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Mapping and identification of genes responsible for less-photosensitive fruit coloration in eggplant

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Abstract

In eggplant (*Solanum melongena*. L), low light during cultivation often hinders proper pigmentation of fruit. While some varieties exhibit less susceptibility to low light for eggplant coloration, however, the genetic basis of such less-photosensitive fruit coloration remains unknown. In this study, we characterized a less-photosensitive eggplant cultivar '609'. Under bagging conditions, fruits of '609' exhibited purple coloration, albeit lighter than fruits grown under natural conditions. Genetic analysis showed that the less-photosensitive trait was controlled by a single dominant gene, designated *SmLP*. Based on BSA and genetic recombination analyses, *SmLP* was mapped to the 7.4–12.5 Mb region on chromosome 10. Within this genetic region, six genes with non-synonymous mutation and seven genes potentially involved in anthocyanin biosynthesis or light signal transduction were identified. Further RT-qPCR analysis revealed that only three out of these genes were differentially expressed in eggplant peel tissues. The three genes *EGP21875*, *EGP21864* and *EGP21911*, encoding MYB domain protein 113, phototropic-responsive NPH3 family protein, and protein with unknown function, respectively, were considered as the putative genes associated with less-photosensitive anthocyanin biosynthesis in eggplant fruits. These results would provide great help in casual gene identification for less-photosensitive trait and promote an understanding of molecular mechanisms underlying less- and non-photosensitive coloration in eggplant.

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Introduction

Eggplant (*Solanum melongena*. L) in the Solanaceae family is a globally and economically important vegetable crop rich in nutrition[1–4]. The color of eggplant fruits, primarily purple, white, or green, is an important quality and commercial trait[5–7]. Purple color varieties, containing high levels of anthocyanins in the fruit peel, are commonly found in the market[8,9]. As a branch of flavonoids in secondary metabolites, anthocyanin is a water-soluble natural pigment responsible for the blue, purple, and red color of many plant tissues[10–12].

The biosynthesis pathway of anthocyanin has been extensively studied in many plant species[13–16]. Both structural and regulatory genes have been found to participate in anthocyanin biosynthesis. Structural genes encode enzymes that directly catalyze anthocyanin biosynthesis, while regulatory genes mainly refer to those coding for transcription factors (TFs) that manipulate the expression of structural genes[17–19]. TFs regulating anthocyanin biosynthesis have also been identified in eggplant, such as SmMYB113, SmGL3[20–23]. In addition, anthocyanin production is affected by various environmental conditions including light and temperature[24–27]. Among them, light is a particular important regulator of anthocyanin biosynthesis[28–31]. However, the anthocyanins-related pigmenting is not or less influenced under darkness in specific materials of sweet cherry, chrysanthemum, mango, turnip, grape, and eggplant[20,32–36].

Fruit color under calyx is an indicator of light requirement in eggplant[37,38]. Specifically, fruits with green peel under the calyx always undergo a color change to white after bagging, which is known as the photosensitive or light-induced type[39,40]. In contrast, fruits with purple peel under the calyx often maintain their purple coloration after bagging, and these eggplants are referred to as the non-photosensitive type[20]. Most quantitative trait locus (QTLs) responsible for purple coloration under calyx in eggplants were detected on chromosome 10[41–45]. In addition, a recent study showed that *SmFTSH 10* (*filamentation temperature sensitive 10*) was the most possible candidate gene of non-photosensitivity in eggplant[45]. Interestingly, we found that some cultivars exhibited light purple under the calyx, while under bagging conditions, the fruits displayed a purple coloration overall, which was significantly lighter compared to the fruits that grew under natural conditions. Therefore, we classify these varieties as the less-photosensitive type.

In this study, two eggplant parental lines with photosensitive and less-photosensitive coloration and their crossing F_2 progenies were used for genetic analysis, bulked sergeant analysis (BSA)-based sequencing and expression analysis to identify the causative genes conferring less-photosensitive anthocyanin biosynthesis. The candidate genes identified in this study would facilitate gene identification for the less-photosensitivity trait and be helpful for the study of the

mechanism of less-photosensitive anthocyanin biosynthesis in eggplant.

Materials and methods

Eggplant varieties and phenotypic assessment

A photosensitive cultivar '749' and a less-photosensitive cultivar '609' were used as parent lines to develop the F_2 population. All eggplants grew in the greenhouse in the experimental fields of Hebei Agricultural University, Baoding, China. Eggplant fruits were bagged on the 5th day after flowering, and fruit color observation were conducted on the 14th day under bagging condition, with the fruits growing in natural conditions as control.

Whole genome sequence of bulked DNA

Total genomic DNA of the two parent lines and the F_2 population was extracted from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method[46]. For genome sequencing, equivalent amounts of DNA from 30 plants with purple-bagged and white-bagged fruits were mixed separately, named 'P Bulk' and 'G bulk' respectively. The qualified DNA was randomly broken into fragments with a length of 350 bp, and sequencing libraries were generated using a TruSeq Nano DNA HT Sample preparation Kit (Illumina USA), followed by Illumina PE150 sequencing.

After quality control, the clean reads of each sample were aligned against the eggplant reference genome[47] using BWA software[48]. The Unified Genotyper function in GATK3.8 software[49] was used to detect SNP and Indel of each sample, and Variant Filtration parameter in GATK was used to filter the SNPs and Indels. Euclidean Distance (ED) algorithm was employed to predicted candidate region associate with less-photosensitivity[50]. Sequencing depth of differential SNPs in each mixed pools were counted to calculate ED on each site. ED was raised to ED^5 to minimize noise of small variation[50].

Obtaining recombinants

To narrow down the candidate region, molecular markers (Supplemental Table S1) were developed based on the SNP sites of the two bulks from the sequencing. All plants in F_2 population and the two parental lines were genotyped using Kompetitive allele specific PCR (KASP) technology with linked SNP markers, that were used to screen recombinants.

Gene expression analysis

Eggplant fruits of '609' and '749' were bagged on the 5th day after flowering, and the fruit peel was harvested on the 14th day after bagging for gene expression analysis, using the peel of fruits grown under natural conditions as control. The expression of candidate genes was detected using qRT-PCR. Primer Premier 5.0 software was used to design the primers, which are listed in Supplemental Table S2. Total RNA of fruit peel was extracted with RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China). A total of 1 μg RNA per sample was reverse transcribed to cDNA using the PrimeScript™ reagent Kit with gDNA Eraser (TaKaRa, Beijing, China) in 20 μL of reaction mixture. The qRT-PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Shanghai, China) in a LightCycler® 96 System (Roche, Basel, Switzerland). The expression of the candidate genes was quantified by $2^{-\Delta C_T}$ method, with *SmGAPDH* (*EGP1067575*) as housekeeping gene. The analysis was performed with three biological replicates. Significant differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) using SPSS 16.0 Statistics (SPSS Inc., Chicago, IL, USA).

Results

Inheritance of the less-photosensitive trait

Under natural growth conditions, both '609' and '749' exhibited dark purple fruit coloration, with the peel under calyx of '609' being light purple and that of '749' being green (Fig. 1). To investigate their responses to darkness, fruits were bagged to block the exposure to light. Under such conditions, '609' plants had significant lighter purple fruits compared to those grown under natural conditions, while '749' plants had white fruits (Fig. 1).

The F_1 hybrids ('609' × '749') and F_2 populations were used for studying the inheritance of less-photosensitive trait. The fruit color after bagging of each plant was visually examined. It was shown that under bagging conditions, the fruits of 15 F_1 individuals generated by crossing '609' and '749' displayed a light purple coloration. Among 178 F_2 individuals, 139 and 39 plants had light purple and white fruits after bagging, respectively, and this rate approximately fitted an expected Mendelian inheritance ratio of 3:1 ($\chi^2 = 0.91 < \chi^2_{0.05,1} = 3.84$) (Table 1). These results indicated that the less-photosensitive trait was controlled by a dominant gene, which was named as *SmLP* hereafter.

Identification of candidate region for *SmLP* through BSA analysis

To map *SmLP*, BSA-based sequencing was carried out by bulking 30 F_2 individuals with white-bagged fruits and purple-bagged fruits, respectively. The high-throughput sequencing generated 78.25 Gb clean data, which comprised 122,455,776

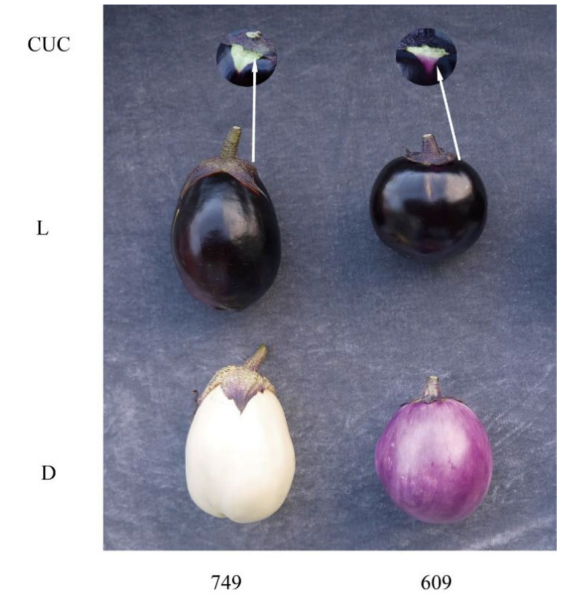

Fig. 1 Fruits of '609' and '749' under (L) natural and (D) bagging conditions. CUC indicates the color under calyx. Eggplant fruits were bagged on the 5th day after flowering, and the picture was taken on the 14th day after bagging, with the fruits growing in natural conditions as control.

Table 1. Genetic analysis of fruit peel pigmentation after bagging in F_2 population.

Generation	Numbers of plants	Number of plants with light purple fruit peel after bagging	Number of plants with white fruit peel after bagging	Expected ratio	χ^2
609	10	10	0		
749	10	0	10		
(609 × 749) F_1	15	15	0		
(609 × 749) F_2	178	139	39	3:1	0.91

Note: $\chi^2_{0.05} = 3.84$, df = 1.

and 138,367,408 high quality reads from 'G bulk' and 'P bulk'. The Q30 ratio was higher than 93.50% (Supplemental Table S3). The mapping rate exhibited an average cover depth of 99.63% (Supplemental Table S4). These results suggested that the sequencing data were reliable and suitable for SNPs and Indels detection.

A total of 60,648 poly morphic sites (43,725 SNPs and 16,923 Indels) were detected in the BSA data. The median plus three standard deviations (SD) of the fitted values at all loci were used as the association threshold for analysis, which was determined to be 0.49. Based on this association threshold, significant associations were detected on Chromosome 10 (Fig. 2a), spanning a total length of 15.82 Mb and located at 4.38–20.15 Mb (Fig. 2b).

Further mapping of *SmLP* by screening recombinants

In order to further determine the positioning range of *SmLP*, 178 individuals in the F_2 population, and 10 KASP primers, which were designed to uniformly cover the preliminary mapping interval, were used to analyze the polymorphism of the two parental lines. The *SmLP* locus was finally mapped to a region between the markers SNP9 and SNP3 (with a physical ranging from 7.4 Mb to 12.5 Mb), based on 52 recombinant individuals (Fig. 3). Within the candidate interval, there were 280 SNPs and 74 Indels in total (Table 2). These SNPs and Indels were associated with 116 genes (Supplemental Table S5),

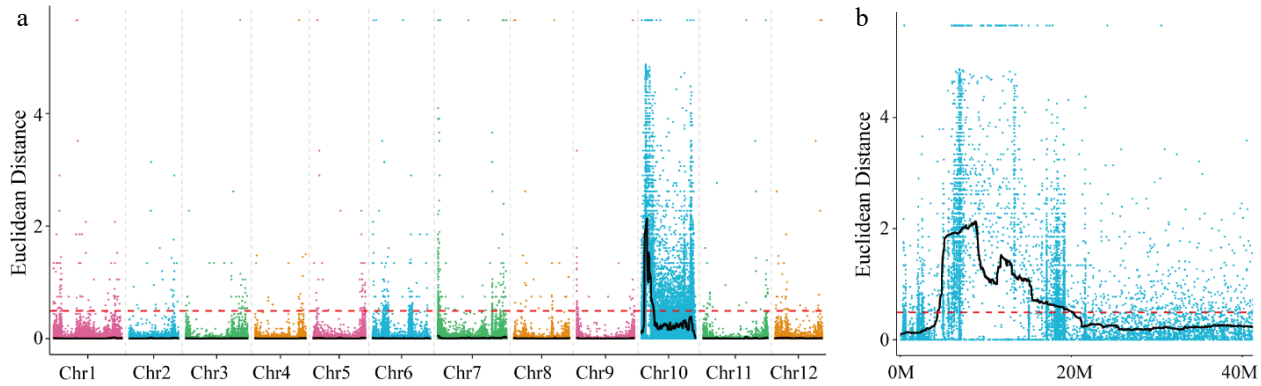

Fig. 2 Distribution of ED-based linkage value on (a) all chromosomes and (b) on Chromosome 10. Each colored dot represents an ED-based linkage value of an SNP site. Black lines represents ED value after fitting. Red dashed lines represents linkage threshold.

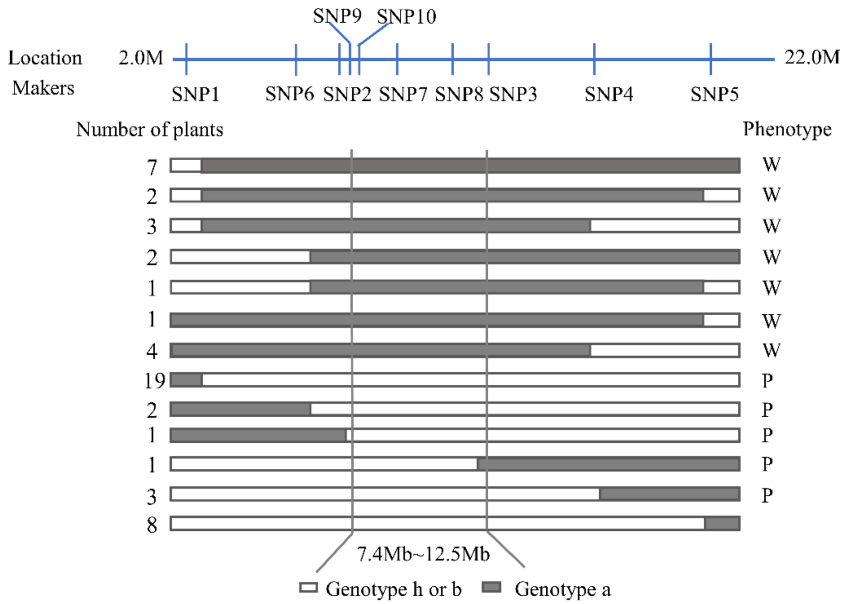

Fig. 3 Genotype and phenotype analysis of recombinant plants in the F_2 population derived from a cross between '609' and '749'. (a) Genotype of the photosensitive parent '749'. (b) Genotype of the less-photosensitive parent '609'. (h) Heterozygote of the '749' and '609'. W, White fruit peel after bagging; P, Purple fruit peel after bagging.

Table 2. Classifications of SNPs and Indels in the candidate region.

Category	The number of SNPs	The number of Indels
Intergenic	222	56
Upstream	23	5
3'UTR	0	1
Non-synonymous	6	0
Synonymous	1	0
Intronic	9	1
Downstream	19	11

Table 3. Nonsynonymous SNPs and their related genes in the candidate region.

Gene ID	SNP loci	Base substitution type	Annotation
EGP21857	7447780	C- > T	Uncharacterized protein LOC102595296
EGP21873	7722116	C- > G	12-oxophytodienoate reductase 1
EGP21911	9870254	C- > T	Undefined
EGP21972	11985911	C- > T	Hypothetical protein BC332_00197
EGP21983	12225282	G- > A	Putative GDSL esterase/lipase-like
EGP22005	12532757	G- > A	MYB domain protein 113

among which six genes had non-synonymous mutations (Table 3).

Identification of candidate genes potentially regulating less-photosensitivity

The biosynthesis pathway of anthocyanin has been studied and characterized very clearly in different plants; related genes controlling the pathway have been classified into structural genes, encoding enzymes that directly catalyze stepwise the anthocyanin biosynthesis process, and regulatory genes controlling the expression of structural genes[13]. The expressions of structural genes and regulatory genes were influenced by both intrinsic biological factors (such as hormones, circadian rhythms) and external environmental factors (such as light, temperature, insects, and fungi)[25,30,51–57]. Based on gene functional annotation and homologous gene functional studies, the *SmLP* region was analyzed in search of anthocyanin biosynthesis-related and light signal transduction-related genes, and there were seven high confidence genes observed in this region (Table 4). Among them, there were three MYB transcription factors. Both *EGP21874* and *EGP21875* encode a TF SmMYB113, and they were homologs of *AtMYB113* in *Arabidopsis*. AtMYB113 was identified as one of the members of the MBW complex, directly regulating the expression of structural genes[58]. *EGP22005* is homologs of the gene *AtMYB16* in *Arabidopsis*, which is involved in controlling trichome maturation and cuticle formation[59,60]. In addition, *EGP21863* encodes auxin response factor 16 (ARF16), and some ARFs were known to negatively regulate the biosynthesis of anthocyanins, such as ARF13 and ARF2[61,62]. *EGP21864* encodes a phototropic-responsive NPH3 family protein. *EGP21891* and *EGP21908* encode phytochrome kinase substrates (PKSs). It was reported that under blue light, phototropins (PHOTs) could interact with NPH3 and PKSs, regulating the bending of the hypocotyl during phototropism[63–65]. Therefore, *EGP21864*, *EGP21891*, and *EGP21908* may be involved in responses to light, particularly to blue light.

The expression analysis of seven genes potentially participating anthocyanin biosynthesis and light signal transduction (Table 4) and six genes with non-synonymous mutations (Table 3) showed that only *EGP21875*, *EGP21864*, and *EGP21911* were expressed in the '609' and '749' peel (Fig. 4, Supplemental Fig. S1). Therefore, these three genes were considered as putative genes controlling less-photosensitive coloration in eggplant.

EGP21875 encodes a MYB TF SmMYB113, which has been reported to participate in regulating the biosynthesis of anthocyanin in eggplant[20,22,23]. In this study, the expression of *SmMYB113* in the '749' peel was significantly downregulated after bagging. Whereas the expression of *SmMYB113* in the '609' peel was not affected by light (Fig. 4). There was one SNP 22.9 kb upstream of the start codon and one SNP 13 kb downstream of the stop codon of *EGP21875*, respectively (Table 5).

EGP21864 encodes a phototropic-responsive NPH3 (Non-Phototropic Hypocotyl 3) family protein. Studies have shown that *AtNPH3* is involved in regulating photomorphogenesis and light-mediated growth responses in *Arabidopsis*[66,67]. After bagging, the expression of *EGP21864* was downregulated in the peel of both '609' and '749'. Notably, under bagging conditions, the expression of *EGP21864* in the peel of '609' was significantly higher than that in '749' (Fig. 4). There was one SNP within the intronic region of *EGP21864* (Table 5).

EGP21911 encodes a protein with unknown function. A SNP (G/A) in '609' identified through sequencing analysis resulted in an amino acid change from Ala-15 to Thr-15 in the first exon (Table 5). Under natural conditions, *EGP21911* showed no distinctively different expression level between the two parental lines. However, under bagging conditions, the expression of *EGP21911* was significantly higher in the peel of '609' compared with '749' (Fig. 4).

Table 4. Candidate genes involved in biosynthesis of anthocyanin and light signal transduction.

Gene ID	SNP category	Indel category	Annotation
EGP21874	Intergenic region in upstream, Intergenic region in downstream	Intergenic region in downstream	MYB domain protein 113
EGP21875	Intergenic region in upstream, Intergenic region in downstream	–	MYB domain protein 113
EGP22005	Upstream, Non-synonymous	–	MYB domain protein 16
EGP21863	Upstream	Intergenic region in upstream	Auxin response factor 16
EGP21864	Intron	–	Phototropic-responsive NPH3 family protein
EGP21891	Intron, Downstream, Intergenic region in downstream	Intergenic region in downstream	Phytochrome kinase substrate 2
EGP21908	Intergenic region in downstream	Intergenic region in downstream	Phytochrome kinase substrate 1

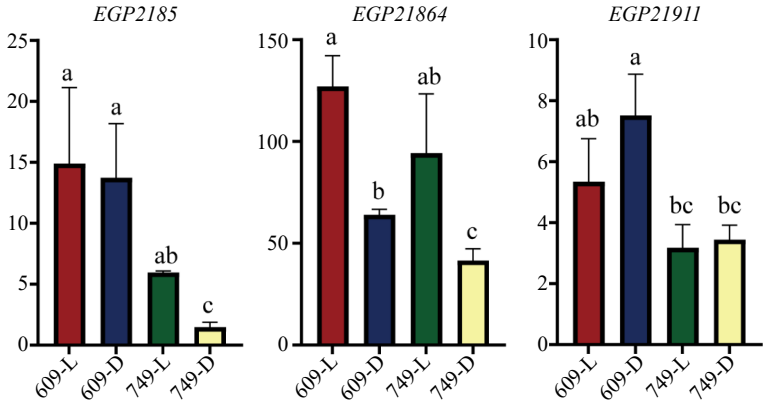

Fig. 4 The transcript level of *EGP21875*, *EGP21864* and *EGP21911* in the fruit peel of '609' and '749'. The y axis indicated the relative expression levels of each gene. L, Under natural conditions; D, Under bagging conditions. The relative expression was determined by $2^{-\Delta CT}$ method. The date are means from three biological replicates with three technical replicates. Error bars indicate SEs. Letters above each column represent significant differences based on one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

Table 5. Candidate genes involved in less-photosensitive anthocyanin biosynthesis in the peel of '609'.

Gene ID	SNP loci	Substitution type	SNP category	Distance	Annotation
EGP21875	7808145 7770881	C- > A T- > C	Intergenic region in upstream, Intergenic region in downstream	22,893 bp 13,006 bp	MYB domain protein 113
EGP21864	7625998	C- > T	Intron	–	Phototropic-responsive NPH3 family protein
EGP21911	9870254	C- > T	Non-synonymous	–	–

Discussion

Eggplant fruit pigmentation is usually affected by low light conditions[39]. While the accumulation of anthocyanins in less- and non-photosensitive varieties was less influenced by darkness (Fig. 1)[20,38], thus identification of the genes responsible for less- and non-photosensitivity is crucial for breeding low light-tolerant varieties. Most previous studies focused on mapping genes related to purple coloration under the calyx, but did not distinguish between less- and non-photosensitivity[41,43,44]. A related study reported that a dominant gene controlling non-photosensitivity in eggplant was mapped between 19.9–20.2 Mb on chromosome 10[45], which is close to, but does not overlap with the candidate region (7.4–12.5 Mb) we identified for controlling less-photosensitivity in this study. Considering the different mapping results and the distinct phenotypes observed after bagging treatment, we speculated that the loci controlling non-photosensitivity and less-photosensitivity in eggplant may be different.

In this study, we identified three putative genes conferring less-photosensitivity in eggplant. *EGP21875* encodes a MYB TF SmMYB113, which plays critical roles in regulating anthocyanin biosynthesis by activating the expression of structural genes[22,23]. Overexpression of *SmMYB113* in eggplant resulted in a significant upregulation of the expression levels of structural genes, such as *SmCHS*, *SmCHI*, *SmF3H* and *SmANS*, and a substantial accumulation of anthocyanins in the regenerating shoots of eggplant[23], indicating that upregulation of the *SmMYB113* may directly increase anthocyanin accumulation. Moreover, the expression level of *EGP21875* in the peel of '609' was significantly higher than that of '749' under bagging conditions (Fig. 4). Therefore, the coloration of the '609' fruit peel under bagging conditions may be associated with the upregulation of *SmMYB113* expression, which may attribute to SNPs in the intergenic region.

Another potential candidate is *EGP21864* encoding a phototropism response NPH3 family protein. Studies have shown *NPH3* is involved in the perception and transduction of light signals, allowing plants to properly orient their growth towards light. The hypocotyl of *nph3* mutant in *Arabidopsis thaliana* failed to exhibit bending towards light and remains mostly straight, resulting in a defective phototropic response[66–68]. The expression of *EGP21864* was down-regulated both in the peel of '609' and '749' under bagging conditions, compared with that in natural conditions. And the expression of *EGP21864* in '609' was significantly higher than that in '749' under bagging conditions (Fig. 4). Therefore, the SNP of *EGP21864* may affect the sensitivity of plants to light. However, there was no literature or report indicating the direct involvement of phototropism response NPH3 family protein in anthocyanin biosynthesis, and future functional studies should be conducted to validate its specific contribution to fruit color determination.

In addition, *EGP21911*, a gene with non-synonymous mutation SNP, encodes a protein with unknown function. Its expression in '609' and '749' peel was not regulated by light. Under bagging condition, the expression in the peel of '609' was significantly higher than that of '749'. Whether this gene is involved in anthocyanin biosynthesis remains to be further studied.

This study focused on mining the genes responsible for less-photosensitivity using BSA analysis, while other reported studies have utilized various methods such as traditional QTL mapping and Genome Wide Association Studies (GWAS) to identify the locus for fruit color under the calyx[41–45]. In BSA analysis, individuals with similar phenotype are grouped together, and molecular markers are used to analyze the pooled samples in order to identify loci associated with the traits of interest. BSA analysis has been widely utilized in gene

mapping due to its advantages of high efficiency, low cost, time-saving, and labor-saving[69–72]. QTL mapping has been proved to be a powerful method to identify regions of genome that co-segregate with a given trait, especially quantitative traits. But QTL mapping is highly time-consuming and labor-intensive[73–76]. Based on linkage disequilibrium, GWAS is an effective analytical tool for deciphering the genetic basis of phenotypic diversity in crops. It possesses significant advantages of high throughput, efficiency, and reduced time consumption[77–79]. The aforementioned studies were primarily conducted on the basis of second-generation sequencing or existing molecular markers, obtaining multiple QTLs, candidate genes, or molecular markers associated with fruit color under the calyx[41–45]. Additionally, except for SNPs and Indels, some tissue color variations in horticultural crops were reported to be related to variations in large genomic segments, such as promoter variations, insertion of transposons, multiple repeats sequence, deletion of segments, etc[80–86]. The detections of such variations may necessitate more refined sequencing techniques, such as third-generation sequencing.

In summary, *EGP21875* encoding a MYB TF, *EGP21864* encoding a phototropic response NPH3 family protein, and *EGP21911* encoding an unknown functional protein were putative genes for regulating less-photosensitive anthocyanin biosynthesis in eggplant. Of note, *EGP21875* (*SmMYB113*) was the best candidate gene. Future studies, including functional validation and genetic mapping, will help to unravel the intricate regulatory networks and molecular mechanisms involved in less-photosensitive coloration in eggplant.

Conclusions

The less-photosensitive pigmentation in eggplant was controlled by a single dominant gene. The causal gene was mapped on chromosome 10, spanning from 7.4 Mb to 12.5 Mb. Three candidate genes, namely *EGP21875* (MYB domain protein 113), *EGP21864* (Phototropic-responsive NPH3 family protein) and *EGP21911* (Unknown protein), were identified as the putative genes conferring less-photosensitive coloration in eggplant.

Author contributions

The authors confirm contribution to the paper as follows: conceptualization, investigation, visualization, writing—original draft, writing—review & editing: Luo L; methodology, software, investigation: Niu Y; conceptualization, visualization, writing—review & editing: Li Q; methodology, software: Xia L, Wang C; investigation, data curation: Luo S; writing—review & editing, visualization: Li N, Xuan S, Wang Y; supervision, funding acquisition: Shen S; writing—review & editing, supervision, funding acquisition: Zhao J, Chen X; data curation: Chen X. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest. Jianjun Zhao is the Editorial Board member of *Vegetable Research* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and the research groups.

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