *et al.* 2013; Guo *et al.* 2022). Zhang H *et al.* (2023) employed those 264 soybean accessions and characterized the levels of arginine, alanine, glutamic acid, and aspartic acid of vegetable soybeans in 2020 and 2021. A total of 89 SNPs significantly associated with those four amino acids in vegetable soybeans were detected over the two years. With ongoing research advancements, GWAS can provide a robust theoretical basis for genetic research and enhanced breeding of vegetable soybeans. While there has been some progress in studying the quality traits of vegetable soybeans, progress in some traits remains sluggish, particularly concerning appearance quality.

In general, most research on vegetable soybeans has focused on health, nutritional, and edible quality traits, with less attention paid to appearance traits. In the current study, we cultivated a natural population of 264 soybean accessions in Nanjing, Nantong and Huai'an, Jiangsu Province, China and collected their pods at the R6 stage. For screening out candidate genes that control pod appearance, we measured pod length, width and color using the PlantPhenoM System and performed GWAS on the three traits using a high-density map containing 2,597,425 SNPs. That analysis identified 525 SNPs and screened out five candidate genes associated with pod length, width, and color. In addition, KASP markers were developed for the SNPs S04_372771 (A/G) (for pod length), S18_51477324 (C/T) (for pod width), and S18_55553200 (G/T) (for pod color), offering significant value for genotyping soybean pod appearance.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, a natural population of 264 soybean accessions (52 landraces, and 212 cultivars from China) was assembled (Zhang *et al.* 2021). In 2023, the soybean materials were planted at Nanjing Luhe Test Base of Jiangsu Academy of Agricultural Sciences (Nanjing, Jiangsu Province, 32.32°N, 118.97°E), Jiangsu Yanjiang Regional Institute of Agricultural Science Test Base (Nantong, Jiangsu Province, 32.01°N, 120.86°E), and Huai'an Academy of Agricultural Sciences Test Base (Huai'an, Jiangsu Province, 33.61°N, 119.01°E). A random block design with three replicates was employed. Each vegetable soybean accession was planted in three rows, with 10 holes per row and two seedlings per hole. Standard field management practices were employed, including appropriate fertilization, watering, regular weeding, and pest control.

2.2. Fresh pod image acquisition and preprocessing

When soybeans reached the R6 stage, three individual plants were randomly chosen from each material, and five fresh pods were randomly selected from each individual plant. An HIK industrial camera (Hikvision, China) was used to capture images of fresh pods, which were subsequently imported into the PlantPhenoM system. Then pod length, width and color of the vegetable soybeans were measured. Due to the potential for distortion in the images caused by scaling and rotation, errors may occur in calculating the phenotypic shape of the fresh pods. Therefore, it is necessary to correct the image before collecting data.

Suppose a raw image represented by $f(x, y)$ becomes $g(x', y')$ due to the geometric distortion, and the coordinates of the pixels before and after the distortion satisfy the following relationship:

$$\begin{cases} x'=h_1(x, y) \\ y'=h_2(x, y) \end{cases}$$

By obtaining the analytic expressions $h_1(x, y)$ and $h_2(x, y)$, the corresponding position (x, y) in the original fresh image could be found by distorting the point (x', y') in the fresh image, in order to correct the geometric distortion of the image. The geometric distortion was changed linearly as follows:

$$\begin{cases} x'=ax+by+c \\ y'=dx+ey+f \end{cases}$$

Using the known position coordinates of the constraint points in the correction template before and after image deformation, the following linear mapping equations were

established:

$$\begin{cases} x'_1=ax_1+by_1+c & y'_1=dx_1+ey_1+f \\ x'_2=ax_2+by_2+c & y'_2=dx_2+ey_2+f \\ x'_3=ax_3+by_3+c & y'_3=dx_3+ey_3+f \end{cases}$$

Then the coefficients were solved to produce the image geometric distortion correction and restore the original spatial relationships of the image.

Fresh pod image data is prone to external environmental interference, including noise, and constant iterative calculations cause errors in the measurement of fresh pod phenotypic parameters. To improve the measurement accuracy, a bilateral filtering algorithm was adopted to filter out noise, and the image output from the filtering algorithm was defined as $g(x, y)$. The formula for calculating the bilateral filtering algorithm was:

$$g(x, y)=\frac{\sum_{i,j} f(i, j)w(x, y, i, j)}{\sum_{i,j} w(x, y, i, j)}$$

where (x, y) represents the current processing point; (i, j) is a point in the vicinity of (x, y); and $w(x, y, i, j)$ represents the weighting coefficient.

The distance between two points and the pixel difference were comprehensively considered according to the following formula:

$$w(x, y, i, j)=e^{-\left[\frac{(i-x)+(j-y)^2}{2\sigma_d^2}\right]}\times e^{-\left[\frac{|f(i, j)-f(x, y)|^2}{2\sigma_r^2}\right]}$$

Based on the above formula, in the two-sided filtering algorithm, the weighted coefficient corresponding to the pixel distant from the edge is the smallest, thereby ensuring that the clarity of the pixel value on the edge of the fresh pod was not impacted.

The output result of bilateral filtering will be directly affected by the choice of parameters σ_d and σ_r. When σ_d and σ_r values are small, the image exhibits a minimal smoothing effect. Controlling the proximity of the control space dictates the number of pixels within the filter window. As this number grows, the influence of distant pixels on the central pixel strengthens, thereby enhancing image smoothness. Moreover, the impact of controlling the gray proximity intensifies with higher values of σ_r. When σ_r increases, the gray difference also increases, leading to a heightened impact of pixels on the central point. When the gray difference exceeds a specific threshold value, those pixels will not be included in the calculation, thereby preserving the gray information at the image edges.

The variable ∇f represents the directional gradient vector of the edge intensity of an image f at position (x, y) with an amplitude expressed by $g(x, y)$ and a direction expressed by $\theta(x, y)$, as in the following formulas:

$$\nabla f=grad(f)=\begin{bmatrix} g_x \\ g_y \end{bmatrix}=\begin{bmatrix} \frac{\partial f}{\partial x} \\ \frac{\partial f}{\partial y} \end{bmatrix}$$

$$g(x, y)=\sqrt{g_x^2+g_y^2}\approx|g_x|+|g_y|$$

$$\theta(x, y)=\arctan\left[\frac{g_y}{g_x}\right]$$

where $grad(f)$ can determine the first partial derivative of the function in the directions of the x and y axes, representing the gradient of f at any (x, y). The sum of the absolute values of the first partial derivative of the function in the directions of the x and y axes represents the amplitude of the gradient.

2.3. Measurement of the phenotypic parameters of vegetable soybeans

After obtaining each completely segmented fresh pod image, the transformational relationship between the image and real coordinate system was obtained using the proportional calculation method. Initially, the split fresh pod image contour was calibrated with the minimum enclosing rectangle. Then the length and width of the minimum enclosing rectangle were calculated, and the actual size of the pod in the real coordinate system was obtained by converting it into the Euclidean distance combined with the proportional calculation method. The formulas employed were:

$$U=\frac{K_\alpha}{K_\beta}$$

$$D=\sqrt{(x_2-x_1)^2+(y_2-y_1)^2}$$

where U represents the calculated ratio of the object size, K_α is the pixel length of the object, K_β represents the true length of the object, and D denotes the Euclidean distance between points (x_2, y_2) and (x_1, y_1).

After preprocessing of the acquired fresh pod image, the maximum width of the fresh pod was obtained by finding and calculating the maximum tangential circle diameter of the fresh pod contour. The definitions of the center coordinates of the maximum inscribed circle of the fresh pod contour were:

$$S=\frac{\sum_i (x_i+x_{i-1})(y_i-y_{i-1})}{2}$$

$$M=\frac{\sum_i (x_i+x_{i-1})^2(y_i-y_{i-1})}{S}$$

$$N=\frac{\sum_i (y_i+y_{i-1})^2(x_i-x_{i-1})}{S}$$

where S is the area of the boundary closed profile; M and N are the horizontal and vertical coordinates of the center of the circle; and x_i and y_i are the horizontal and vertical coordinates of a point on the boundary closed profile. After determining the center of the maximum inscribed circle, the radius of the maximum inscribed circle was obtained by calculating the distance between each point on the boundary closed contour and the center of the circle to obtain the width of the vegetable soybean. The formulas for determining the maximum inscribed circle radius were:

$$R=\sqrt{(x_i-m)^2+(y_i-n)^2}$$

$$D=2R$$

where R is the radius of the maximum inner inscribed circle of the pod; x_i and y_i are the horizontal and vertical coordinates of the boundary closed contour; m and n are the horizontal and vertical coordinates of the center of the circle; and D is the pod width.

2.4. Fresh pod color similarity matching

Pod color was identified as the most conspicuous external indicator of the freshness of fresh pods. The fresher the pods, the greener the color, whereas aging pods transition to black or yellow hues. In this study, the pod color gradient was categorized into seven reference samples as described by Zhang *et al.* (2021) (Fig. 1).

For pod color similarity matching, a color similarity matching algorithm was used. First, the fresh pod image was segmented into RGB three-color channels, then the histograms of each channel were calculated, and the histogram of the measured fresh pod image was compared with those seven reference samples based on numerical values. Finally, the matching results of the three-color channels were averaged. The higher the average matching degree, the more similar the pod color is to the reference pod color. The score of the final matching result between the tested and reference pod color was quantified. Scores of 1 to 7 were assigned to the green to black fresh pod color gradient (Fig. 1).

2.5. GWAS

A natural population of 264 soybean accessions was employed for target trait identification and GWAS, and the resequencing information of the population was reported in our previous article (Zhang *et al.* 2021). The overall performances were predicted by the best linear unbiased prediction (BLUP) implemented in the "lme4" R package. The "GAPIT" R software package was used to calculate the GWAS of pod length, width, and color of the vegetable soybeans at the R6 stage. A mixed linear model (MLM) was employed for GWAS to control the false-positive association sites.

2.6. Identification of candidate genes

The linkage disequilibrium (LD) decay distance of the population used in this study was 120 kb (Zhang *et al.* 2021). The candidate region based on pairwise LD correlation was calculated by the R package "LDBlockShow". Through GWAS and the examination of significant SNPs, candidate genes related to the target traits were pinpointed within a 120 kb range around these SNP sites, as guided by the LD decay range of the soybean population. Candidate gene information referenced the Phytozome 13 soybean genome database (https://phytozome-next.jgi.doe.gov/info/ Gmax_Wm82_a2_v1). Candidate gene expression levels were analyzed by the expression data obtained from the publicly available database at https://phytozome-next.jgi.doe.gov (accessed on 19 October 2023) and modified by the $\log_2$ratio.

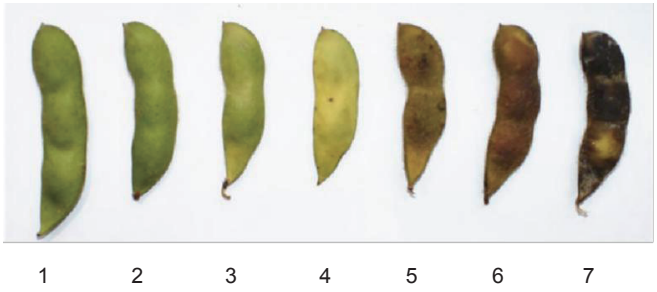


Fig. 1 Schematic diagram of the color gradient of vegetable soybean fresh pods (Zhang *et al.* 2022). Different pod colors were defined as 1 through 7, respectively.

2.7. Development of KASP markers

Using the primer-BLAST function of NCBI (https://www.ncbi.nlm.nih.gov/), primers were designed for S04_372771, S18_51477324, and S18_55553200. Each primer set comprised two specific forward primers F1 and F2, and a universal reverse primer R. F1 included a FAM fluorescent joint sequence, while F2 contained a HEX fluorescent joint sequence (underlined). The primers were synthesized by Qingke Biotech (Nanjing, China).

2.8. PCR for genotyping using KASP markers

The reaction conditions of KASP-PCR were set as follows: pre-denaturation at 94°C for 15 min; denaturation at 94°C for 20 s, followed by ten extension cycles, with each cycle cooling by 0.6°C from 61°C to 55°C for 60 s each; then an additional denaturation at 94°C for 20 s, followed by ten extension cycles at 55°C for 60 s. After completion of the reaction, a QuantStudio5 real-time fluorescence quantitative PCR instrument was used to obtain fluorescence data.

3. Results

3.1. Descriptive statistical and variance analysis of vegetable soybean pod length, width and color

In this study, fresh pods of vegetable soybeans were gathered at the R6 stage from Nanjing, Nantong, and Huai'an in 2023, and three appearance quality traits, namely pod length, pod width, and pod color, were investigated. The three traits of 264 soybean accessions were examined *via* descriptive statistics and variance analysis (Tables 1 and 2).

The coefficients of variation (CV) for the primary appearance quality traits of the 264 materials ranged from 8.64 to 30.00%, suggesting wide genetic diversity, which was helpful in identifying the related genetic loci of appearance quality traits. Pod lengths ranged from 2.82 to 7.26 cm, with an average of 4.27 cm; pod widths ranged from 1.29 to

Table 1 Descriptive statistics of vegetable soybean pod length, width and color at the pod-filling stage (R6) stage[1)]

Trait	Region[2)]	Max	Min	Mean	SD	CV (%)
Pod length	Nanjing	7.26	3.52	4.27	0.43	9.36
	Nantong	5.39	3.22		0.40	9.80
	Huai'an	6.12	2.82		0.43	10.43
Pod width	Nanjing	2.50	1.36	1.77	0.17	10.40
	Nantong	2.53	1.51		0.17	8.64
	Huai'an	2.43	1.29		0.18	10.70
Pod color	Nanjing	6.87	1.47	3.60	0.89	27.40
	Nantong	5.27	1.43		0.66	19.57
	Huai'an	7.00	1.50		1.24	30.00

[1)] Max, maximum; Min, minimum; SD, standard deviation; CV, coefficient of variation.
[2)] Nanjing, Nantong, and Huai'an, Jiangsu Province, China.

Table 2 Variance analysis of vegetable soybean pod length, width and color at the pod-filling stage (R6) stage[1)]

Trait	Variation source	*DF*	SS	MS	*F*-value	*P*-value
Pod length	Variety	1	9.64	1.61	2.77	0.013
	Environment	2	13.47	6.74	11.59	0.000
	Variety×Environment	12	21.48	1.79	3.08	0.001
Pod width	Variety	6	1.57	0.262	2.96	0.009
	Environment	2	7.17	3.587	40.44	0.000
	Variety×Environment	12	4.38	0.365	4.11	0.000
Pod color	Variety	7	31.16	4.45	2.42	0.022
	Environment	2	290.28	145.41	79.06	0.000
	Variety×Environment	14	204.39	14.60	7.95	0.000

[1)] *DF*, degree of free; SS, stdev square; MS, mean square.

2.53 cm, with an average of 1.77 cm; and pod color scores ranged from 1.47 to 7, with an average of 3.60. The CVs of appearance quality traits ranged from 9.36 to 27.40% in Nanjing, 8.64 to 19.57% in Nantong, and 10.43 to 30.00% in Huai'an (Table 1). The CVs of appearance quality traits in Huai'an were higher than those in Nanjing and Nantong, suggesting that this environment exerted a specific influence on the variation of appearance quality traits of vegetable soybeans. Each pod trait at the different test sites showed significant differences with each other, which indicates that these traits can be affected by environment (Fig. 2).

The variance analysis results indicated that the appearance quality-related traits at the R6 stage had significant differences among the various genotypes, different environments, and genotype-environment interaction effects. These findings suggested that the appearance quality traits of vegetable soybeans with different genotypes were impacted by environmental factors and may change in response to environmental fluctuations (Table 2). Therefore, it is feasible to effectively employ genetic variation to exclude genotypes with undesirable appearance quality traits in natural populations of vegetable soybeans, and the screening results will also be influenced by environmental factors.

3.2. GWAS of vegetable soybean pod length, width and color

Microsoft Excel 2016 was employed to determine the pod length, width, and color frequencies across 264 vegetable soybean accessions in Nanjing, Nantong, and Huai'an, and the frequency distribution and density curves were plotted (Fig. 3). The histograms of phenotypic data exhibited the characteristics of approximately normal distributions, indicating that the natural population of vegetable soybeans was rich in genetic variation and suitable for subsequent GWAS.

The phenotypic results of vegetable soybean pod length, width, and color were integrated with the genotype data acquired by resequencing, and MLM was employed to perform GWAS *via* the "GAPIT" R software package. When the threshold of SNP $-\log_{10}(P)\geq 5$ was met, the marker was considered significantly associated with the trait. Manhattan plots were constructed using the "CMplot" R package (Figs. 4–6).

Overall, a total of 525 SNPs significantly associated ($-\log_{10}(P)\geq 5$) with the appearance quality traits of vegetable soybeans at the R6 stage were detected across the three regions in 2023 (Tables 3–5). Among these, the number of SNPs associated with pod length was the largest, and they were primarily distributed on chromosome 4.

The phenotypic variation explained (PVE) of SNPs associated with pod length ranged from 7.34 to 10.52%, and S04_372771 held the highest PVE of 10.52% (Table 3). The PVEs of SNPs associated with pod width ranged from 8.21 to 9.90%. Among them, S18_51477324 could explain 9.90% phenotypic variation. The three SNPs that had the highest PVEs for each trait were selected for the subsequent analysis.

3.3. Haplotype analysis and candidate gene discovery

To examine the phenotypic influence of allelic variations, genotypic haplotypes were analyzed for S04_372771 (A/G), S18_51477324 (C/T), and S18_55553200 (G/T), which were significantly linked to the fresh pod length, width, and color of vegetable soybeans, respectively. A significant correlation site for vegetable soybean pod length, S04_372771, was identified. The pod length of fresh vegetable soybeans with the genotype GG was greater than that of genotype AA (Fig. 7-A). S18_51477324 was significantly associated with

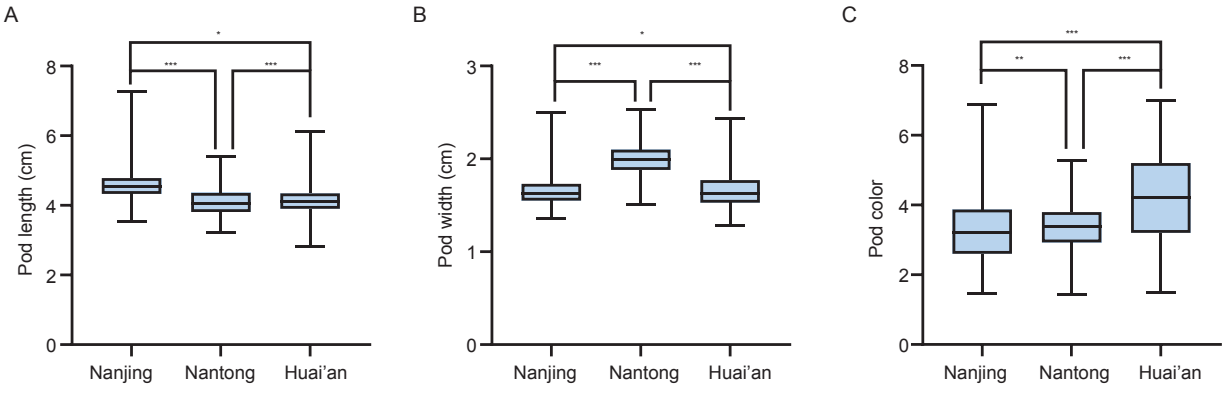


Fig. 2 Comparisons of pod length, width and color in different environments. A, B, and C show the comparisons of soybean pod length, pod width, and pod color in Nanjing, Nantong, and Huai'an, Jiangsu Province, China, respectively. Bars mean SD (n=10). *, P<0.05; **, P<0.01; ***, P<0.001.

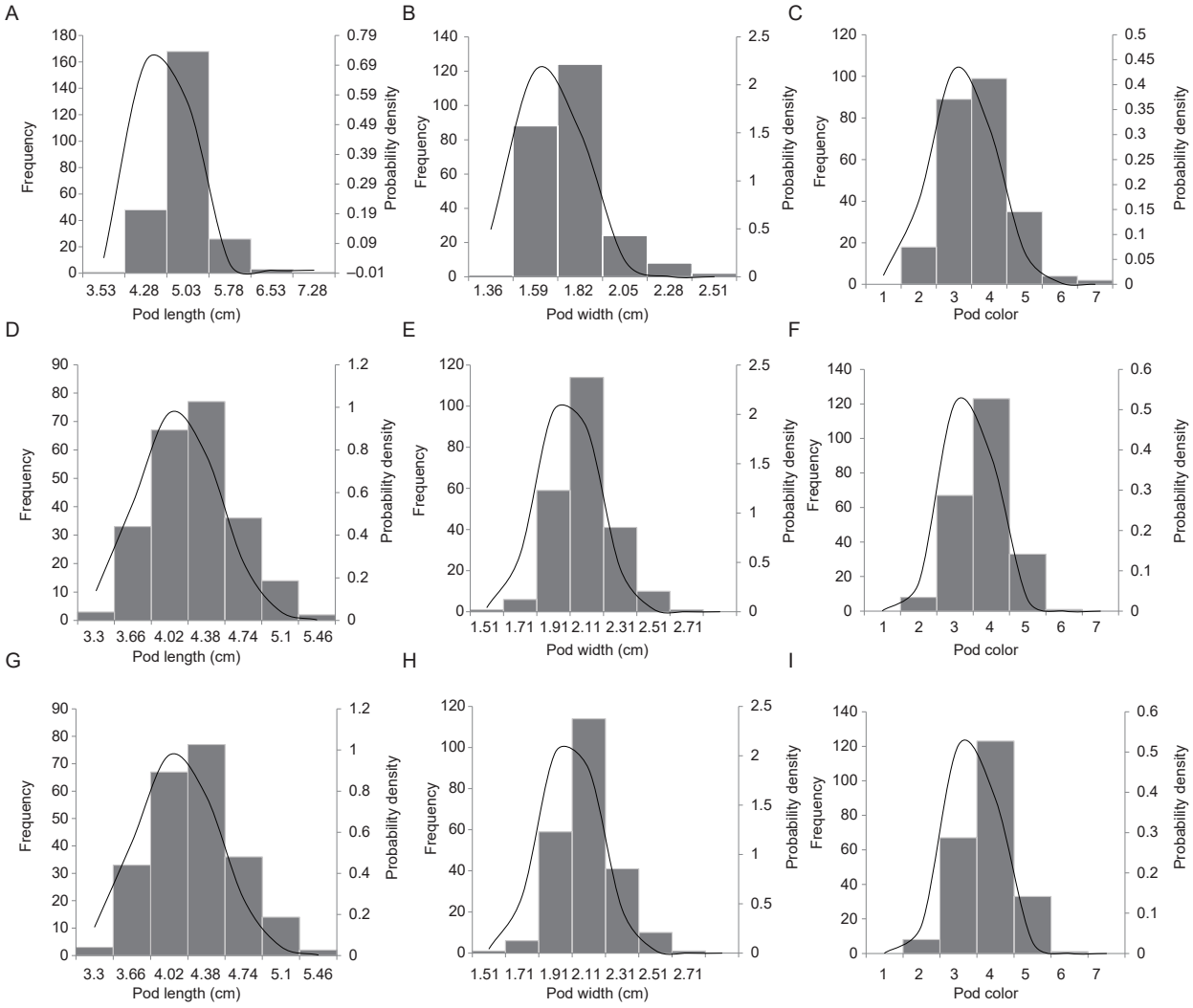


Fig. 3 Frequency distributions of three soybean pod traits in Nanjing, Nantong and Huai'an, Jiangsu Province, China. A, B and C show the frequency distribution maps of pod length, pod width, and pod color in Nanjing, respectively. D, E and F show the frequency distribution maps of pod length, pod width, and pod color in Nantong, respectively. G, H and I show the frequency distribution maps of pod length, pod width, and pod color in Huai'an, respectively.

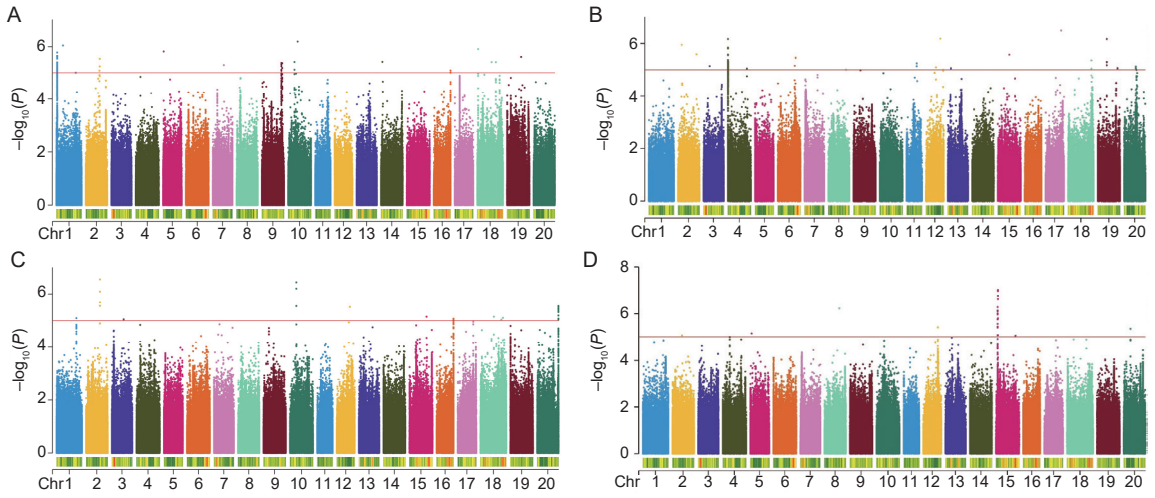


Fig. 4 Genome-wide association study (GWAS) of soybean pod length at the pod-filling stage (R6) stage. A, B, and C show the GWAS results of pod length in Nanjing, Nantong and Huai'an, Jiangsu Province, China, respectively. D, GWAS for the average SNP of the best linear unbiased prediction (BLUP). Red lines denote the significance threshold of $-\log_{10}(P)=5.0$.

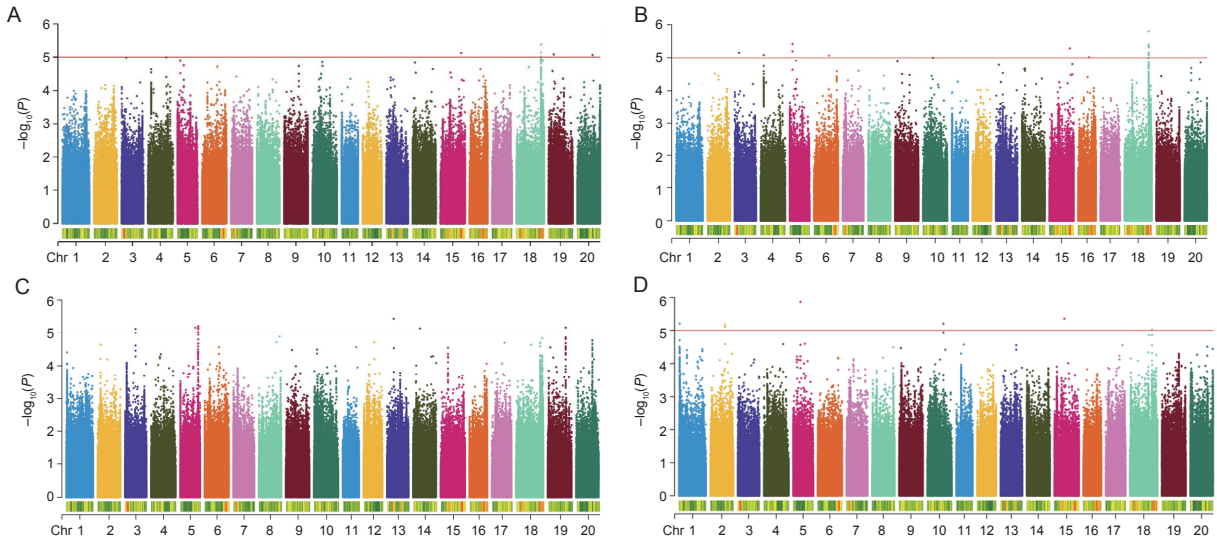


Fig. 5 Genome-wide association study (GWAS) of soybean pod width in the pod-filling stage (R6) stage. A, B, and C show the GWAS results of pod width in Nanjing, Nantong, and Huai'an, Jiangsu Province, China respectively. D, GWAS for the average SNP of the best linear unbiased prediction (BLUP). Red lines denote the significance threshold of $-\log_{10}(P)=5.0$.

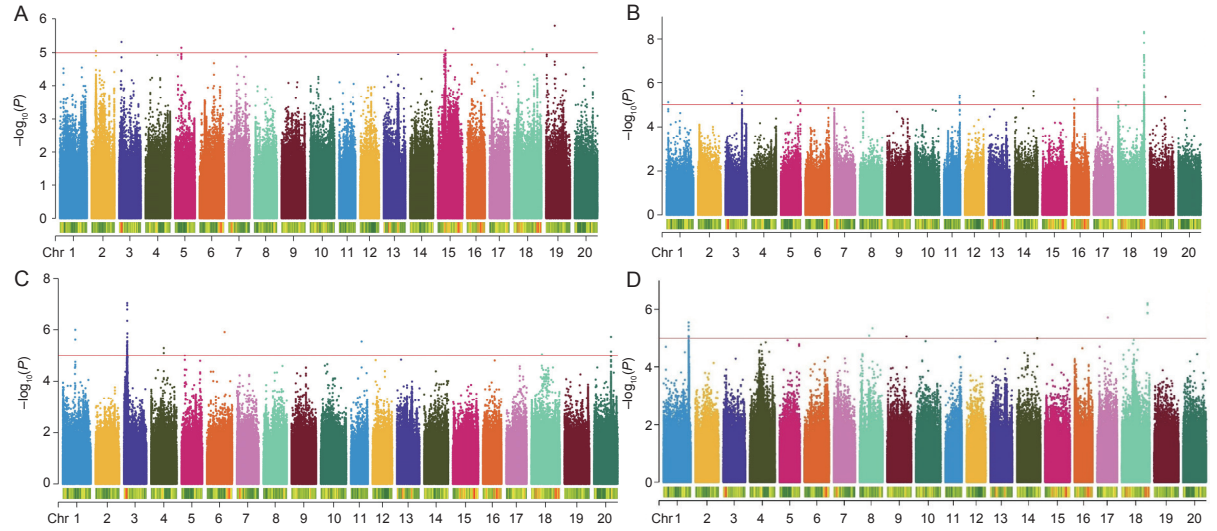


Fig. 6 Genome-wide association study (GWAS) of soybean pod color at the pod-filling stage (R6) stage. A, B, and C show the GWAS results of pod color of Nanjing, Nantong, and Huai'an, Jiangsu Province, China, respectively. D, GWAS for the average SNP of the best linear unbiased prediction (BLUP). Red lines denote the significance threshold of $-\log_{10}(P)=5.0$.

pod width, and the pod width of fresh vegetable soybeans with the TT genotype was greater than the CC genotype (Fig. 7-B). The significant correlation locus with pod color was S18_55553200, and the pod color of vegetable soybeans with genotype GG was closer to the standard pod color than fresh pods with genotype TT (Fig. 7-C).

The SNPs S04_372771, S18_51477324, and S18_55553200 were significantly associated with the appearance quality traits of vegetable soybean ($-\log_{10}(P)\geq5.0$), and the functional prediction of candidate genes was conducted within the range of 120 kb. Local LD analysis was conducted to select candidate genes (Fig. 8-A–D). According to gene functional annotation, five candidate genes associated with the three appearance quality traits of vegetable soybeans were identified (Table 6). All of them have high expression levels in the pods (Fig. 7-D). Among them, *Glyma.04G004700* encodes a PHD family protein expressed in multiple tissues, and *GmPHD34* is highly expressed in fresh pods (Yang *et al.* 2019). Glyma.15G051600 is a BRI1-associated receptor kinase. The GSK3/shaggy-like kinase, encoded by *Glyma.18G225700*, promotes cell elongation and operates in various specific tissues at certain developmental stages (Li C *et al.* 2021). Glyma.18G225900 is the homologue of AT4G00740, which is a pectin methyltransferase that plays a role in the growth of various organs during plant development (Du *et al.* 2020). Finally, *Glyma.18G272300* encodes a brassinosteroid-6-oxidase 2.

3.4. Development and application of KASP markers

The extracted soybean genomic DNA was employed as

Table 3 Significant SNPs associated with vegetable soybean pod length in the three regions of Jiangsu Province, China

Region[1)]	Chromosome	SNP number	Peak SNP	$-\log_{10}(P)$ max	R^2 (%)
Nanjing	1	74	S01_1598368	5.79	9.29
	2	3	S02_31571156	5.52	8.79
	5	1	S05_1492289	5.83	9.36
	7	1	S07_25211618	5.29	8.36
	9	10	S09_44002957	5.38	8.51
	10	1	S10_13964963	5.41	8.57
	14	1	S14_3116244	5.41	8.58
	16	3	S16_37635491	5.09	7.98
	18	2	S18_32369426	5.40	8.56
	19	1	S19_31688704	5.60	8.92
Nantong	2	3	S02_42073508	5.58	9.99
	3	1	S03_13998407	5.13	9.05
	4	119	S04_372771	5.84	10.52
	6	2	S06_40601182	5.45	9.71
	11	2	S11_22718809	5.24	9.27
	13	1	S13_7143311	5.05	8.88
	15	1	S15_26042304	5.56	9.95
	18	2	S18_52756901	5.35	9.50
	19	3	S19_20452733	5.28	9.36
	20	2	S20_26240654	5.11	9.00
Huai'an	1	1	S01_448003	5.09	7.42
	2	3	S02_31121582	5.69	8.47
	3	1	S03_26392515	5.04	7.34
	10	1	S10_13965033	5.53	8.19
	12	1	S12_29345943	5.50	8.13
	15	1	S15_38501099	5.14	7.51
	16	2	S16_37778272	5.06	7.38
	18	8	S18_30297119	5.14	7.51
	20	11	S20_47604962	5.53	8.18
BLUP	2	1	S02_21646978	5.07	15.66
	5	1	S05_2722448	5.16	15.80
	8	1	S08_33219205	6.23	17.65
	12	1	S12_32697689	5.56	16.24
	15	31	S15_4070413	7.01	19.04
	20	1	S20_14666796	5.35	16.13

[1)] BLUP, the best linear unbiased prediction.

Table 4 Significant SNPs associated with vegetable soybean pod width in the three regions of Jiangsu Province, China

Region[1)]	Chromosome	SNP number	Peak SNP	$-\log_{10}(P)$ max	R^2 (%)
Nanjing	15	1	S15_43602583	5.14	9.29
	18	3	S18_51548538	5.12	9.24
	19	1	S19_10606185	5.08	9.16
	20	1	S20_32056635	5.06	9.11
Nantong	3	2	S03_9596163	5.16	9.50
	4	1	S04_6771759	5.09	9.34
	5	1	S05_6057129	5.20	9.59
	6	1	S06_31924567	5.07	9.31
	10	1	S10_20939390	5.00	9.16
	15	1	S15_43602583	5.29	9.79
	16	1	S16_22933921	5.02	9.20
	18	14	S18_51477324	5.34	9.90
Huai'an	3	3	S03_223778753	5.57	9.18
	5	2	S05_38143965	5.06	8.21
	10	1	S10_47411241	5.11	8.31
	14	1	S14_15076683	5.47	9.00
	19	2	S19_11756498	5.16	8.41
BLUP	1	1	S01_1074167	5.21	13.10
	2	2	S02_31121578	5.18	13.04
	5	1	S05_15320596	5.87	14.27
	10	2	S10_33856485	5.21	13.10
	15	1	S15_21011238	5.36	13.37
	18	1	S18_46703985	5.02	12.77

[1)] BLUP, the best linear unbiased prediction.

Table 5 Significant SNPs associated with vegetable soybean pod color in the three regions of Jiangsu Province, China

Region[1)]	Chromosome	SNP number	Peak SNP	$-\log_{10}(P)$ max	R^2 (%)
Nanjing	3	1	S03_5046678	5.10	8.33
	4	2	S04_24632212	5.16	8.44
	15	1	S15_32385607	5.41	8.93
	19	1	S19_18192857	5.80	9.63
Nantong	1	6	S01_3410897	5.10	8.25
	3	5	S03_34999177	5.62	9.25
	5	2	S05_36217869	5.16	8.36
	11	6	S11_33290458	5.41	8.83
	14	2	S04_40405336	5.61	9.22
	16	1	S16_6399204	5.22	8.47
	17	16	S17_8088168	5.73	9.46
	18	75	S18_55553200	8.32	14.61
	19	1	S19_33294096	5.33	8.69
Huai'an	1	2	S01_25243559	6.00	13.50
	3	36	S03_6533734	7.07	13.69
	4	2	S04_25671614	5.31	9.78
	5	1	S05_6570011	5.01	9.14
	6	1	S06_35956589	5.92	11.12
	11	1	S11_21846554	5.56	10.33
	18	1	S18_21344690	5.05	9.24
	20	2	S20_34435148	5.74	10.73
BLUP	1	7	S01_52276554	5.55	10.43
	8	2	S08_26747991	5.34	10.05
	9	1	S09_40317577	5.06	9.53
	17	1	S17_22121738	5.71	10.74
	18	6	S18_53636031	6.21	11.67

[1)] BLUP, the best linear unbiased prediction.

the template, and the corresponding primers F1, F2, and R (Table 7) designed for S04_372771 (A/G), S18_51477324 (C/T), and S18_55553200 (G/T) were used for PCR amplification with the QuantStudio5 real-time fluorescence quantitative PCR instrument. In this study, a number of vegetable soybeans randomly selected from the population were subjected to amplification and genotyping using KASP molecular marker primers on the QuantStudio5 real-time fluorescence quantitative PCR instrument. As shown in Fig. 8, all the three KASP markers can clearly distinguish the two genotypes.

KASP markers were designed to target S04_372771, which was significantly linked to vegetable soybean pod length. The red dots near the *x*-axis of Fig. 9-A are relatively clustered, indicating vegetable soybeans with the T allele variation. In contrast, blue represents vegetable soybean germplasms with the A allele variation near the *y*-axis (Fig. 9-A).

S18_51477324 is specifically associated with the width of vegetable soybean pods and was also targeted with KASP markers. The red dots near the *x*-axis in Fig. 9-B represent vegetable soybeans with the T allele variation, and they have relatively wide fresh pods. In contrast, blue represents the vegetable soybeans with the C allele variation near the *y*-axis (Fig. 9-B).

S18_55553200 was significantly linked to vegetable pod color and was also targeted by a KASP marker. The blue dots near the *y*-axis of Fig. 9-C are relatively clustered to represent the germplasms of vegetable soybeans with the G allele, and they have a fresh pod color closer to the standard pod color. Red represents the vegetable soybean

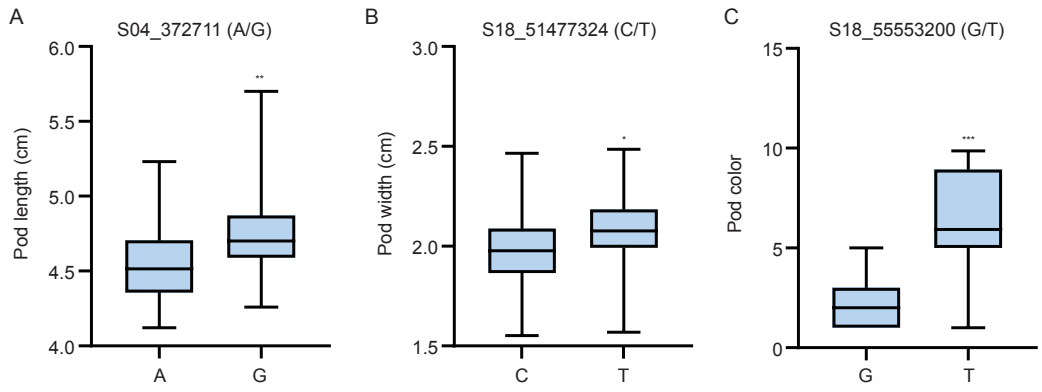


Fig. 7 Significant SNP haplotypes of appearance quality-related traits in a natural population of vegetable soybeans. Bars mean SD (n=10). *, P<0.05; **, P<0.01; ***, P<0.001.

germplasms near the x-axis carrying the T allele, with a pod color far different from the standard pod color (Fig. 9-C).

4. Discussion

China possesses extremely rich vegetable soybean germplasm resources. These vegetable soybean germplasms have a broad genetic foundation, rich genetic variation, and contain numerous favorable gene types for the breeding of new vegetable soybean varieties and novel gene mining. In this study, the phenotypic information collection and analysis system of vegetable soybeans was employed to characterize the appearance quality of vegetable soybeans, and GWAS was performed to explore potential loci and genes associated with vegetable soybean appearance quality.

4.1. Pod length, width and color of vegetable soybeans showed significant differences in different environments

Regional crop testing is the most widespread method for determining crop variety characteristics, but the interaction effect between genotype and environment is common in crops grown in different environments (Xu *et al.* 2017). Several studies have confirmed the influence of genotype and environmental interaction on crop traits. For example, such interactions greatly influenced the accuracy of predicting the dry matter quality of perennial ryegrass, and several perennial ryegrass varieties with high dry matter quality were identified under three environments (Zhu *et al.* 2023). As an oilseed crop, sesame seed yield is highly impacted by the environment. By examining the seed yields of 20 sesame genotypes under three fertilization environments, stable genotyped sesame seeds were chosen (Patel *et al.* 2024). In this study, the appearance quality-related traits of 264 vegetable soybeans from Nanjing, Nantong, and Huai'an were assessed in 2023. The variation ranges of fresh pod length, pod width, and pod color score in Nanjing were 3.52 to 7.62 cm, 1.36 to 2.50 cm, and 1.47 to 6.87, respectively (Table 1). In Nantong, the variation ranges of pod length, pod width, and pod color score were 3.22 to 5.39 cm, 1.51 to 2.53 cm, and 1.43 to 5.27, respectively (Table 1). The ranges of fresh pod length, pod width, and pod color score in the Huai'an area were 2.82 to 6.12 cm, 1.29 to 2.43 cm, and 1.5 to 7, respectively (Table 1). The variation range of the appearance quality traits was 5.94 to 30.00%, with significant differences among genotypes and influences exerted by the environment (Table 1). This result was further confirmed by ANOVA, which demonstrated that appearance quality-related traits at the R6 stage had significant differences among genotypes, between different environments, and between genotypes and environments (Table 2).

4.2. GWAS for pod length, width and color

GWAS is the most commonly employed and effective method for the analysis of important agronomic traits. Currently, GWAS is also widely employed in vegetable soybeans, primarily for yield traits such as 100-grain weight, node number of the main stem, and nutritional quality features such as protein and fat. For instance, one study used 55,159 SNPs and phenotypic data from 298 vegetable soybean germplasms to perform GWAS and identified 40 SNPs significantly associated with seed protein content that were distributed across 17 different genomic regions (Hwang *et al.* 2014). Another study (Zatybekov *et al*. 2017) examined plant height, node number of the main stem, and height of the first branch across 113 vegetable soybean materials and performed a GWAS analysis based on genomic data. A total of six SNPs were found to be significantly correlated with the height of the first branch, node number of the main stem, and height of the first branch (Zatybekov *et al.* 2017). Thus, this technique can assist in developing large grain varieties with high oil and low protein contents. In this study, high-density SNP physical maps integrated with phenotypic and genotype data were employed for GWAS. At a significance level of $-\log_{10}(P)\geq 5.0$, extremely significant associations of SNP loci with pod length, pod width, and pod color were detected.

4.3. Candidate genes of pod length, width and color

SNPs S04_372771, S18_51477324, and S18_55553200 exhibited PVEs of 10.52, 9.90 and 14.61%, respectively. In the 120 kb region upstream and downstream of these three loci, a total of five candidate genes were identified. The gene *Glyma.04G004700* encodes a PHD family protein which is a homologue of AT1G14770. PHD proteins are reportedly

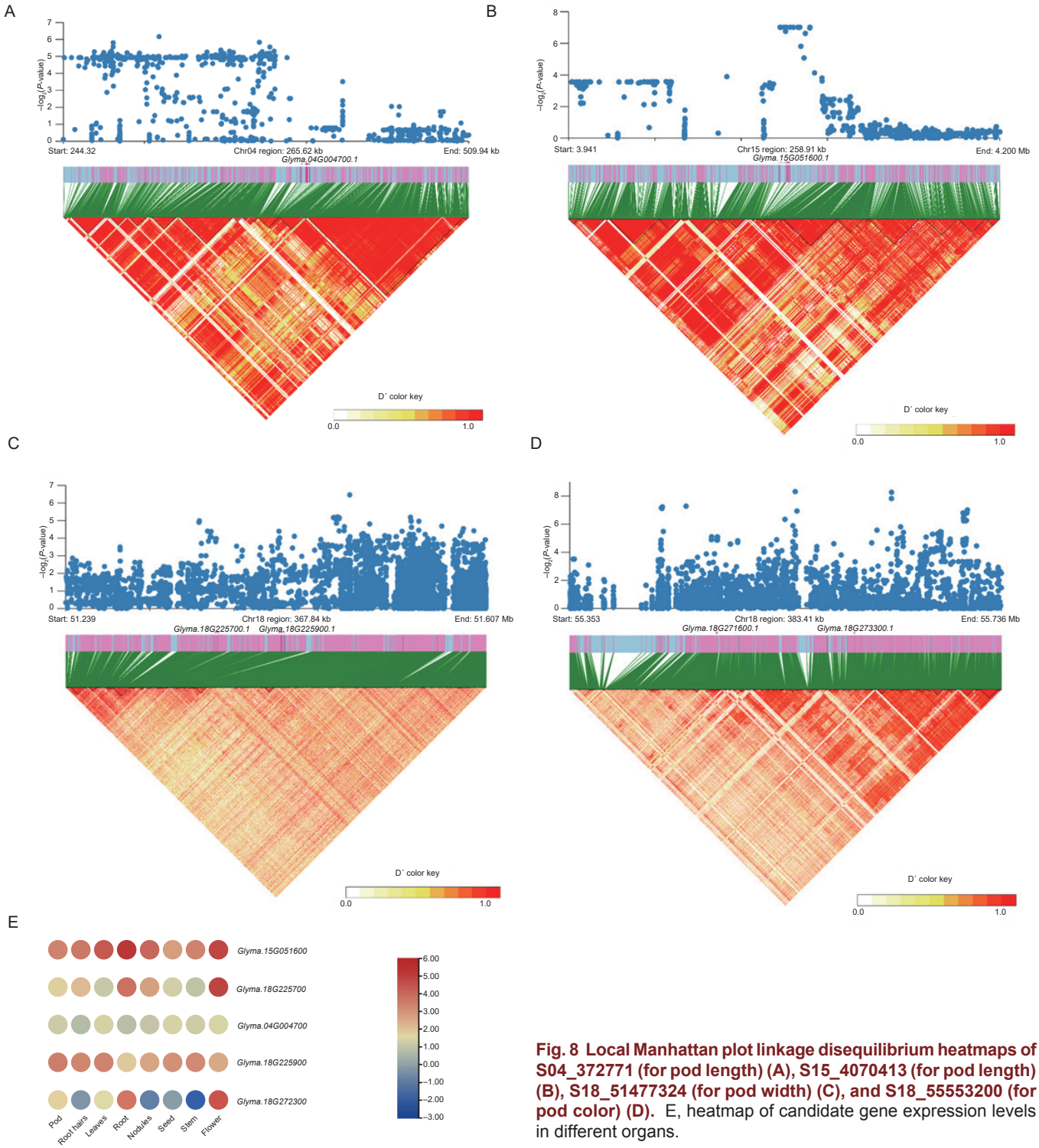


Fig. 8 Local Manhattan plot linkage disequilibrium heatmaps of S04_372771 (for pod length) (A), S15_4070413 (for pod length) (B), S18_51477324 (for pod width) (C), and S18_55553200 (for pod color) (D). E, heatmap of candidate gene expression levels in different organs.

involved in multiple plant biological processes, including plant growth and development, responses to abiotic stresses and regulation of flowering time (Quan *et al.* 2023). *GmPHD34* exhibits high expression levels in fresh soybean pods, thereby promoting their growth (Yang *et al.* 2019). Glyma.15G051600 is a BRI1-associated receptor kinase (BAK), and its homologue ATBAK1 can interact with brassinosteroid-insensitive 1 (BRI1) to modulate brassinosteroid signaling (Li *et al.* 2002; He *et al.* 2013). Overexpression of *ATBAK1* results in elongated organs, while *atbak1* displays dwarfing and reduced sensitivity to brassinosteroids (Li *et al.* 2002). *Glyma.18G225700*, a homologue of *AT4G00720*, encodes a GSK/Shaggy-like kinase. This gene was reported to negatively regulate the expression of downstream BR (brassinosteroid) response genes and be involved in plant development (Tong *et al.* 2012; Li C *et al.* 2021). In rice, OsOML4 is a negative regulator of grain size and plant height. GSK2 can phosphorylate OML4 and regulate the stability of OML4 to regulate rice grain size by inhibiting cell expansion (Lyu *et al.* 2020). GSK3/SHAGGY-LIKE KINASE2

Table 6 Candidate genes for the appearance quality traits of vegetable soybean

Gene ID	Trait	Homologs in *Arabidopsis thaliana*	Functional annotation
Glyma.04G004700	Pod length	*AT1G14770*	PHD
Glyma.15G051600	Pod length	*AT4G33430*	BRI1-associated receptor kinase
Glyma.18G225700	Pod width	*AT4G00720*	GSK/Shaggy-like kinase
Glyma.18G225900	Pod width	*AT4G00740*	Pectin methyltransferase
Glyma.18G272300	Pod color	*AT3G30180*	Brassinosteroid-6-oxidase 2

Table 7 Specific primers for the KASPs[1)]

Primer name		Primer sequence (5′→3′)
S04_372771	F1	**GAAGGTGACCAAGTTCATGCT**AATGTTCTTGGACCTGCAGT**A**
	F2	**GAAGGTCGGAGTCAACGGATT**AATGTTCTTGGACCTGCAGT**G**
	R	ATAAGCACGTCGTTCCAAAGT
S18_51477324	F1	**GAAGGTGACCAAGTTCATGCT**CTAAGCGTATCTGCCTCGC**T**
	F2	**GAAGGTGACCAAGTTCATGCT**CTAAGCGTATCTGCCTCGC**C**
	R	AACCACTTCAGCTTCAAGCCA
S18_ 55553200	F1	**GAAGGTGACCAAGTTCATGCT**GTGAGCATGCATGCTGTCA**G**
	F2	**GAAGGTGACCAAGTTCATGCT**GTGAGCATGCATGCTGTCA**T**
	R	TTTCCCTTAAAACATTATAA

[1)] Bold and underlined regions represent fluorescent adapters, and bold bases represent the target SNP site.

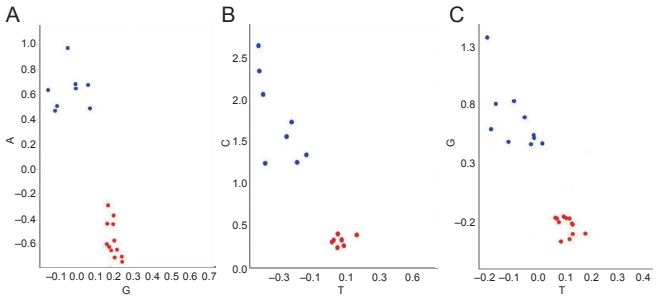


Fig. 9 Genotyping of KASP markers. A, B and C represent the genotypes of S04_372771, S18_51477324, and S18_ 55553200, respectively.

phosphorylates and stabilizes *OsMADS1* transcriptional factor, which targets the TERMINAL FLOWER1-like gene *RICE CENTRORADIALIS2*. The tissue-specific activation of BRD3 in the secondary branch meristems enhances panicle branching, minimizing the negative effects on grain size and improving grain yield (Zhang *et al.* 2024). GSK3 phosphorylates and regulates the Green Revolution protein Rht-B1b to reduce plant height in wheat (Dong *et al.* 2023). Therefore, this gene has great potential for regulating pod width and influencing various plant traits. The pectin methyltransferase (QUA2) encoded by *Glyma.18G225900* is involved in cell elongation and plays a role in the growth of multiple organs during plant growth and development (Du *et al.* 2020). *Glyma.18G272300* (brassinosteroid-6-oxidase) encodes a cytochrome P450 enzyme that catalyzes the last reaction in the production of brassinolide (TAIR) and participates in BR signaling (Liu *et al.* 2021). Brassinolide is the most active BR (Wang *et al.* 2021), and BR was reported to function in maintaining plant color (Xue *et al.* 2021; Li *et al.* 2022; Xu X *et al.* 2022). Thus, Glyma.18G272300 may regulate pod color through BR signaling. In summary, all five genes may participate in regulating the appearance traits of vegetable soybeans, and BR may be one of the important factors regulating pod appearance quality.

4.4. KASP markers for pod length, width and color

Three corresponding KASP markers of S04_372771 (A/G), S18_51477324 (C/T), and S18_55553200 (G/T) were found to be effective in predicting the appearance quality traits of vegetable soybeans (Fig. 9). This establishes the research foundation for further investigations of genes related to the appearance quality of vegetable soybeans, and molecular marker-assisted breeding is expected to be applied to the breeding of vegetable soybeans. This study offers valuable genetic resources and a robust theoretical basis for the breeding of vegetable soybeans with optimal appearance quality. In addition, the phenotype collection and analysis system of vegetable soybeans constructed in this study can be applied to the establishment of a standardized phenotype database for vegetable soybeans.

5. Conclusion

In this study, we found significant differences in the appearance quality traits of vegetable soybeans among different genotypes and environments. We identified 525 SNPs and five candidate genes associated with pod traits at the R6 stage based on GWAS in Nanjing, Nantong and Huai'an. Furthermore, we developed KASP markers for S04_372771, S18_51477324, and S18-55553200.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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