Characterization of a dominant *SmNac-like* gene as a candidate for photosensitivity in the fruit peel of eggplant

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Running title: *SmNac-like* gene controls photosensitivity trait in eggplant

ABSTRACT

Anthocyanins in the fruit peel of photosensitive eggplants exhibit a different distribution pattern compared to the photo-insensitive ones. The latter exhibits a uniform anthocyanin content, whereas photosensitive eggplants lack anthocyanin accumulation in areas not exposed to light, such as under the calyx, or have lower concentrations in less-exposed areas. In the current research work, genetic analysis of F1 and F2 populations revealed that the non-photosensitive phenotype in eggplants follows an autosomal dominant inheritance with a 3:1 ratio, indicating that the photosensitive trait is regulated by a single dominant gene. To locate and narrow down the position of the gene underlying photosensitivity, an F2 population segregating for photosensitivity and presence of anthocyanins was used for bulked segregant analysis sequencing (BSA-seq), and RNA-seq analysis of bagged photosensitive and non-photosensitive eggplants was performed. As a result, we identified the *SmNAC1-like* gene as a candidate for fruit photosensitivity trait in the eggplant materials used in this study. In the photo-insensitive eggplants (DH-ECAVI and IVIA-371) two different high-impact mutations were identified leading to light-insensitive and uniform anthocyanin content. The mutation in DH-ECAVI causes a premature stop codon, while in IVIA-371 an indel mutation occurs in the 3'-UTR sequence. No high-impact mutations were found in *SmNAC1-like* gene in photo-sensitive eggplants. These findings provide new insight into the regulation of the molecular mechanisms of anthocyanin biosynthesis in non-photosensitive eggplant and have important implications for marker-assisted selection and breeding.

KEYWORDS

Solanum melongena, *SmNAC1-like*, Anthocyanin Biosynthesis and Photosensitivity

INTRODUCTION

Eggplant (*Solanum melongena* L.) is a solanaceous crop with high economic value and characterized by the presence of high concentrations of anthocyanins in the fruit peel of some cultivars. Anthocyanins are water-soluble pigments derived from anthocyanidins by the addition of sugars, which make flowers and fruits appear red, blue or purple (Takos et al., 2006 and Mattioli et al., 2020). In addition, anthocyanins offer several benefits to plants, such as protection against UV radiation, extreme temperatures, and pathogen attacks (Lorenc-Kukula et al., 2005; Liu et al., 2013 and Ren et al., 2014). Finally, anthocyanins contribute to human health, since they present strong antioxidant properties, with beneficial effects on human well-being (Muñoz-Falcón et al., 2009; Speer et al., 2020).

Light plays a major role in the synthesis of anthocyanins in the fruit peel of eggplants. Previous research in photosensitive eggplants demonstrated that light activates the anthocyanin biosynthetic pathway leading to the accumulation of anthocyanins in the fruit peel in a time-exposure manner (Jiang et al., 2016; Li et al., 2017; Sang et al., 2022, Li et al., 2023). However, non-photosensitive eggplants are able to synthesize large amounts of anthocyanins under shaded conditions (Jiang et al., 2016 and Sang et al., 2022). A special case is the fruit peel under the eggplant calyx, which is always shaded and therefore remains uncolored (either partially or completely) in photosensitive fruit peels compared to non- photosensitive ones, indicating that light is required for the development of purple pigmentation in eggplant (Li et al., 2017; Li et al., 2018 and Zhang et al., 2019). Consequently, breeding for non-photosensitive eggplant varieties is an effective method to maintain a stable anthocyanin accumulation independently of environmental conditions. Given the relevance of the non-photosensitivity trait in eggplant breeding, some studies have focused on mapping the genes responsible for photoinsensitivity. These studies have identified different QTLs associated with non-photosensitivity across different chromosomes, likely due to the use of diverse eggplant materials, research backgrounds and eggplant reference genomes (Qiao et al., 2022; He et al., 2022; Mangino et al., 2022; Luo et al., 2023a-2023b and You et al., 2023). Several genes are involved in the regulation of anthocyanin biosynthesis in eggplant where the structural genes in the anthocyanin biosynthetic

pathway include enzyme-encoding genes such as *CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR* and *ANS* (Jaakola et al., 2013 and Liu et al., 2018). Those genes are regulated by transcription factors (TFs), several of which have been postulated as candidates to regulate anthocyanin gene expression (Li et al., 2017; Li et al., 2018; Qiao et al., 2022; He et al., 2022; Mangino et al., 2022; Zhou et al., 2022; Li et al., 2023; Luo et al., 2023a-2023b You et al., 2023). A common feature of the candidate TFs is that they activate R2R3-MYB, basic helix-loop-helix (bHLH) or WD repeat proteins (WDR), which form the MYB-bHLH-WDR (MBW) complex to regulate anthocyanins structural biosynthesis genes (Ramsay et al., 2005; Yan et al., 2021). Researching features connected to anthocyanins fruit peel pigmentation can help us comprehend this characteristic of eggplants and create new kinds of eggplants with varied anthocyanin coloration.

In this study, we investigated the photosensitivity trait in local eggplant varieties from País Valencià. To map non-photosensitivity trait, we evaluated an F2 population derived from a cross between a photosensitive cultivar (*S. melongena* ASI-S-1) and a non-photosensitive variety (*S. melongena* IVIA-371). This population also segregates for anthocyanin and clorophyll pigmentation. Bulked segregant analysis by sequencing (BSA-seq), combined with RNA-seq, was applied to identify candidate loci, *SmNAC-like protein*, for photosensitivity (PS) in the F2. The presence of DNA mutations in the locus identified was in agreement with the genotype of non-photosensitive phenotypes (e.g., IVIA-371 and DH-ECAVI) and photosensitive phenotypes (e.g., AN-S-26, H15, and ASI-S-1). Two different mutations in the down-regulated *SmNac1-like* gene, a transcription factor, were found in IVIA-371 and DH-ECAVI but not in the photosensitive accessions. The identification of *SmNac1-like* as a candidate gene for the eggplant photosensitive trait is useful to understand the genetic basis of the regulation of the anthocyanin pigmentation in the eggplant fruit peel, as well as for eggplant breeding.

MATERIAL AND METHODS

1.- Plant materials and cultivation conditions.

The *S. melongena* accessions ASI-S-1 (anthocyanin photosensitive, non-striped and with presence of chlorophyll pigmentation in the peel) and IVIA-371 (anthocyanin non-photosensitive, striped and no chlorophyll pigmentation in the peel) were used as parents to develop an F2 population of 120 individuals segregating for the photosensitivity trait (PS), presence of fruit anthocyanin (FA) and chlorophyll content (CC) (Figure 1 Panels A and B). PS was categorized as PUC (presence of purple pigmentation under the calyx, i.e., negative for PS) or puc (absence of purple pigmentation under the calyx, i.e., positive for PS. In addition to the eggplant accessions ASI-S-1 and IVIA-371, two sub-introgression lines (IL-69(6)5-12-Ch10 and IL-69(6)5-8-Ch10, derived from the introgression line IL-M5-19) plus accessions DH-ECAVI (photoinsensitive-anthocyanin uniform pigmentation), H15 and AN-S-26 (photosensitive- anthocyanins in the fruit peel exposed to sunlight) were used in the current study (Mangino et al., 2022). With regards to the phenotype of IL-69(6)5-12-Ch10 and IL-69(6)5-8-Ch10 showed a complete loss of anthocyanins content in the fruit peel (Figure S1). The introgression line IL-M5-19 introgression line derives from the MEL5 eggplant (photosensitive) with an introgression from the eggplant wild relative *S. insanum* in chromosome 10.

Seeds of all the accessions were germinated in Petri dishes following the Ranil et al. (2015) protocol and transferred to seedling trays in a climatic chamber under a photoperiod 16 h light/ 8 h dark and temperature regime of 22 ± 2 ºC. The plantlets were grown for four weeks (up to the fifth true leaf development stage) in a climatic chamber before acclimatization for 48 hours in a benched glasshouse. Plantlets were then transplanted to 15 L pots filled with coconut fiber and grown in a pollinator-free benched glasshouse of the Universitat Politècnica de València (UPV), Valencia, Spain. Plants were spaced 1.2 m between rows and 1 m within the row, fertirrigated using a drip irrigation system and trained with vertical strings. Pruning was done manually to regulate vegetative growth and flowering. Phytosanitary treatments were performed when necessary.

2.- Generation of a F2 population and BSA-Seq analysis of PUC vs puc bulks.

The F2 population obtained by the intraspecific cross between ASI-S-1 and IVIA-371 accessions was phenotyped for the PS and FA fruit traits. A Chi-square test was performed to assess the goodness-of-

fit to a monogenic dominant inheritance pattern (3:1 segregation model). The F2 population was used for a BSA-Seq for the PS trait. The PS have been scored in 94 plants out of the 120 of the total population, as 26 plants did not display anthocyanin pigmentation. The PUC and puc bulks consisted of 15 F2 individuals. Young leaves were harvested from the entire F2 population (120 F2 individuals) as well as from six individual plants for each of the two parents. Their DNA was isolated, quantified and tested for quality prior to select six plants from each of the parents and 30 individual F2 plants based on their different PS phenotype (PUC vs. puc). Genomic DNA was extracted using the silica matrix (SILEX) protocol (Vilanova et al., 2020) and checked for integrity by agarose electrophoresis. NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) ratios 260/280 and 260/230 were used to test the DNA quality, while its concentration was estimated with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Pools for the PUC and puc phenotypes bulks were made by mixing in a equimolecular from each of selected F2 individual and shipped to Novogene (Novogene Europe, Cambridge, United Kingdom) where genomic libraries (PE 150, insert 350 bp) were constructed and sequenced. All raw reads were trimmed using the fastq-mcf tool from the Ea-utils package (Aronesty, 2013) with “q 30 -l 50” parameters, and the overall quality was checked using FastQC v.0.12.1 (Andrews et al., 2010). Clean reads were mapped against the 67/3 v3 (for QTL comparison and DNA variant analysis in reported eggplant populations with PS trait) and v.4.1 (for data cross-referencing with RNA-seq) eggplant reference genome (Barchi et al., 2019). To map the clean reads BWA-MEM v.0.7.17–r1188 was used with default parameters (Li and Durbin et al., 2009). The ΔSNP-index was estimated using the QTL-seq software v.2.2.3 (Takagi et al., 2013) from BAM files.

3.- RNA-seq analysis of F2 population.

RNA-seq analysis was conducted on bagged fruits from the F2 population to ascertain the gene expression profile of eggplant fruits with and without PS. Eggplant flowers from each plant were manually pollinated and kept in opaque paper bags for five days in the dark. The time lapse was selected, as it was found to be the shortest time for the appearance of anthocyanin pigmentation. Subsequently, a set of eggplant fruit peels was gathered by cutting them with a surgical blade

avoiding as much as possible to collect eggplant flesh, and subsequently frozen in liquid nitrogen before being kept in storage at -80ºC. The eggplant fruit peels were grouped in three randomized replicates for each phenotype of PS trait (PUC and puc). For the PUC samples, each replicate consisted of 4 fruit peels. However, for the puc samples, due to the limitation in the number of fruits available, each replicate consisted of three samples. The RNA extractions were done with RNAqueous kit and Ambion Plant RNA isolation aid reagent (Invitrogen™ AM1912 and AM6990) following the manufacturer instructions. The RNA integrity and quality were checked by 1.5% agarose gel and with the spectrometer Nanodrop 1000 (Thermo Scientific). RNA concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen) and an AccuBlue ® Broad Range RNA quantitation kit (Biotium). The samples with the highest values of 260/280 and 260/230 ratios were selected, normalized for their concentration and randomly pooled.

RNA samples were shipped to Novogene Europe (Cambridge, UK) for library preparation and sequencing. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Then, mRNA was fragmented and the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. After adapter ligation, fragments were size-selected, PCR-amplified, and purified. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms (NovaSeq 6000), according to effective library concentration and data amount (9 GB of data). With regards to RNA-seq read processing, clean reads were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data through Novogene in-house perl scripts and Q20, Q30 and GC content were calculated. All the downstream analyses were based on clean, high-quality data (Table S1). Reference genome (*S. melongena* 67/3 v4.1) and gene model annotation files were downloaded from Sol Genomics Network (SGN) website. Index of the reference genome was built and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5 (Zhang et al., 2021). This tool was selected for mapping as it can generate a database of splice junctions based on the gene model annotation file, giving a better mapping result than other non-splice mapping tools. Reads numbers mapped to each gene were

counted using featureCounts v1.5.0-p3 (Liao et al., 2014). Then, FPKM was calculated based on the length of each gene and reads mapped to it.

Differential expression analysis was performed using the DESeq2 R package (1.20.0), which uses a statistical model based on the negative binomial distribution for determining differential expression (Love et al., 2014). The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling false discovery rate (Benjamini and Hochberg., 1995). Genes with an adjusted P-value $\leq$ 0.05 were assigned as differentially expressed. Finally, Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEG) was implemented by the clusterProfiler R package (Yu et al., 2012; Wu et al., 2021; Xu et al., 2024), which corrects gene length bias. GO terms with corrected P-value < 0.05 were considered significantly enriched by differentially expressed genes. The clusterProfiler R package was used to test the statistical enrichment of differential expression genes in KEGG pathways database (http://www.genome.jp/kegg/).

4. Evaluation of DNA sequence variants in photo-sensitive and photo-insensitive cultivars from different eggplant population.

To determine the presence of DNA sequence variants in the DEGs located inside the QTL identified at chromosome 10 at photo-in/sensitive eggplant cultivars analyzed against v4.1 eggplant reference genome (Barchi et al., 2019), firstly the genomic DNA chromosome 10 (63.4 – 67.3 Mb) from the eggplant reference genome “67/3” v4.1 versus v3 were compared with D-Genies (Cabanettes, Klopp., 2018). As a result, no major breaks and inversions were detected. So, the gene synteny remains constant at the genomic DNA region under consideration. Secondly of all the clean reads of ASI-S-1 and IVIA-371 obtained in a previously research (Mangino et al., 2022) was mapped against the eggplant reference genome “67/3” v3 with BWA-MEM and only the uniquely aligned reads were selected. Regarding the variant calling was carried out again, due that the reported sequence changes reported by genes Mangino et al. (2022) were not validated by Sanger Sequencing (Table S2). With regard the new variant calling filters for the PS trait in the F2 interspecific cross between ASI-S-1 and IVIA-371 were done with FreeBayes (Garrison and Marth., 2012) with the following parameters: --min coverage 10, --limit coverage 60 amd –min alternate fraction 0.2. The predicted effects of the

sequence changes detected were evaluated with SnpEff (Cingolaniet et al., 2012). The DNA variants that affect the regulatory sequences (5'-UTR and 3'-UTR) and/or the coding sequences (i.e., frameshift, indels, splicing variants, etc) for the PS trait in homozygous allelic variants were classified as “high impact”. The Integrative Genomics Viewer (IGV) tool (Robinson et al., 2022) was used to validate the variants detected by the combination of FreeBayes + SnpEff softwares. To expand the number of eggplants cultivars evaluated, eggplants with PS trait (DH-ECAVI, ANS-S-26 and H15) previously reported (Mangino et al., 2022) were evaluated. As previously indicated clean reads of the respective eggplants accessions was mapped against the eggplant reference genome “67/3” v3 with BWA-MEM and only the uniquely aligned reads were selected. Then the variant calling were done with FreeBayes (Garrison and Marth., 2012) with the following parameters: --min coverage 10, --limit coverage 60 amd –min alternate fraction 0.2. The predicted effects of the sequence changes detected were evaluated with SnpEff (Cingolaniet et al., 2012). “High impact” variants were selected and validated with IGV

RESULTS

1.- Identification and inheritance of a QTL linked to photoinsensitivity.

1.1.- Generation and inheritance of the scored traits in the F2 population.

To determine the PS trait segregation pattern, an F2 population was produced by mating the *S. melongena* parents ASI-S-1 (puc, non-striped, and with presence of chlorophyll pigmentation in the peel) with IVIA-371 (PUC, striped, and with absence of chlorophyll pigmentation in the peel). Regarding the presence of plant anthocyanin pigmentation in the parents, ASI-S-1 displayed anthocyanin in leaves and stem, meanwhile IVIA-371 only showed anthocyanin content in the stem but not in the leaves (Figure 1 Panel A). Regarding the F1, it displayed the PUC phenotype and FA phenotype with uniform (non-striped) anthocyanin pigmentation and also showed background chlorophyll presence in the peel. In the F2 generation, the PS, FA and CC traits segregated resulting in several phenotypic combinations (Figure 1 Panel B) for the three traits present in the eggplant cultivars used in this study.

The segregation pattern of the PS trait showed an autosomal dominant inheritance (Table 1), being eggplants with PUC and non-striped phenotype the most abundant (Figure 1). Plants producing fruits without anthocyanin pigmentation (i.e., green or white fruits), were not scored for the PS phenotypes (as the phenotype could not be determined) (Figure 1 Panel B). These results suggested that the PS trait in our IVIA-371 x ASI-S-1 population are controlled each by one dominant gene. On the other hand, although the FA and CC traits segregate in the F2 population were not used in this study as they were independent traits in relation to the PS trait (data not shown).

1.2.- Identification of the genomic loci linked to PS trait by bulk segregation analysis in the F2 population

To narrow down the genomic loci linked to the PS trait, two DNA pools from the IVIA-371 x ASI-S-1 F2 population (15 plants with the PUC phenotype and 15 with the puc phenotype) were sequenced. This sequencing yielded an average of 154 million 150-bp raw reads per bulk, with a mean coverage of 33.3x. Using ASI-S-1 as the reference sequence, the ΔSNP-index was calculated for 535,083 SNPs, getting a QTL on chromosome 10 between 62.9-69.7 Mb with a 99 % confidence interval (CI) for the

PS trait. Similarly, when the ΔSNP-index was calculated for 562,169 SNPs using IVIA-371 as the reference sequence, a region between 63,4-67,5 Mb was identified on the same chromosome 10 with a 99 % CI (Figure 2, Figure S2 and Table S3). As a result, 102 genes were identified within the common candidate region (Table S4). Variant calling and prediction of the sequence changes were evaluated in both candidate regions of ASI-S-1 and IVIA-371, no DNA variants in the coding sequences (frameshift, indels, splicing variants, etc.) were detected**2.- Genetic evidences of QTl for photoinsensitivity in different eggplant populations.**

In order to determine the relevance of the QTL on chromosome 10 in the photosensitivity trait, it was compared with previous QTLs identified in different eggplant populations (Table 2). To be able to compare with the previous data, the BSA-seq analysis was re-analyzed against the v3 of the eggplant reference genome. As a result of the re-analysis a QTL in the chromosome 10 for ASI-S-1 between 84,1-87,9 Mb with 99 % CI and a region between 83,5-97,7 Mb with 95 % CI for IVIA-371 were identified (Table S5, Table S6, Figure S2 and Figure S3). Firstly different sub introgression lines derived from the IL-M5-19 introgression line (introgressed DNA of Solanum insanum into MEL5 eggplant) were evaluated. As a result, the sub introgression lines (IL-69(6)5-12-Ch10 and IL-69(6)5-8-Ch10) with introgressed DNA at chromosome 10, IL-69(6)5-12-Ch10 at 84,4-102,8 Mb and IL-69(6)5-8-Ch10 at 105,8-106,6 Mb respectively (Table 2), showed complete loss of anthocyanins content in the fruit peel (Figure S1). With regards the S3MEGGIC population for FA (91.1-94.8 Mb) and PUC (91.1-94.8 and 98.2-100.5 Mb) showed significant SNPs stacked at the end of chromosome 10 (Table 2).

Taking all the QTLs identified in the different populations together, suggest that the end of the chromosome 10 as a hotspot related with anthocyanin related traits. Six of QTL reported showed overlap (total or partial) along the different eggplant populations (Table 2). Of these six reported QTLs, two groups can be observed: the first one consists of four overlapping QTLs (IL-69(6)5-12-Ch10 84,4-102,8 Mb, FA 91.1-94.8 Mb, PUC 91.1-94.8 Mb, ASI-S-1 84,1-87,9 Mb and IVIA-371 83,5-97,7 Mb), while the second one consists of two QTLs with partial overlap (IL-69(6)5-12-Ch10 at

84,4-102,8 Mb and PUC 98.2-100.5 Mb). As a summary two common DNA regions across all the QTLs were identified (QTLs_ASI-S-1 84.1-87.9 (3.80 Mb) and PUC 98.2-100.5 (2.3 Mb)).

3.- Differential expression analysis of bagged eggplants

The gene expression analysis between PUC and puc bagged eggplant fruits of F2 eggplant population was performed by RNA-seq of the fruit peel. The results indicate that from a total of 177 DEGs, 53 DEGs were up-regulated in the fruit peel of eggplants with PUC phenotype, while 124 DEGs were down-regulated in the fruit peel of eggplants with puc phenotype (Figure 3 and Table S7-9). The 177 DEGs identified from the eggplant fruit peel for the PS trait can be grouped in four different subclusters (Table S9 and Figure S4). The subclusters 1 and 3 are made of the DEGs expressed mainly in IVIA-371 (PUC phenotype) with a total of 53 genes, that constitute the whole up-regulated DEGs identified by RNA-seq. The subclusters 2 and 4 contain 124 genes expressed totally in ASI-S-1 (puc phenotype), and constitute all the down-regulated DEGs reported for the eggplant fruit peel (Table S2 and Table S8). Concerning the functional and gene enrichment analysis of up- and down-regulated genes in eggplant fruit peel with PUC and puc phenotypes, few DEGS were identified due to the fruit peel characteristics. For the up-regulated DEGs expressed in IVIA-371, the gene set showed an enrichment in KEGG terms related to flavonoid and phenylpropanoid biosynthesis (Table S7), mostly located in the subcluster 1 (Table S8) and in agreement with the presence of anthocyanins in the fruit peel of eggplant (Figure 3 and Table S8). The following up-regulated genes in flavonoid biosynthesis were identified in IVIA-371: SMEL4.1_09g021180.1, SMEL4.1_05g002700.1, SMEL4.1_08g022350_1, SMEL4.1_02g020840_1, SMEL4.1_05g002760_1, SMEL4.1_05g018170_1 and SMEL4.1_12g016460_1. Regarding the GO annotation terms, an enrichment in terms like small molecule metabolic process, transferase activity of acyl and hexosyl groups, or coenzyme bonding and iron ion binding was observed. Those functions are consistent with the activity of the up-regulated genes identified by the KEGG terms.

Concerning the down-regulated genes in IVIA-371 and, converserly, over-expressed in ASI-S-1, the gene set showed an enrichment in KEGG terms related to MAPK signaling pathway, phenylpropanoid biosynthesis, cysteine and methionine metabolism, zeatin and alanine biosynthesis, and aspartate and

glutamate metabolism (Table S7). The enrichment in those KEGG terms in the MAPK signaling pathway and zeatin biosynthesis are involved in plant growth and fruit enlargement (Schäfer et al., 2015 and Jagodzik et al., 2018) in agreement with the fact that the eggplant fruits were growing. About the KEGG terms of cysteine and methionine metabolism plus alanine, aspartate and glutamate metabolism are reported to play a role in the generation of secondary metabolites involved in plant growth, fixing inorganic sulfur, precursors of several biosynthesis pathways, and plant protection against abiotic stress (Moormann et al., 2022). Finally, the accumulation of KEGG terms in phenylpropanoid biosynthesis indicated an accumulation of the biosynthesis precursors of flavonoids (Vogt, 2010; Dong et al., 2021 and Liu et al., 2021). With respect to the GO annotation terms an enrichment in terms like coenzyme binding, carbohydrate metabolic process, regulation of biological quality, or carbon-oxygen lyase activity was observed. Those functions are consistent with the activity of the up-regulated genes identified by the KEGG terms since they are involved in biosynthetic pathways.

4.- Identification of genes expressed in photo-insensitive fruits in the dark conditions, *SmNac1*

4.1- Evaluation of the DEGs inside of the candidate region acotated in the F2 population

To narrow down the number of putative candidate genes within the common candiate region of ASI-S-1 and IVIA-371 (102 genes), their expression levels (Table S9) were evaluated given that no DNA variant were found in the variant calling plus prediction of the sequence changes (see above – section 1.2). As a result, five genes were differentially expressed (*SMEL4.1_10g016630.1*, *SMEL4.1_10g014730.1*, *SMEL4.1_10g013610.1*, *SMEL4.1_10g014530.1*, and *SMEL4.1_10g019230.1*) (Table 3). The *SMEL4.1_10g016630.1* codifies for a CHI-like protein, essential enzyme in the anthocyanin synthesis pathway, is up-regulated. This is in agreement with the fact that the PUC and puc pools showed anthocyanin content in the fruit peel (Figure 2). With regards the down-regulated genes (*SMEL4.1_10g014730.1*, *SMEL4.1_10g013610.1*, *SMEL4.1_10g014530.1*, and *SMEL4.1_10g019230.1)*, *SMEL4.1_10g014730.1* and *SMEL4.1_10g014530.1* codifies for a

NAC1-like protein (a transcription factor) RAD5B-like protein (DNA repair protein) respectively. The remaining down-regulated genes encode proteins of unknown function (Table 3).

Since the differentially expressed genes did not show mutations in the coding sequences, the regulatory regions (5'UTR and 3'UTR) were analysed. As a result the NAC-1 like protein (*SMEL4.1_10g014730.1*) showed an indel in the 3'UTR (Reference: ATATTATTATTATTATTATC, Alternate variant: ATATTATTATTATT---ATC) (File S1 and FileS2).

4.2- Presence of DNA mutation of *SmNac1* in other eggplant accessions photosensible (H15 and ANS-S-26) and photoinsenstible (DH-ECAVI)

With the aim to known of the DNA mutation in the *SMEL4.1_10g014730.1* was present in other eggplant accessions previously reported in the v3 genome eggplant consortium (Mangino et al., 2022). Firstly the equivalent of the five genes differentially expressed were located in the v3 of the eggplant refence genome. All of them were inside of the common DNA region of ASI-S-1 (84,1-87,9 Mb) and IVIA-371 (83,5-97,7 Mb) (Table S6). Secondly the coding sequences,5'UTR and 3'UTR sequences were reevaluated in photosensitivy (H15, ANS-S-26 and ASI-S-1) and photoinsensitivity (DH-ECAVI and IVIA-371) eggplant accessions (Table 4).

As a result, in the photoinsensitivity eggplant accessions only *SMEL4.1_10g014730.1* showed the same disruptive inframe insertion at 3'UTR in IVIA-371, plus DH-ECAVI showed a disruptive in frame insertion that causes a frameshift in the coding sequence (Table 4 and File S1 and FileS2). With regards the photosensitivy (H15, ANS-S-26 and ASI-S-1) eggplant no DNA mutations were detected in *SMEL4.1_10g014730.1* and only for H15 *SMEL4.1_10g014530.1* showed a conservative in frame insertion that do not change the open reading frame of RAD5B-like protein.

DISCUSSION

Plant domestication in the Solanaceae family resulted in a great diversity of fruit shapes, sizes, and fruit colours. Despite this, modern breeding programs have generally favored eggplants with constant and intense dark and purple pigmentation in the fruit peel (Samuels et al., 2015; Wang et al., 2015 and Taher et al., 2017). In western markets, eggplants with black or purple peel are of higher economic value (Muñoz-Falcón et al., 2009). In our work, different eggplant populations were used to evidence that regarding to antocyanin related traits (complete absence of antocyanin, photsensitivy and fruit anthocyanin) the chromosome 10 is a candidate DNA region to take into account. Anthocyanins have a strong antioxidant effect, so their high content in the fruit peel of eggplant improves nutrition. So, a research to determine the genetics basis of the PS is needed to set the basis of a new generation of eggplants cultivars with high content of secondary metabolites beneficial for human health. In the current research the PS showed an inheritance in agreement with a monogenic dominant gene, following 3:1 ratio in and F2 generation, consistent with previous research data (Honda et al., 2014; He et al., 2022; Qiao et al., 2022; Luo et al., 2023ab). These eggplant accessions were selected because, based on the corrected data reported by the GWAS study of the MAGIC population, neither of the two accessions presented mutations in the CDS of the candidate regions, suggesting that expression must have been altered. Regarding the fruit pigmentation of the F2 generation is probably due to the heterozygosity of the parentals for the genes that controlled photosensitivity, plant anthocyanin, and fruit anthocyanin.

The reported studies on the PS trait used commercial varieties of eggplant (Honda et al., 2012) or non-photosensitive eggplants varieties (He et al., 2022; Qiao et al., 2022; Luo et al., 2023ab) as parental donors for the PUC phenotype. Meanwhile for the puc phenotype present in photosensitive eggplants is recessive compared to the dominant allele of non-photosensitive eggplant varieties (Honda et al., 2012; He et al., 2022; Qiao et al., 2022; Luo et al., 2023a and Luo et al., 2023b). It should be noted that two commercial eggplant varieties with the PUC phenotype (IVIA-371) and (ASI-S-1) were used to produce the F2 population.

As opposite what happens with other fruit colour traits (such as the green fruit neeting (Arrones et al., 2022 and 2024, Toledo Garcia, et al., 2024)), that the phenotype present in the commercial eggplant

varieties involves loss of gene function and recessive inheritance, so the PS trait results in a dominant inheritance and a gain of function. That phenomenon is quite uncommon since domestication usually involves the loss of gene function or regulation (Lester and Hasan, 1991).

Regarding the localization of the reported QTLs (He et al., 2022; Qiao et al., 2022; Luo et al., 2023a, Luo et al., 2023b) for the PS at the end chromosome 10 using the eggplant genome 67/3 v3 as a reference with complete different eggplant accession is in agreement with our results, validating that region as a promising localization to perform fine-tune mapping or expression analysis to identify gene candidates.. However, other QTLs for the PS trait have been reported in chromosome 1 and 3 (Mangino et al., 2022). Here, we confirmed the end chromosome 10, using v3 eggplant genome as a reference, as candidate region related with anthocyanin content traits, identifying 84 potential candidates genes for ASI-S-1 and 550 for IVIA-371. To filter the number of candidates genes identified by the BSA-seq analysis, the list of genes was compared with the identified DEG from the RNA-seq of F2 population. Out of the five genes identified as differentially expressed, the *SmNAC1-like* (SMEL4.1_10g014730.1) is mutated in eggplant varieties with PUC phenotype (DH-ECAVI and IVIA-371) but not in puc phenotype (ANS-S-26. H15 and ASI-S-1) eggplant varieties. All these results together provide strong evidence of *SmNAC1-like* gene as candidate gene controlling the PUC trait in our eggplant varieties.

Regarding gene regulation of the anthocyanin biosynthetic pathway, previous studies suggest that light and cold induce anthocyanin biosynthesis from the moment of exposure until saturation through a highly dynamic range of TFs (Jiang et al., 2016; Li et al., 2017; Sang et al., 2022, Li et al., 2023 and Zhou et al., 2020). Specifically, *SmCry1*, *SmCry2*, *SmCry3* and *SmUVR8* are involved as photoreceptors in response to light. Meanwhile *SmCBF1*, *SmCBF2* and *SmCBF3* have been reported as sensors in response to low temperature. Both sets of genes activate the signaling pathway to produce anthocyanins (Li et al., 2017; Li et al., 2023 and Zhou et al., 2020). Regarding the different downstream stages of the anthocyanin synthesis pathway, there is consensus on the necessary participation of the pathway's structural genes: *SmCHI*, *SmF3H*, *Sm3GT*, *Sm5GT*, *SmDFR* and *SmANS*) (Jaakola et al., 2013 and Liu et al., 2018. For the intermediate steps, several TFs and their interactions have been proposed in different eggplant populations (R2R3-MYB transcription factor

[*SmMYB113*, *SmMYB86*, *SmMYB75*, *SmMYB35*], basic helix–loop–helix [*SmbHLH1*, *SmbHLH13*], *SmHY5*, *SmTT8*, *SmGL3*, *SmTTG1*, *SmCIP7*, *SmGL2*, *SmGATA26*, *SmCOP1*, *SmSPA* and *SmFTSH10*) (Jiang et al., 2016; Li et al., 2017; Li et al., 2018; Qiao et al., 2022; He et al., 2022; Mangino et al., 2022; Zhou et al., 2022; Li et al., 2023; Luo et al., 2023ab and You et al., 2023). Specifically, any mutation in *SmMYB113* or pathway's structural genes generate phenotypes without anthocyanin content (Mangino et al., 2022; Luo et al., 2023ab and You et al., 2023). Therefore, for the PS trait the TFs proposed (*SmHY5*, *SmTT8*, *SmGL3*, *SmTTG1*, *SmCIP7*, *SmGL2*, *SmGATA26*) are in the first steps of the signaling pathway and always upstream of the *SmMYB113* (Mangino et al., 2022, Luo et al., 2023ab and You et al., 2023). In that sense, *SmNAC1-like* agrees with the previous candidate TFs proposed. The homologous of *SmNAC1-like* in *Prunus persica* (*PpNAC1*) and *Malus domestica* (*MdNAC1*) are located upstream of the *SmMYB113* homologous in *P. persica* (*PpMYB10.1*) and *M.* domestica (MdMYB10) (Liu et al., 2023 and Meng et al., 2023).

By using different eggplant materials, we have identified *SmNAC1-like* gene as a promising candidate for the PS trait, with disruptive mutations associated with non-photosensitivity phenotypes. The eggplant PS trait has significant implications for fruit visual quality, as it results in uniform peel pigmentation, which is highly desirable for fruit commercialization. The study of this relevant trait could be of interest for future eggplant breeding projects with the aim of increasing the diversity of the fruit colour palette. In addition, due to a higher concentration of anthocyanin contents and their effect as an antioxidant, *SmNAC1-like* could also be a valuable target for controlling the gene expression of the anthocyanin content in the fruit peel of the eggplants.

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CONTRIBUTIONS

JG-C, SM, PG, SV, MP and JP conceived the idea. JG-C and SM supervised and designed the manuscript. JG-C, SM, AA and MDT-T developed the F2 population for BSA-seq and RNA-seq. EG-P developed the introgression lines. JG-C, SM, JL, JS-P, MDT-T performed DNA and RNA extraction. JG-C wrote the manuscript. All the authors reviewed and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI SRA repository, BioProject IDs for BSA-seq and RNA-se analysis are XX and XX, respectively.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation if this manuscript or in any of the figure and table attached to the manuscript.

SUPPLEMENTARY MATERIALS

File S1. DNA sequence modification in SMEL4.1_10g014730.1 in 67/3 v3, DH-ECAVI and IVIA-371.

File S2. cDNA and protein sequence of SMEL4.1_10g014730.1 in 67/3 v3 and DH-ECAVI.

Table S1. RNA quality data of the samples used in the RNA-seq data of PUC and puc.

Table S2. Evaluation of the gene related with anthocyanin biosynthesis in the QTLs determined by Mangino et al., 2022.

Table S3. Results of bulk segregation analysis in ASI-S-1 and IVIA-371 with a confidence interval of 95% and 99%, respectively. Eggplant genome reference v4.1

Table S4. List of genes in the candidate region acotated by BSA-seq in eggplant varieties ASI-S-1 and IVIA-371. Eggplant genome reference v4.1

Table S5. Results of bulk segregation analysis in ASI-S-1 and IVIA-371 with a confidence interval of 95% and 99%, respectively. Eggplant genome reference v3

Table S6. List of genes in the candidate region acotated by BSA-seq in eggplant varieties ASI-S-1 and IVIA-371. Eggplant genome reference v3

Table S7. List of differentially expressed genes in the eggplant varieties IVIA-371 (PUC positive trait) versus ASI-S-1 (puc negative trait).

Table S8. List of the subcluster identified in the heatmap of the differentially expressed genes of light-independent versus light-dependent eggplant varieties.

Table S9. List of the enrichment analysis of the GO and KEGG terms of the up- and down-regulated genes in PUC versus puc eggplant fruit peel

Figure S1. Phenotype of the two sub-introgression lines (IL-69(6)5-12-Ch10 and IL-69(6)5-8-Ch10) derived from the introgression line IL-M5-19.

Figure S2. Delta of SNP-Index of the bulk segregation analysis of light-dependent and light-independent eggplant varieties

Figure S3. Number of differentially expressed genes (DEG) in the four subclusters identified in the clustering heatmap PUC vs puc. The DEG in the subcluster 1 and 3 were up-regulated in the light-independent (IVIA-371) eggplant (Panel A and B) meanwhile the DEG in the subcluster 2 and 4 were up-regulated in the light-dependent (ASI-S-1) eggplant.

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TABLES

Table 1. Genetic analysis of the segregation of photosensitivity in the F2 population from the cross of ASI-S-1 x IVIA-371. For photosensitivity, only the plants yielding fruits with anthocyanic pigmentation were used.

Cross	Population	Photosensitivity		Total	Expected segregation	Chi-square	p value
		Presence	Absence				
[ASI-S-1xIVIA371] self-crossed	F2	76	18	94	3:1	1.58	0.20

Table 2. QTLs related with anthocyanin biosynthesis (fruit anthocyanin and purple under calyx traits) in different eggplant materials used in the current research and in Mangino et al. (2022), which used as parents of a MAGIC population some of the accessions used in this study.

Type of material	Trait	Accession	Genome version	Chromosome	QTL anthocyanin pigmentation (Mb)		Size (Mb)	Reported
					Start	End		
Introgression Line	Purple under calyx and fruit anthocyanin	IL-69(6)5-12-Ch10	v3	10	84.38*	102.79*	18.41	Current study
Introgression Line	Purple under calyx and fruit anthocyanin	IL-69(6)5-8-Ch10	v3	10	105.85	106.64	0.79	Current study
MAGIC population	Fruit anthocyanin	S3MEGGIC population	v3	1	5.11	5.88	0.77	Mangino et al., 2022
				9	16.2	17.4	1.20	
				10	91.08*	94.81*	3.73	
	Purple under calyx	S3MEGGIC population	v3	1	5.11	5.88	0.77	
				3	7.22	8.65	1.43	
				10	31.54	40.09	8.55	
					2.08	2.67	0.59	
					3.94	4.94	1.00	
					91.08*	94.81*	3.73	
					98.25*	100.55*	2.30	
F2 ASI-S-1 x IVIA-371	Purple under calyx	ASI-S-1	v3	10	84.1*	87.9*	3.80	Current study
		IVIA-371			83.5*	97.7*	14.20	

* The QTLs of the IL-69(6)5-12-Ch10, S3MEGGIC population for fruit anthocyanin and Purple under calyx overlap with the candidate regions obtained by BSA-seq in the eggplant accessions IVIA-371 and ASI-S-1 as a reference.

Table 3. List of differentially expressed genes in the QTL of chromosome 10 delimited by bulk segregation analysis in the eggplant varieties IVIA-371 (photoinsensitive) and ASI-S-1 (photosensitive).

Regulation	RNA-seq data			Genome version	Gene description
	Gene Name	log2FoldChange	p value		
UP	*SMEL4.1_10g016630.1*	577,158,472,269,854	6.03E-09	v4.1	CHI-like. Chalcone flavonone isomerase of Nicotiana tabacum
DOWN	*SMEL4.1_10g014730.1*	-8,997,230,728,622	1.40E+04	v4.1	NAC1-like. NAC domain-containing protein 1 of Solanum lycopersicum
	SMEL4.1_10g013610.1	-231,064,107,425,366	7.57E+09	v4.1	Protein of unknown function
	SMEL4.1_10g014530.1	-128,031,116,712,945	0.00011	v4.1	RAD5B-like. DNA repair protein RAD5B of Arabidopsis thaliana
	SMEL4.1_10g019230.1	-114,443,348,682,244	0.00031	v4.1	Protein of unknown function

Table 4. DNA high-impact sequence variants present in the differentially expressed genes located in the QTL of chromosome 10 delimited by bulk segregation analysis in the photoinsensitive and photosensitive eggplant varieties used in this study

Gene Name*	Gene position†		PUC			*puc*	
	Start	**End**	**DH ECAVI**	**IVIA-371**	**AN-S-26**	**H15**	**ASI-S-1**
SMEL4.1_10g016630.1 (SMEL_010g353630.1)	96300106	96301916	-	-	-	-	-
SMEL4.1_10g014730.1 ***(SMEL_010g350010.1)***	88316013	88317850	Position: 88,316,597-602 Reference: AAAGGT Alternate: AGATCGGAAGAGCGTCGT Type: INDEL; Disruptive inframe insertion	Position: 88,317,680-699 Reference: ATATTATTATTATTATTATC Alternate: ATATTATTATTATTATC Type: INDEL; 3_prime_UTR_variant	-	-	-
SMEL4.1_10g013610.1 (SMEL_010g349620.1)	87113226	87117639	-	-	-	-	-
SMEL4.1_10g014530.1 (SMEL_010g349810.1)	88067120	88069892	-	-	-	Position: 88,069,753-756 Reference: TAGG Alternate: AGATCGGAAGAGC Type: INDEL; Conservative_inframe_insertion	-
SMEL4.1_10g019230.1 (SMEL_010g351900.1)	92381275	92381400	-	-	-	-	-

*The gene names between brackets belong to the genome version v3 of *Solanum melongena* from Eggplant Sequencing Consortium

† The gene position of the DEG located in the QTL of chromosome 10 belong to the genome version v3 of *Solanum melongena* from Eggplant Sequencing Consortium

FIGURES

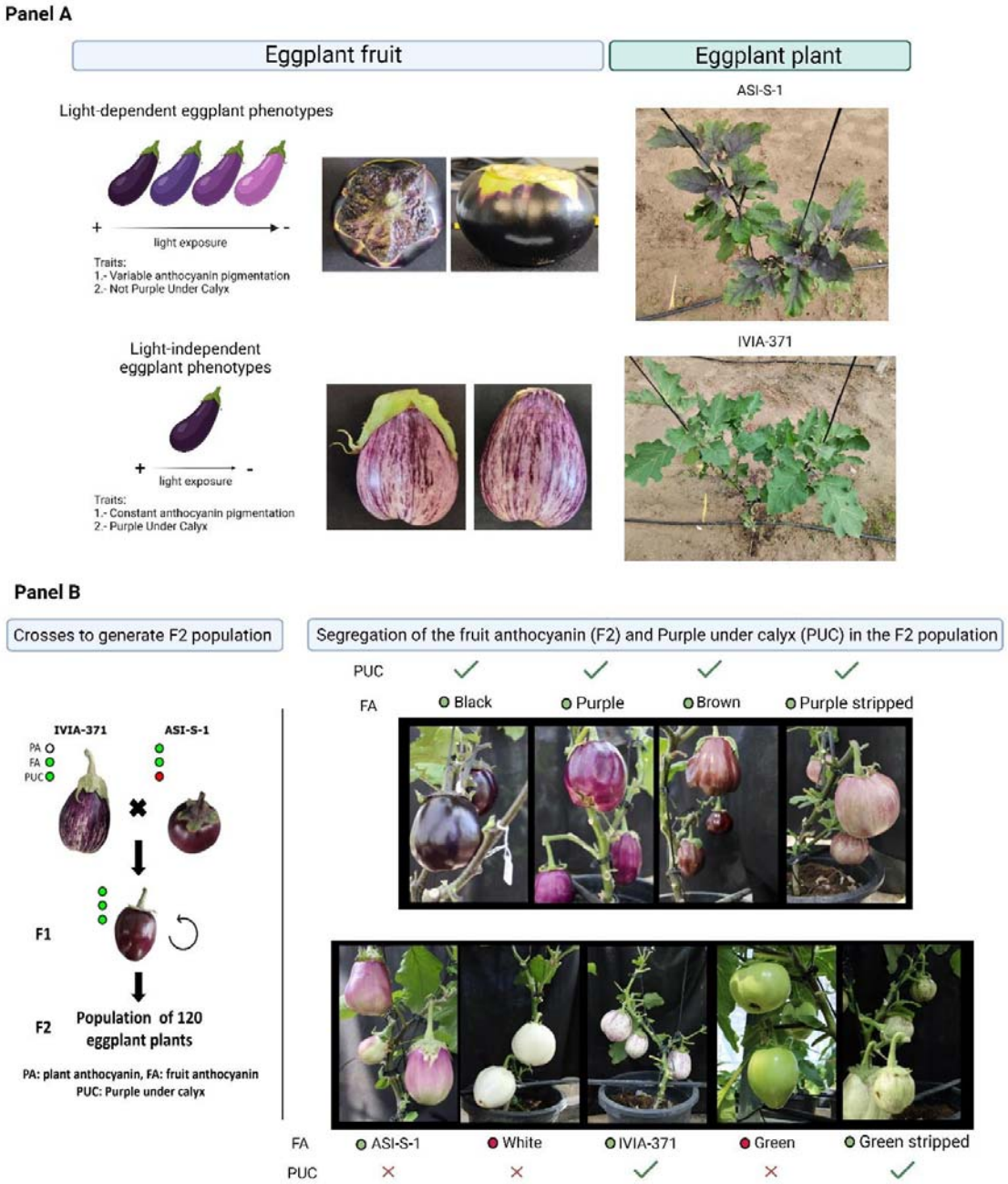

Figure 1. Phenotype of the *Solanum melongena* varieties photosensitive (ASI-S-1) and photoinsensitive (IVIA-371) and F2 population. Panel A: Phenotype of the eggplant fruit of ASI-S-1 and IVIA-371. Panel B: Crosses performed to develop the F2 population and the different F2 phenotypes obtained for fruit anthocyanin (FA), purple under calyx (PUC) and chlorophyll content (CC).

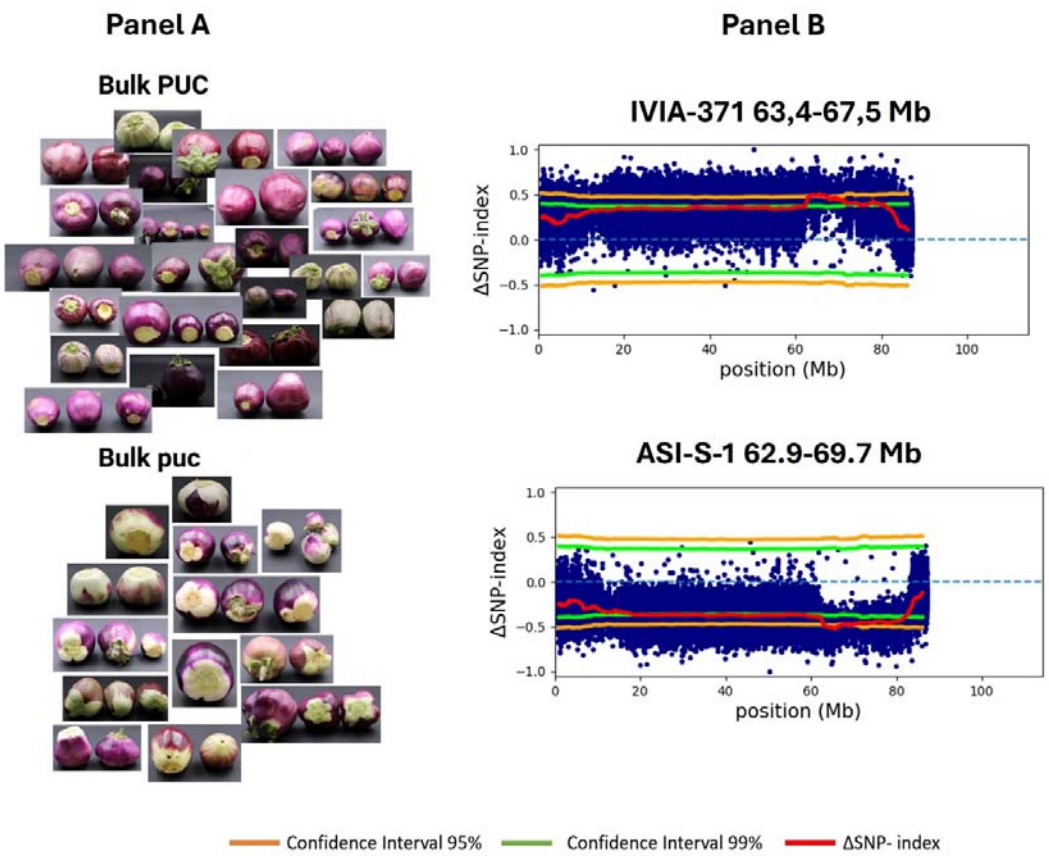

Figure 2. Results of the bulk segregation analysis in the F2 population of IVIA-371 (photoinsensitive) x ASI-S-1 (photosensitive). Panel A: Phenotype of the eggplants used to generate the bulks. Panel B: ΔSNP-index and the position of the respective QTL for IVIA-371 and ASI-S-1, respectively.

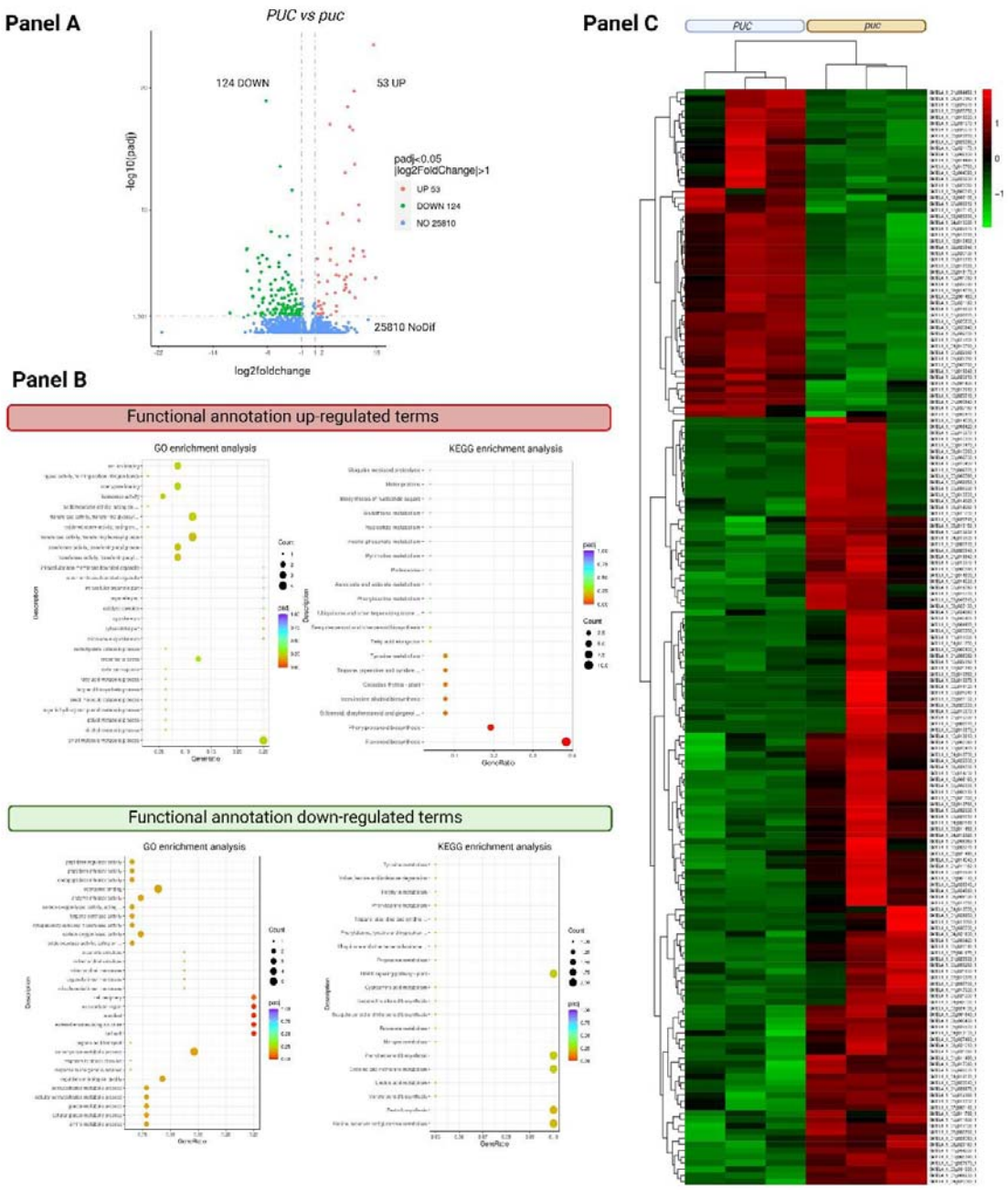

Figure 3. Differentially expressed genes (DEG) in early bagged photoinsensitive (IVIA-371) versus photosensitive (ASI-S-1) eggplants. Panel A: Vulcano plot of the up- and down-regulated of the fruit peel in early developed bagged eggplants. Panel B: Enrichment analysis of the up- down-regulated terms in GO and KEGG terms. Panel C: Clustering heatmap among the PUC vs puc and the DEG expressed in the eggplant fruit peel.