Understanding the mechanism of red light-induced melatonin biosynthesis facilitates the engineering of melatonin-enriched tomatoes

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Understanding the mechanism of red light-induced melatonin biosynthesis facilitates the engineering of melatonin-enriched tomatoes

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Abstract

Melatonin is a functionally conserved broad-spectrum physiological regulator that exists in most biological organisms in nature. Enrichment of tomato fruit with melatonin not only enhances its agronomic traits but also provides extra health benefits. In this study, we elucidate the full melatonin biosynthesis pathway in tomato fruit by identification of novel biosynthetic genes that encode caffeic acid O-methyltransferase 2 (SlCOMT2) and N-acetyl-5-hydroxytryptamine-methyltransferases 5/7 (SlASMT5/7). We further revealed that red light supplement has significantly enhanced melatonin content in tomato fruit. This induction relies on the "serotonin—N-acetylserotonin—melatonin" biosynthesis route via the SlphyB2-SlPIF4-SlCOMT2 module. Based on the regulatory mechanism, we targeted the binding motif of SlPIF4 in the promoter of *SlCOMT2* to design the gene-editing strategy and significantly enhanced the production of melatonin in tomato fruit. Our study provides a good example of how the understanding of plant metabolic pathways responding to environmental factors can guide the engineering of new health-promoting food.

Keywords: metabolic engineering, tomato, melatonin, biosynthesis, light signal, gene editing

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine compound found in all organisms from plants to animals. It was first discovered in the pineal gland of cattle in 1958, also known as epiphysin[1, 2], and has been proven to be the most powerful endogenous free radical scavenger known at present[3, 4]. In animals and humans, melatonin has the functions of improving sleep, delaying aging, alleviating allergic symptoms, and regulating the immune system[5, 6]. Some studies also showed the oncostatic property of melatonin on different types of tumors, as well as reducing the damage resulting from inflammation[7, 8].

In plants, melatonin mainly functions as a growth promoter and antioxidant[9]. It has the activities of delaying senescence, enhancing photosynthesis, regulating photoperiod, affecting seed germination and root morphogenesis, regulating flowering and fruit ripening, removing free radicals and alleviating stress damage, and can give plants the ability to resist adverse environments, Conducive to plant survival and reproduction[9, 10].

As the world's favorite fruit, tomato is the ideal target for plant metabolic engineering[11]. Synthetic strategies have been successfully

applied to tomato metabolic engineering. Fruit-specific expression of transcription factors *AmDel* and *A*m*Ros 1* leads to the upregulation of genes required for anthocyanin biosynthesis and results in increased anthocyanin levels and higher total antioxidant capacity[12, 13]. The fruit-specific expression of *AtMYB12* could be used to enhance the demand for aromatic amino acids biosynthesis, and it can be applied as an effective tool to engineer palpable levels of novel phenylpropanoids in tomato[14, 15]. During the past several years, the rapid development of genome-editing technology provides new ideas for creating excellent tomato germplasm. The accumulation of provitamin D3 in tomatoes was engineered by genome editing, which provides a biofortified food with the added possibility of supplement production from waste material[16]. By inducing mutations at the C-terminal region of *GAD* genes utilizing the CRISPR/Cas9 system, the content of γ-aminobutyric acid (GABA) was greatly increased in the tomato leaves and red-stage fruits[17]. In 2021, the world's first GABA-enhanced genome-edited tomato ‘Sicilian Rouge’ made with CRISPR-Cas9 technology was launched into the open market[18].

Previously, tomato fruit treated with exogenous melatonin was found to show higher levels of nutrients (such as carotenoids, flavor, etc.) with better fruit yields compared to nontreated plants[19, 20]. Moreover,

melatonin treatments effectively promote fruit ripening, while maintaining the sensory and nutritional attributes of fruit via enhancing antioxidant capacity in ripening fruit, which refers to delaying fruit senescence and extending shelf life[19-21]. Therefore, increasing the content of melatonin in tomato fruit may improve both the nutrition and agronomic traits.

Previous studies have shown that the synthesis of melatonin in plants starts from the synthesis of tryptophan, which requires four consecutive enzymatic reactions. Tryptophan decarboxylase (TDC) and tryptophan-5-hydroxylase (T5H) are key enzymes in the first two steps of melatonin synthesis, catalyzing the production of serotonin (5-hydroxytryptamine), 5-hydroxytryptamine-N-acetyltransferase (SNAT) and n-acetyl-5-hydroxytryptamine-methyltransferase (ASMT) / caffeic acid-o-methyltransferase (COMT) catalyzing the final formation of melatonin from serotonin. Studies have found that there are at least four possible melatonin synthesis routes in plants, and TDC and SNAT may be the rate-limiting enzymes in the process of melatonin synthesis[22, 23]. However, it has also been suggested that ASMT may also be the rate-limiting enzyme in the process of melatonin synthesis[24-27]. On the other hand, COMT can effectively catalyze the production of melatonin, showing strong ASMT activity.

Previous study showed melatonin contents have been significantly reduced in *Arabidopsis comt* knockout mutants [28].

At present, it has been found that there are at least five *TDC* candidate genes in tomato, of which *SlTDC3* is expressed in almost all tissues, and *SlTDC1* and *SlTDC2* are only expressed in tomato fruits and leaves, respectively, indicating that the expression of *TDC* genes may be tissue-specific, and the expression of these genes may play different roles in plant growth and development or resistance [29, 30]. However, the full melatonin biosynthesis pathway, especially in tomato fruit, is yet to be elucidated.

The synthesis and signal transmission of melatonin in plants are significantly affected by environmental factors (such as light, temperature, etc.) [31, 32]. The regulation of melatonin synthesis by light signal has been well-studied in animals. Studies in mice have shown that melatonin synthesis depends on the rhythm clock and the core regulators cry1/2 of the light-sensing signal[33]. However, the research on plants is lagging behind. How different light signals coordinate the synthesis and metabolism of melatonin has been unclear.

Here, the melatonin synthesis pathway in tomato fruit was completely elucidated. The functions of biosynthetic genes (*SlSNAT*,

SlASMT5, *SlASMT7* and *SlCOMT2*) were identified. We also found that red light treatment could significantly promote melatonin synthesis in tomato fruit via the SlphyB2-SlPIF4-SlCOMT2 module. Based on the regulatory mechanism, we targeted the binding motif of PIF4 in the promoter of *SlCOMT2* to design the gene-editing strategy and significantly enhanced the production of melatonin in tomato fruit. Our data not only expand our current knowledge of how environmental factors affect the biosynthesis of key metabolites, but also provide a good example of how to use the regulatory mechanism to guide the breeding of crops with enhanced nutrition.

Results

Screening of melatonin biosynthetic genes in tomato fruit

To elucidate the full melatonin biosynthesis pathway in tomato, we BLASTed the tomato genome for homologous genes of known melatonin biosynthetic genes: *TDC, T5H, SNAT, ASMT* and *COMT*. In total, 17 candidates were identified (Fig S1a). During the tomato fruit development process, the content of melatonin increases significantly at the breaker stage (Fig. 1a). Using the transcriptome data of the MicroTom Metabolic Network (MMN)[34], We further conducted correlation (Fig. S1b, 1b) and quantitative (Fig. S1c) analysis to narrow down to 10 candidate genes with reasonable expression levels in tomato fruit (Fig 1b).

All these 10 candidate genes were then verified by transient overexpression in tobacco (*Nicotiana Benthamiana*) leaves and silencing in tomato fruits. We found, *Solyc07g054280*, which encodes SlTDC2, is responsible for the first step (from tryptophan to tryptamine) in melatonin biosynthesis. *Solyc09g014900*, which encodes SlT5H, catalyzes the next step from tryptamine to serotonin (Fig 1c, Fig S2, S3, S4, S5 and S6).

Previous studies indicate that from serotonin, melatonin biosynthesis might have alternative routes, which require the participation of

SNAT, ASMT or COMT[9, 22, 23]. We transiently overexpressed/silenced the remaining 7 genes to check the contents of melatonin. Transient overexpression of *SlASMT5* (Solyc03g097700), *SlASMT7* (Solyc06g064500), *SlCOMT2* (Soluc10g85830) and *SlSNAT* (Solyc10g074910) can significantly induce the production of melatonin in tobacco leaves. While silencing of these genes can significantly reduce melatonin content in tomato fruit (Fig 1c, Fig S2, S3, S4, S5 and S6). Notably, the expression level of *SlASMT7* was found to be associated with the 5-methoxytryptamine route of melatonin biosynthesis (Fig 1c). All these data indicate SlSNAT, SlCOMT2, SlASMT5 and SlASMT7 are involved in the biosynthesis of melatonin.

Functional verification of the roles of SlSNAT, SlCOMT2 and SlASMT5/7 in melatonin biosynthesis

The expression levels of *SlASMT5*, *SlASMT7*, *SlCOMT2* and *SlSNAT* in different tissues were measured quantitatively. While *SlSNAT* was expressed in all tested tissues, *SlASMT7, SlASMT5* and *SlCOMT2* were mainly expressed in fruits after the breaker stage (Fig. S7). The expression levels of these genes matched with previous transcriptome data (Fig S1a). Fig. S8 shows the expression of these four genes in the Tomato Expression Atlas database[35] and MMN database[34]. This is consistent with melatonin content (Fig 1a). The

localization experiment using *Arabidopsis* protoplasts showed that SlSNAT was localized in the chloroplast. While SlASMT5, SlASMT7 and SlCOMT2 were in the nucleus and cytoplasm (Fig. S9). This is consistent with the previous reports[22, 23, 30].

We then generated stable overexpression and RNAi lines for *SlASMT5*, *SlASMT7*, *SlCOMT2* and *SlSNAT*, respectively (Fig S10, S11). Compared to WT fruit, overexpression of these four genes individually can significantly enhance the production of melatonin, while silencing of these genes can reduce the content of melatonin (Fig 2a, Fig S12).

In vitro enzyme assays using the recombinant proteins from *E. coli* confirmed that SlSNAT can catalyze the production of N-acetylserotonin from serotonin, as well as the synthesis of melatonin from 5-methoxytryptamine (Fig 2b and Fig. S13). This indicates the biosynthesis of melatonin in tomato fruit may have two possible routes: one is through the "serotonin—N-acetylserotonin—melatonin" route; the other one is the "serotonin—5-methoxytryptamine—melatonin" route. We then incubated the recombinant SlASMT5, SlASMT7 and SlCOMT2 proteins with either serotonin or N-acetylserotonin. The recombinant SlASMT7 can catalyze the formation of 5-methoxytryptamine from serotonin but failed to produce melatonin from N-acetylserotonin

(Fig 2b and Fig. S13). This indicates SlASMT7 is involved in the 5-methoxytryptamine route of MT biosynthesis. On the other hand, both recombinant SlASMT5 and SlCOMT2 can catalyze the production of melatonin from N-acetylserotonin while failing to produce 5-methoxytryptamine from serotonin (Fig 2b and Fig. S13). We further verified the function of *SlASMT5*, *SlASMT7* and *SlCOMT2 in vivo* by RNAi and found only the silencing of *SlASMT7* can significantly reduce the contents of 5-methoxytryptamine (Fig S14). All these indicate SlASMT5 and SlCOMT2 catalyze the N-acetylserotonin route of MT biosynthesis. While SlASMT7 is involved in the 5-methoxytryptamine route (Fig 2c).

Melatonin biosynthesis in tomato fruit is significantly induced by red light treatment

Red light supplements were reported to introduce excellent characteristics such as early ripening, enhanced nutrients and delayed senescence to tomato fruit[36-39]. We found when tomato plants were provided with the red light supplement, the expression levels of *SlASMT7*, *SlASMT5* and *SlCOMT2* were induced and the content of melatonin was significantly increased during the ripening process (Fig. 3a, S15). We also found that red light supplements significantly increased the content of *N*-acetylserotonin but not

5-methoxytryptamine (Fig. 3b, S16). It seems like the red light-induced melatonin biosynthesis relies on the activation of the *N*-acetylserotonin route via SlCOMT2 and SlASMT5.

To test this hypothesis, we repeated the red light supplement experiment to *SlASMT7*, *SlASMT5* and *SlCOMT2* RNAi lines. Compared to WT, the fruit of RNAi-*SlASMT5* and RNAi-*SlCOMT2* lines contained significantly lower melatonin under the red light supplement. While the RNAi-*SlASMT7* line still responded well to red light treatment (Fig 3c). All these data indicate red light supplement enhances melatonin biosynthesis in tomato fruit via activation of the expression level of *SlASMT5* and *SlCOMT2*.

SlPIF4 directly inhibits the expression of *SlCOMT2* to suppress melatonin biosynthesis in tomato fruit

To investigate the molecular mechanism of red light-induced melatonin biosynthesis, we scanned the promoter region of *SlASMT5* and *SlCOMT2*. And series of light signal-related G-box elements were found in both promoters (Fig. S17a, 4a). On the other hand, the *proSlASMT5* and *proSlCOMT2* were used respectively as baits to screen yeast one-hybrid libraries. A cDNA fragment showing homology to *phytochrome-interacting factors 4* (*SlPIF4*) was

identified to bind to both *proSlCOMT2* and *proSlASMT5* (Supplementary file 1). PIF4 has been reported to play vital roles in light response and is capable of binding to the G-box domain[40]. Therefore, we hypothesize that SlPIF4 is a potential regulator for MT biosynthesis.

To investigate whether SlPIF4 can directly bind to *proSlCOMT2* and *proSlASMT5*, we first performed the yeast one-hybrid (Y1H) assay. Three G-Box elements of the *SlCOMT2* genome sequence were selected as possible binding sites (P1-P3) (Fig. 4a). The results showed that SlPIF4 could bind to the P2 element of the *SlCOMT2* promoter (Fig. 4b). A G-Box was also predicted on the promoter of *SlASMT5*, but it could not be bound by SlPIF4 (Fig. S17).

We further approved SlPIF4 could repress the activity of the *SlCOMT2* promoter using the Dual-Luc system in both tobacco leaves (Fig. 4c) and tomato protoplast (Fig. 4d). When the P2 motif was mutated, the inhibition of SlPIF4 on *proCOMT2* was released (Fig 4f). The EMSA assay with normal and mutation probes with the CArG motif in the promoter (P2 and mP2) of *proSlCOMT2* also suggested that SlPIF4 directly binds to the *SlCOMT2* promoter (Fig. 4e).

To further examine the direct binding of SlPIF4 to the *proSlCOMT2 in vivo*, we generated FLAG-tagged *SlPIF4* overexpression tomato lines

(Fig S18, S19). By ChIP-qPCR, we found that SlPIF4 directly binds to the G-Box element in the P2 site of *proCOMT2*. While P1 and P3 are invalid sites for SlPIF4 binding, which is consistent with the results found above (Fig. 4f).

Together, all these data suggest SlPIF4 can suppress the expression of *SlCOMT2* through the interaction with the P2 site of *proSlCOMT2*. Under normal growth conditions, the expression of *SlCOMT2* was significantly up-regulated in the *slpif4* RNAi lines, together with significant induction of the MT content (Fig. 4g, h, i). After the red light supplement, although there was still significant induction of MT contents in the transgenic lines (27% and 16% increase, respectively), their MT content enhancement ratios were significantly lower than that of the WT fruit (63%) (Fig. 4i, S20). These data indicate SlPIF4 is a negative regulator for MT biosynthesis. And it is involved in the red light-mediated regulation of MT biosynthesis.

The SlphyB2-SlPIF4-SlCOMT2 module mediates the red light-induced melatonin biosynthesis in tomato fruit

As one of the key plant phytochrome photoreceptors, phytochrome B2 (phyB2) plays an important role in red light response signaling[41-43]. In the MMN database[34], the expression of *SlphyB2* in different developmental stages of tomato fruits presents highly

consistent with melatonin, while *SlphyB2* and *SlPIF4* show opposite trends (Fig. S21). We first checked whether SlphyB2 can inhibit the expression of *SlPIF4*. The Dual-Luc assay using tomato protoplast indicated SlPIF4 could bind to its own promoter to achieve self-activation. Although SlphyB2 didn't inhibit the activity of *proPIF4* alone, it could inhibit the self-activation of *SlPIF4* (Fig. 5a).

Previous studies suggest phyB2 activates thermo-response by regulating the PIF4 stability[44, 45]. The firefly luciferase complementation imaging assays were performed to identify the interaction between SlPHYB2 and SlPIF4 (Fig. 5b, S22). And when *SlPIF4-LUC* was expressed together with *SlphyB2* in tobacco leaves, the luciferase signal was significantly decreased. This inhibition can be removed by adding proteasome inhibitor MG132 (Fig. 5c). This indicates SlphyB2 might facilitate the degradation of SlPIF4.

To verify SlPHYB2 can regulate SlPIF4 at the protein level *in vivo*, we transiently overexpressed FLAG-tagged *SlPIF4* in both WT and RNAi-*SlphyB2* tomato fruit. In the WT fruit, compared to fruit stored in dark, the SlPIF4 protein content in agroinfiltrated fruit under light was significantly reduced. This phenotype can be effectively blocked by infiltrating the proteasome inhibitor MG132 into fruit (Fig. 5d, S23, S24). In the RNAi-*SlphyB2* tomato fruit, however, the degradation of SlPIF4 under red light supplement has been effectively inhibited.

These data indicate SlphyB2 can regulate the SlPIF4 stability via the 26S proteasome pathway. Consequently, in the RNAi-*SlphyB2* lines (Fig S25), the expression level of *SlCOMT2* has been inhibited and the melatonin content was significantly decreased, and red light treatment was no longer effective (Fig. 5e, f, S26).

To sum up, SlPIF4 negatively regulates melatonin biosynthesis in tomato fruit via direct inhibition of *SlCOMT2* expression. Under the red light supplement, the activation of SlphyB2 facilitates the degradation of SlPIF4 via the 26S proteasome pathway. Therefore the inhibition of *SlCOMT2* expression has been released and the biosynthesis of melatonin is enhanced (Fig. 6).

Engineering new melatonin-enriched tomatoes

To test whether this regulatory mechanism can be used for breeding new tomato varieties with enhanced melatonin production, two gene editing strategies are designed. One strategy is to directly knock out *SlPIF4* (Fig. 7a), and the other one is to mutate the SlPIF4 recognition site on *proSlCOMT2* (Fig. 7b). Both methods can significantly enhance the production of melatonin (Fig. 7c). However, gene editing targeting the SlPIF4 recognition site on *proSlCOMT2* can induce much stronger melatonin accumulation under normal growth conditions: Compared with WT, the melatonin content of the two strains of CR-*slpif4* (12# and 15#) increased by about three folds. However, the melatonin

322 content in the two CR-*proslcomt2* strains (5# and 8#) increased by
323 8.75 and 12.64 folds respectively (Fig. 7c, S27).

324

Discussion

Melatonin is an indoleamine compound found in all organisms from plants to animals[32, 46]. Unlike animals, whose melatonin biosynthesis pathway has been thoroughly investigated[47, 48], the melatonin biosynthesis pathway in most plants remains uncharacterized[9, 49]. In this study, the biosynthetic pathway of melatonin in tomato was fully elucidated. We found alternative melatonin biosynthesis routes co-exist in tomato. One is through the "serotonin—N-acetylserotonin—melatonin" route, in which SlASMT5 and SlCOMT2 are the key enzymes (Fig 1c). The other one is the "serotonin—5-methoxytryptamine—melatonin" route, in which SlASMT7 is the core enzyme (Fig 1c)

As sessile photoautotroph organisms, plants are constantly challenged by diverse external environmental conditions. To develop resisting capacity, plants produce various environment-induced metabolites such as nutrients, anti-nutrients, and phytohormones[50, 51]. In this study, we found that red light treatment at the fruit development stage can effectively induce the synthesis of melatonin in tomato fruit. Although there are alternative routes for melatonin biosynthesis in tomato (Fig 1c), the red light-induced melatonin enhancement mainly relies on the

346 “serotonin—*N*-acetylserotonin—melatonin” route (Fig 3b) via the
347 activation of *SlCOMT2* and *SlASMT5* (Fig 3a,c).

348 We further found that SlPIF4 can directly inhibit the expression of
349 *SlCOMT2*. And under red light supplement, the activation of SlphyB2
350 facilitates the degradation of SlPIF4. Therefore the inhibition of
351 *SlCOMT2* has been released (Fig. 3-6). Previous studies have shown
352 that PIFs are bHLH family transcription factors that can bind to
353 photoreceptor phytochrome proteins (PHYs), and phytochrome can
354 accelerate the degradation of PIFs-dependent 26S proteasome by
355 promoting the phosphorylation of PIFs under red light[41-43]. Studies in
356 *Arabidopsis* show that PIF4 is a negative regulator of plant light signal
357 transduction and can antagonize and regulate plant signal
358 transduction[52, 53]. Tomato phytochrome interaction factor PIF4
359 regulates tomato plants’ response to temperature stress by
360 integrating light and temperature hormone signals[54]. SlPIF4 has close
361 homology with *Arabidopsis* AtPIF4, while AtPIF4 is not only a
362 transcription factor necessary for the process of light signaling but
363 also can positively regulate the synthesis of anthocyanins[55, 56]. The
364 joint cross-response of multiple environmental factors is the general
365 trend of future research on plant growth and development and
366 quality formation. Light and temperature often act on plants together,

and PIF4, as an important transcription factor of light and temperature signals, may be a link for further exploration of other regulatory genes and pathways. And indeed, we did find some other TFs including bHLH, bZIP, WRKY, MYB, *etc* families in the Y1H screen library (Supplementary file 1), which we will further investigate in the following studies. It has been reported that *HsfA1a* in tomato plants can promote the synthesis of melatonin to confer cadmium tolerance [57]. In cassava, MeHsf20, MeWRKY79 and MeRAV1/2 are able to induce melatonin production by binding in the promoters of melatonin biosynthesis genes [58]. However, most studies on involved TFs are related to stress response, and more TFs affecting melatonin synthesis need to be identified [59].

Recent studies reveal that effective tomato metabolic engineering can be achieved by gene-editing targeting key biosynthetic genes [17, 60]. Our data indicate that silencing and knockout *SlPIF4* can significantly enhance the production of melatonin in tomato fruit (Fig 4g-i) even under normal growth conditions. However, as the vital functions of SlPIF4 in various signaling pathways[56, 61], it is not wise to simply knock down/out this master regulator. Alternatively, we targeted the SlPIF4 recognition site in *proSlCOMT2* to design gene-editing strategies. By doing this, we can also significantly enhance melatonin

production in tomato fruit. As previous studies indicated that during tomato fruit ripening, DNA methylation is the key regulatory component [62, 63]. The DNA methylation rate of SlCOMT2 was checked from the green stages to the ripening stages in our unpublised Database. We found that at the green stage, the *proSlCOMT2* was highly methylated (Fig. S28). Therefore, even without the inhibition of SlPIF4, the expression of *SlCOMT2* is low during green fruit stages in *slpif4* or *proslcomt2* mutants. Actually, this is the key advantage of the gene-editing for *proCOMT2*, which only removed the SlPIF4 inhibition during the ripening stages without changing its expression pattern in other stages. Notably, compared to directly knocking out *PIF4*, the *proslcomt2* mutations have significantly higher melatonin production than the *pif4* mutants (Figure 7c), this was possibly due to other unknown TFs (Supplementary file 1) interacting with the mutated G-box motif.

In summary, this study elucidated the full melatonin biosynthesis pathway in tomato fruit. We also uncovered the mechanism of red light induction of melatonin biosynthesis and successfully developed melatonin-enriched tomato varieties through gene editing. Our findings demonstrate that understanding the mechanisms by which environmental factors regulate key metabolism can be used to create novel nutrient-enriched crops.

Materials and Methods

Plant materials, growth conditions and light treatments

Tomato (*Solanum lycopersicum* L. cv. MicroTom) seeds (Pan American Seed, Inc., Hillsborough, FL, USA) were grown in a standard greenhouse under 16 h photoperiod (16 h light/8 h dark at 23 °C，relative humidity 70%). The light intensity indicated as PPFD (photosynthetic photon flux density), was set at 250μmol m^{-2} s^{-1} above the plant canopy and maintained by adjusting the distance of 15 cm from LEDs to the canopies. Red light refers to replacing 30% white light with red light, which means 30% red light at a wavelength of 657nm and 70% white light at a multiwavelength, white light as a control. The collected tissues were frozen in liquid nitrogen and stored at -80°C freezer until further investigation. Three biological replicates, each of which was a pooled sample of 10-12 individual fruits were analyzed.

Melatonin and metabolic intermediates extraction and analysis

The tomato tissues from three independent biological samples were ground into a fine powder and used for melatonin and metabolic intermediates measurement based on the AB SciexQTRAP 6500 LC-MS/MS platform. In short, 200mg tomato powder was extracted with 1.0 mL 80% aqueous methanol by ultrasonic for 20 min at 4 °C.

The supernatants were transferred into new Agilent tubes after 10 min centrifugation at 10,000 g for LC-MS/MS analysis. ACCUCORE C30 chromatographic column was used with the mobile phase of acetonitrile (solvent A)-methanol (solvent B)-ultrapure (solvent C) water (v/v/v). The column temperature was set to 18℃ and the injection volume was 2μL. The gradient elution procedure with 1mL/min flow velocity was as follows, time (1, 2, 4.5, 7.5, 8, 10 min)/mobile phase (90%A-10%C, 100%A, 85%A-15%B, 100%A, 90%A-10%C, 90%A-10%C). Fragment XICs were extracted using SCIEX OS software (version 1.7). And the same method was used for calibrating and quantifying the mass spectrum peaks of melatonin.

Coexpression/coregulation identification and analysis

The Tomato Expression Atlas database[35] and the MicroTom Metabolic Network (MMN) database[34] were used for the preliminary identification of melatonin biosynthesis-related genes according to coexpression/coregulation analysis. Heat maps created by R (v3.6.0) displayed for high-throughput of the expression levels of the coexpression genes.

Plasmid construction and generation of transgenic lines

The subject sequence was introduced into the relevant vector by a homologous recombination system (ClonExpress® IIOne Step Cloning Kit, C211, Vazyme) or restriction endonuclease reaction. pEAQ (for overexpression) and pTRV (pTRV1 and pTRV2 vectors, for Virus-induced gene silencing, VIGS) were used for transient transformation, respectively. pCAMBIA1306 (35S::3×FLAG) was used for constitutive expression, pBWA(V)HS-RNAi for RNA interference construction. Take pHSbdcas9i as the vector backbone for a one-step CRISPR/Cas9 binary constitutive. A plasmid with the correct insertion was introduced into *Agrobacterium tumefaciens* strain EHA105.

In vitro enzyme activity verification

The assay was performed according to the method described by Fu et al [65, 66]. The subject sequence was introduced into the *pDEST17* vector by the Gateway system. Methyltransferase and acetyltransferase were selected for enzyme activity verification, and heat shock transformation was carried out with *Escherichia coli* BL21. The single colonies were selected and cultivated in LB liquid medium with corresponding resistance at low speed for 3-5 hours at 37 ℃. The positive strains were obtained by polymerase chain reaction. Subsequently, 20 μL bacterial solution was taken to the LB medium

containing antibiotics and incubated overnight at 37 ℃ until the OD_{600} reached 0.5~1.0. IPTG was added to a final concentration of 0.5~1.0 mM and induced at 28 ℃ for 8 hours. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine whether the protein was expressed.

The *Escherichia coli* liquid with the target protein was centrifuged at 4 ℃ at 5,000×g for 10 min. The collected solution was resuspended with 10 mL 1X PBS buffer, which was mainly composed of Na_2HPO_4 and KH_2PO_4. An enzyme activity reaction was taken after being treated by ultrasonic wave. Adding 400μL methanol to stop the reaction after incubation at 30℃ for 1 hour. Then centrifuged at 4℃ at 20,000×g for 10 minutes, and the supernatant was used for mass spectrometry.

Subcellular localization

The full-length coding region without the termination codon was amplified with 35S::GFP (*pCAMBIA1302*). It was then transformed into protoplasts of *Arabidopsis* after incubation for 12 h at 28 °C. A confocal laser scanning microscope was used for GFP fluorescence detection.

Construction and screening of yeast library

500 μg high-quality total RNA was extracted from tomato tissues, and Gateway technology was used for yeast library construction. The cDNA library was prepared by Yuanbao Biotech (Nanjing, China). SD/-His-Leu-Trp deficient 3AT culture screening plate and Y187 yeast strain were used to screen the yeast library. Each obtained more than 600 clones, and high-throughput sequencing was performed after plaque collection.

Yeast one-hybrid assays

The promoter fragments were amplified and cloned into the pLacZi vector, and the CDS of *SlPIF4* was fused to pB42AD. The constructs were then transformed into the yeast strain EGY48, and yeast cells were inoculated on a selective medium for 3 days at 28 °C and transferred to the SD/-Ura-Trp medium. Yeast colonies would turn blue with X-gal if there was an interaction between the factors.

Transient Dual-luciferase reporter assay

The fragment of the *SlCOMT2* or *SlASMT5* promoter was cloned into the pGreenII0800-LUC vector. *A. tumefaciens* strain GV3101 harboring targeted fragments were grown in infiltration medium (2 mM Na_3PO_4, 50 mM MES, and 100 mM acetosyringone) to an OD_{600} of 0.5 and then introduced via a syringe into the leaves of a

4–5-week-old *N. benthamiana* plant. After 48–96 h, a CCD camera was used to observe luciferase activity. 2-3 weeks of tomato leaves were used for protoplast separation. After enzymatic hydrolysis, a vacuum was applied for 30 minutes, followed by the addition of W5 and resuspended in an ice bath. Add 200 μ L protoplast to the target plasmid. After 40% PEG-mediated transformation, the protoplasts were placed in a dark environment at 24°C for 20 h. The Dual-Luciferase Reporter Assay System (Promega, cat. #e1910, Madison, USA) was used to measure the fluorescence intensity of luciferase and renilla (REN). The relative LUC/REN ratios were used to represent the activity of the promoters.

ChIP-qPCR assay

The transgenic lines 35S::FLAG-*SlPIF4* was assessed by ChIP-qPCR assays. The plant tissues were crosslinked in 1% formaldehyde, and chromatin was isolated. Anti-green fluorescent (GFP) protein antibody (Thermo Fisher Scientific) was added to sonicated chromatin, followed by overnight incubation to precipitate bound DNA fragments. DNA was eluted and amplified with primers corresponding to the gene of interest. The EpiTect ChIP OneDay Kit (Qiagen) was used according to the manufacturer's instructions.

Primers used for the assay are listed in Table S3, and each was repeated at least three times.

Electrophoretic mobility shift assay

The fusion proteins of SlPIF4 were generated through prokaryotic expression in vitro. The CDSs of *SlPIF4* were cloned into the PGEX-5T vector containing a GST target and expressed in *Escherichia coli* strain BL21. IPTG was used to induce protein production. MagneGST™ Pull-Down System (Promega) was used for protein purifying, and LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher, New York) was used for the subsequent EMSAs. Unlabeled probes were used for probe competition. Then loaded it onto a pre-run native 6.5% polyacrylamide gel with TBE buffer as the electrolyte. After electro-blotting onto a nylon membrane (Millipore, Darmstadt, Germany) and UV cross-linking (2000 J for 5 min), the membrane was incubated in blocking buffer for 30 min and rinsed in washing buffer. A CCD camera was used to visualize the chemiluminescent signal.

Floated-leaf Luciferase complementation imaging assay

SlPHYB2 was cloned into 35S::GFP (pCAMBIA1302), while *SlPIF4* was constructed with a luciferase vector. To investigate whether SlPHYB2

interacts with SlPIF4 in vivo, we used the pCABIA1300-cLUC and pCABIA1300-nLUC vectors by FLuCI assay. SlphyB2 was fused to the C-terminal fragment of luciferase (cLUC), while SlPIF4 was fused to the N-terminal fragment of luciferase (nLUC). The interactions between nLUC and SlPHYB2-cLUC as well as SlPIF4-nLUC and cLUC were used as negative controls. The final constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101 and different combinations of plasmids were co-infiltrated into tobacco (*N. benthamiana*) leaves. After incubation in the dark for 12-14 h and then in light for 48 h, the tobacco leaves were sprayed with 100 mM D-luciferin and kept in the dark for 5-10 min, a CCD camera.

Validation of ubiquitination degradation

The ubiquitination and degradation of SlPIF4 by SlphyB2 were validated with the help of proteasome inhibitor MG132 (Beyotime, S1748), which can effectively block the proteolytic activity of the 26S proteasome complex. 80mM MG132 (10mM MgCl2, 50μM MG132) and its reference solution were injected 6h before collection. A CCD camera was used to observe luciferase activity in tobacco leaves. For the western blot, 30 DPA tomato fruits were selected from RNAi-*SlphyB2* and wild-type plants, and inject infection solution from the bottom of the fruit until there is liquid leaching at the stem.

Incubate the infected fruits in the dark for 24 hours, followed by 3 days of dark cultivation. Half of the plants with the injected MG132 or its reference solution were treated with red light for 30 minutes. BCA was used for the determination of the total protein concentration. SDS-PAGE electrophoresis was performed with the consistent protein content of each sample.

Total RNA isolation and qRT-PCR analyses

Samples were harvested and ground into a fine powder using liquid nitrogen. Total RNA was extracted using RNAiso reagent (BIOFIT, RN33050) as recommended by the manufacturer. 1μg RNA was used for the first-strand cDNA by the PrimeSciptTMRT reagent Kit containing gDNA eraser (Takara, Kusatsu, Japan). qRT-PCR was performed using the Bio-Rad CFX384 Real-Time System (1725124) according to the manufacturer's instructions. The relative expression level of each gene was calculated using the ΔCt method as described previously [34], *SlUBI* act as an internal control. Average values were calculated by 3 biological replicates (n=3). One biological replicate is the pool of 10-12 samples.

Statistical analysis

At least three biological replicates were included in the data, the statistical significance of differences was determined by ANOVA followed by the post-hoc Tukey's test or the Student's *t*-test (GraphPad Prism version 8).

Accession numbers

The assession numbers of genes are as follows: *SlTDC1* (Solyc07g054860), *SlTDC2* (Solyc07g054280), *SlT5H* (Solyc09g014900), *SlSNAT* (Solyc10g074910), *SlASMT7* (Solyc06g064500), *SlASMT5* (Solyc03g097700), *SlCOMT2* (Solyc10g085830), *SlPHYB2* (Solyc05g053410), *SlPIF4* (Solyc07g043580).

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613 **Author contributions**

614 YZ and ZXZ conceived and designed the experiments; ZXZ, XZ, YTC
615 and WQJ performed most of the experiments; JZ, JYW, and YJW
616 provided technical support; XY provided technical support on light
617 treatment; SCW and MCL provided conceptual advice; WQJ and XZ
618 contributed to plants transformation; ZXZ, XZ and YZ analyzed the
619 data and wrote the manuscript with inputs from all authors.

620 **Conflicts of interest**

621 The authors declare that they have no conflicts of interest.

622

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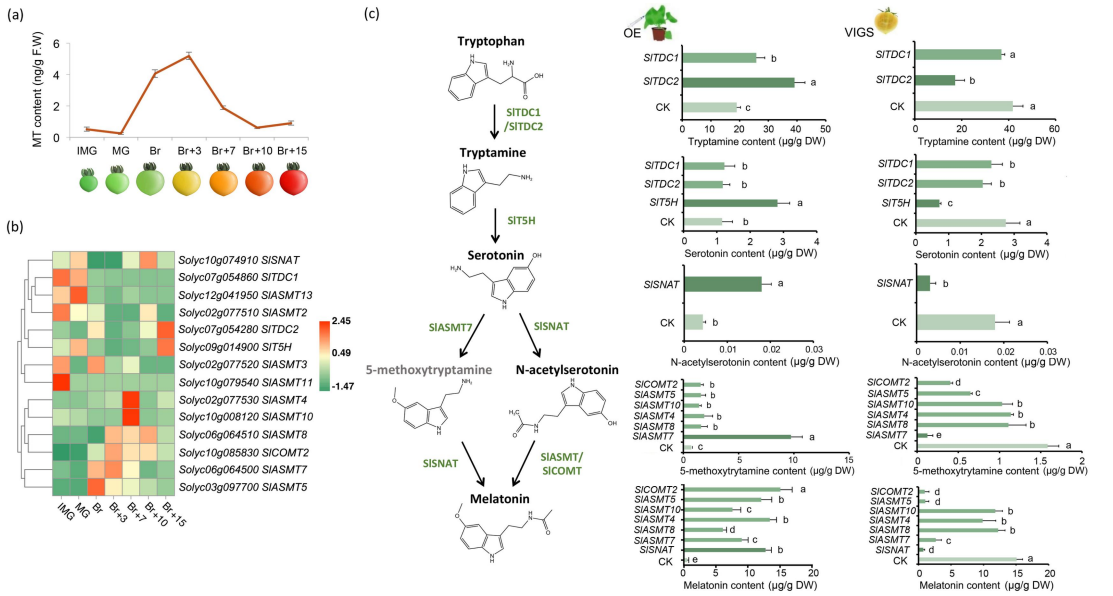
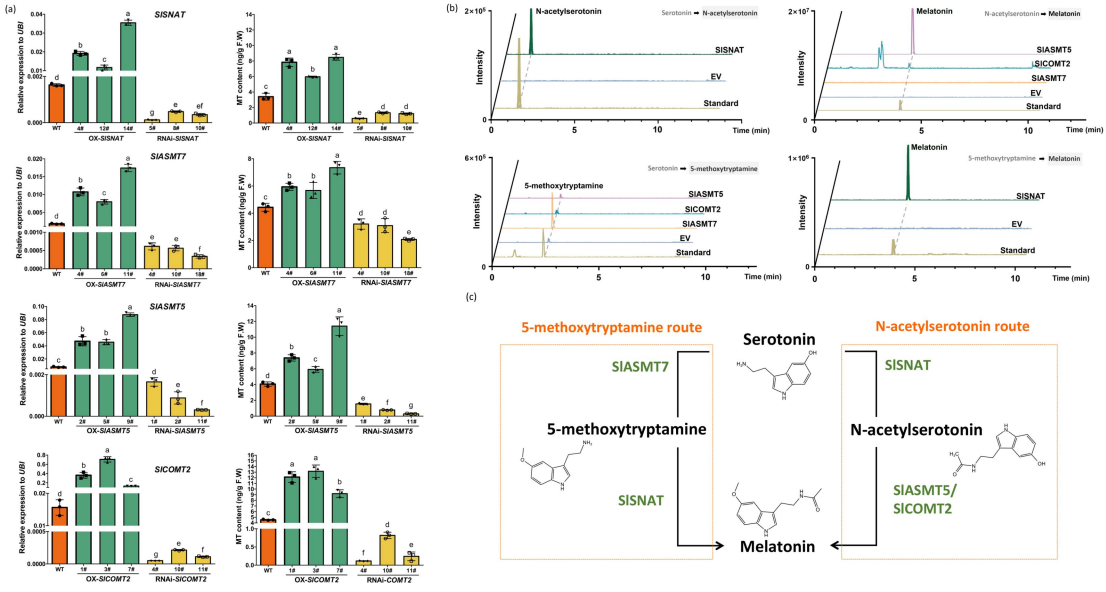


Fig. 1 Preliminary screening of key structural genes for MT synthesis.

(a) Determination of melatonin content in tomato fruit at different development stages. IMG (immature green), MG (mature green), Br (breaker), Br+n (breaker plus n days). (b) The expression analysis heat map of the melatonin synthetic genes was obtained by screening from the MMN Database. (c) Melatonin synthetase gene obtained by instantaneous verification screening. 'OE' indicates gene transient overexpression (injection into tobacco leaves); 'VIGS' indicates gene transient silencing (injection into tomato fruits). CK indicates the determination result after injection of the corresponding empty carrier. 10-12 individual leaves (for transient expression) or 10-12 individual tomato fruits (for VIGS) with uniform sizes were pooled as one biological replicate. Data are represented as Mean ± SEM (n=3). The P values indicate the results from pairwise comparisons of one-way ANOVA tests. Different letters represent a significant difference at $P < 0.05$.



828

829 **Fig. 2 *In vivo* and *in vitro* verification of melatonin biosynthetic genes**

830 (a) Determination of gene expression and melatonin content in stable transgenic
831 tomato. OX-*SlGENE* represents the overexpression lines, and RNAi-*SlGENE*
832 represents the silencing lines. 10-12 individual tomato fruits at the Br+3 stage
833 were pooled as one biological replicate. Data are represented as Mean ± SEM
834 (n=3). The P values indicate the results from pairwise comparisons of one-way
835 ANOVA tests. Different letters represent a significant difference at $P < 0.05$. (b) *In*
836 *vitro* enzyme activity verification of key structural genes. Different proteins were
837 incubated with different substrates (serotonin, N-aceytlserotonin and
838 5-methoxytrptamine, respectively) to detect the production of N-aceytlserotonin,
839 5-methoxytrptamine, and melatonin, respectively. 'EV' indicates the empty vector
840 for negative control. (c) The roles of SlSNAT, SlCOMT2 and SlASMT5/7 in
841 melatonin biosynthesis.

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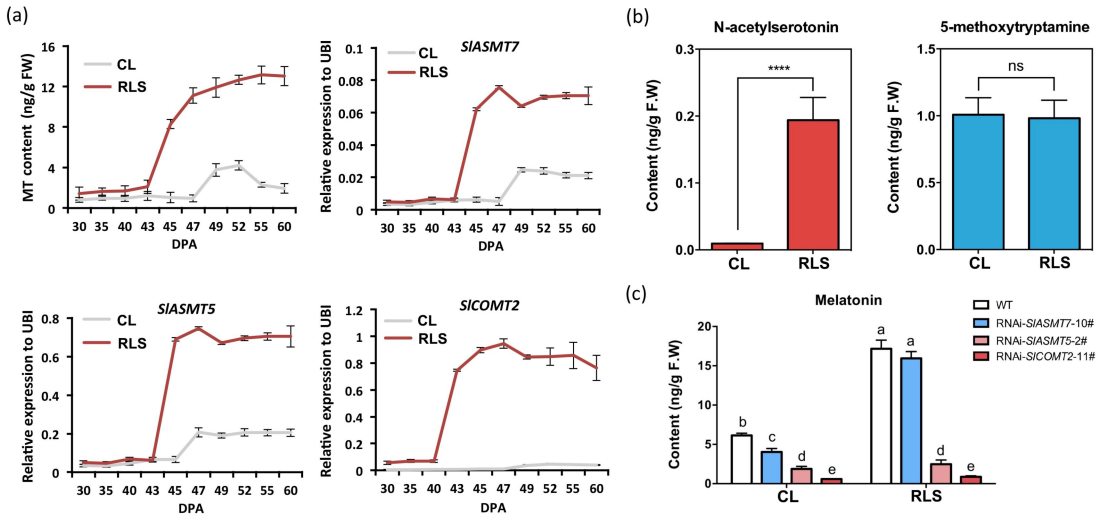


Fig. 3 Red light supplement induced melatonin biosynthesis in tomato fruit.

(a) Red light can significantly induce melatonin synthesis in tomato fruit. Determination of melatonin content and quantification of synthase gene in tomato fruit at 10 different development stages under control light (CL) and red light supplement (RLS). Data are represented as Mean ± SD (n=3). In which 10-12 individual fruit were pooled as one biological replicate. (b) Content of N-acetylserotonin and 5-methoxytryptamine in wild-type tomato fruit after red light treatment. Data are represented as Mean ± SD (n=3). In which 10-12 individual fruit were pooled as one biological replicate. ****P < 0.0001, ns. not significant (Student's t-test). (c) Melatonin content in fruit of transgenic tomato lines after red light treatment. Data are represented as Mean ± SEM (n=3). In which 10-12 individual fruit were pooled as one biological replicate. The P values indicate the results from pairwise comparisons of one-way ANOVA tests. Different letters represent a significant difference at P < 0.05.

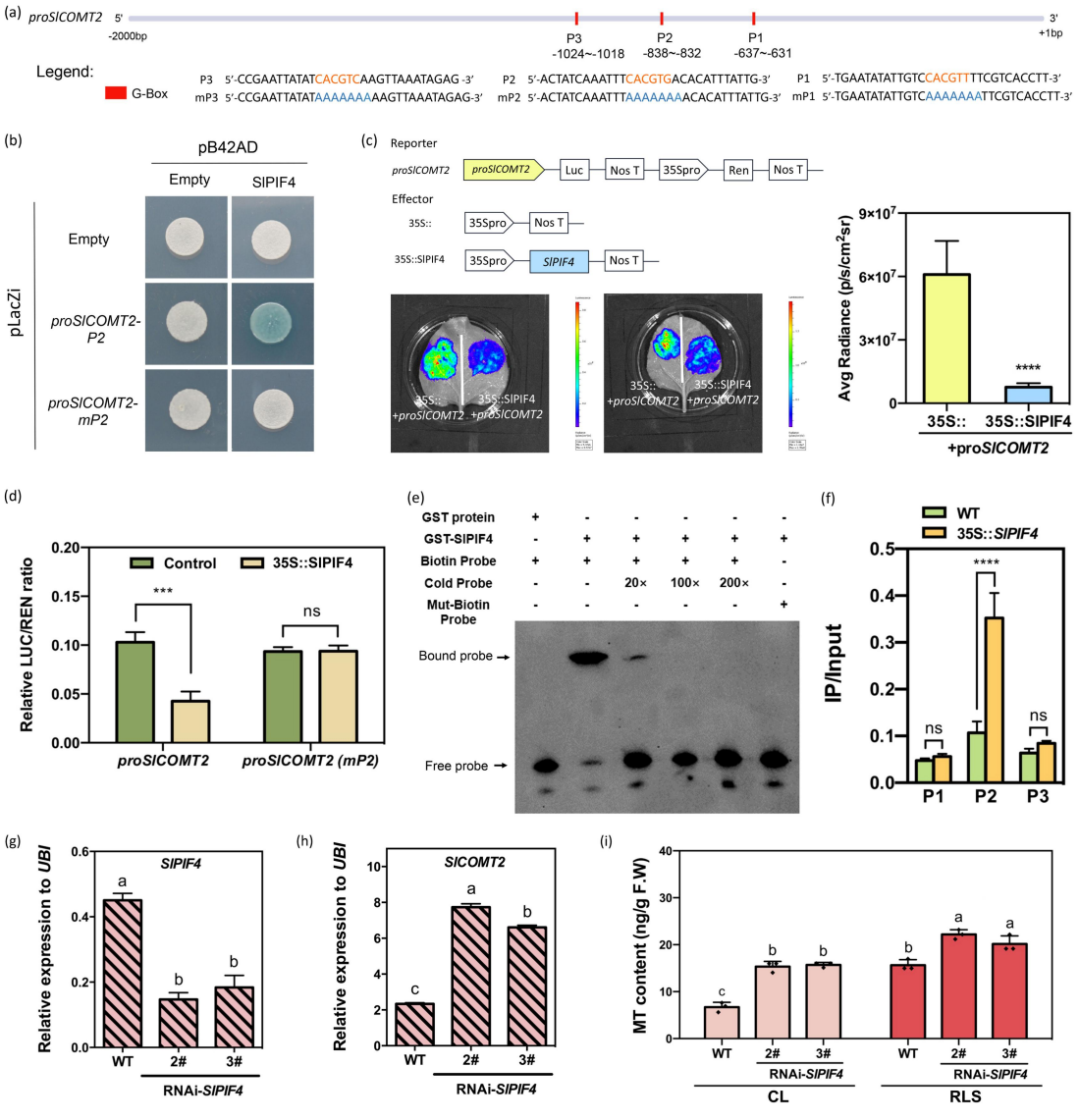


Fig. 4 SlPIF4 directly binds to the G-Box domain (P2) of *proSlCOMT2* to inhibit its expression.

(a) Schematic diagrams showing the SlASMT5 and SlCOMT2 genomic regions. The position of G-BOX is indicated by a red BOX. (b) Interactions between SlPIF4 proteins and *SlCOMT2* promoters with P2 and P2 mutation (mP2) in yeast cells. A blue plaque indicates binding. (c) Interactions of SlPIF4 protein and the promoters of *SlCOMT2* confirmed with dual luciferase reporter assays in *Nicotiana benthamiana* leaves. 35S::+*proSlCOMT2* were used as controls. The

right column chart shows the quantitative fluorescence intensity. (d) SlPIF4 binding to the regions of *proSlCOMT2* in the WT and transgenic lines of 35S::*SlPIF4*. '*proSlCOMT2(mP2)*' is a 2000 bp promoter sequence with a mutation in the P2 domain. LUC/REN is the average ratio of the bioluminescence of firefly luciferase to that of Renilla luciferase. (e) EMSA of SlPIF4 binding to the P2/mP2 fragment. SlPIF4 binds to the P2 fragment of *proSlCOMT2*, while the mutant of P2 (mP2) does not present binding. '+' indicates presence; and '-' indicates absence. (f) ChIP analysis of SlPIF4 binding to the regions of SlCOMT2 in the WT and transgenic lines of 35S::*SlPIF4*. Data in (c), (d) and (f) are represented as Mean ± SD (n=3). ***P < 0.001, ****P < 0.0001, ns. not significant (Student's t-test). (g) Transcript level of *SlPIF4* in the fruit of RNAi-*SlPIF4* transgenic lines as well as WT. (h) Transcript level of *SlCOMT2* in the fruit of RNAi-*SlPIF4* transgenic lines as well as WT. (i) Melatonin content in fruit of RNAi-*SlPIF4* transgenic lines as well as WT after red light treatment. Data in (g), (h) and (i) are represented as Mean ± SEM (n=3). In which 10-12 individual tomato fruits at the B+3 stage were pooled as one biological replicate. The P values indicate the results from pairwise comparisons of one-way ANOVA tests. Different letters represent a significant difference at P < 0.05.

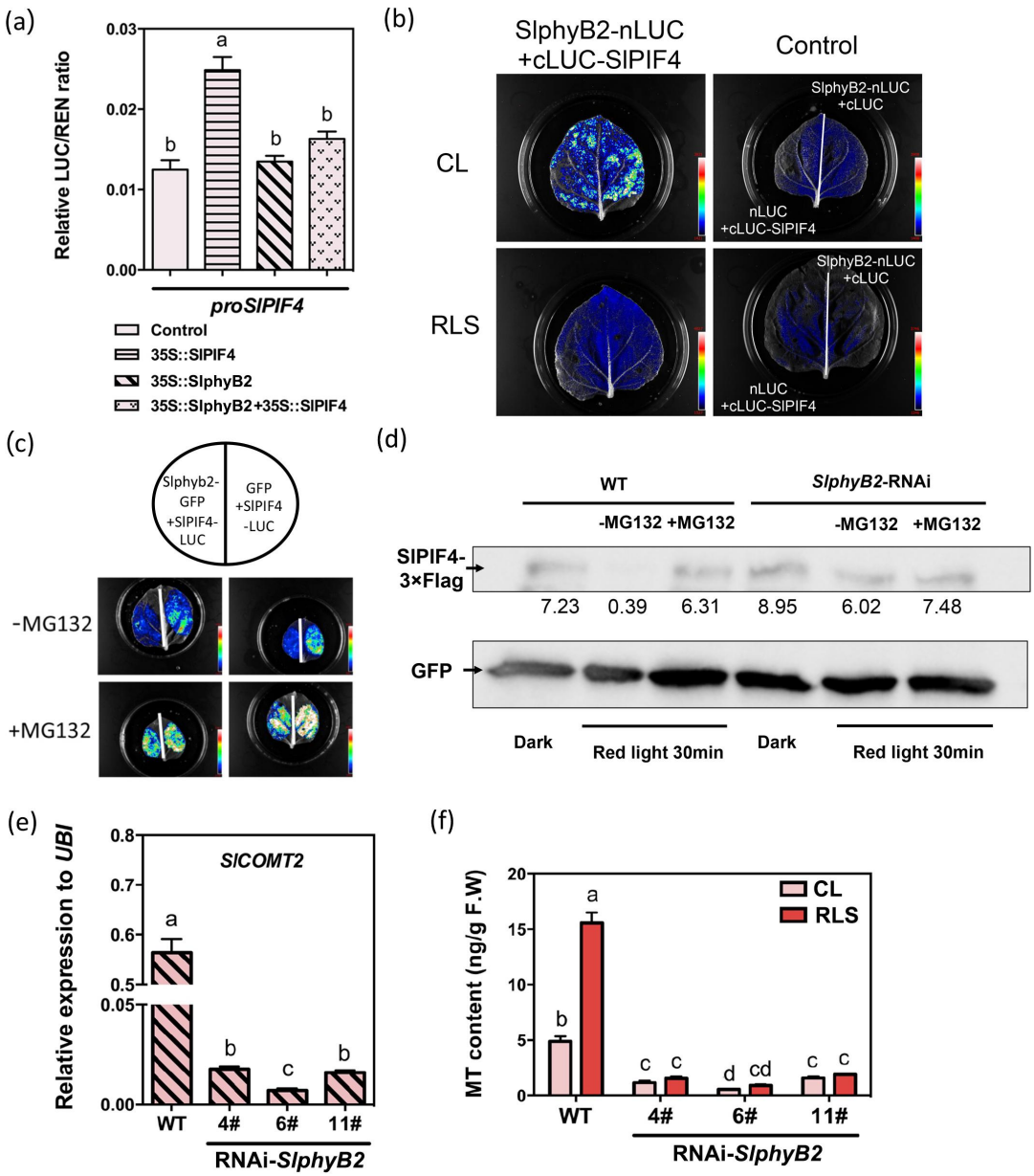
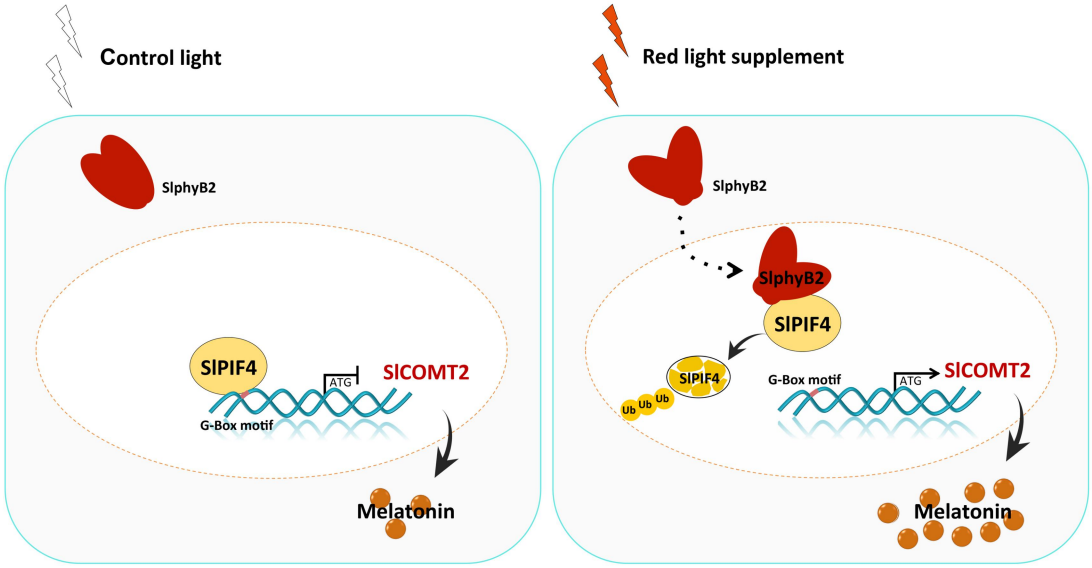


Fig. 5 Red light response of melatonin mediated by SlphyB2-SlPIF4-SlCOMT2.

(a) The transcriptional regulation relationship between *SlphyB2* and *SlPIF4*. The dual-LUC experiment proves that SlphyB2 can inhibit the self-activation of SlPIF4 on its own promotor. Data are represented as Mean ± SEM (n=3). (b) Quantitative analysis of luminescence intensity showing the interaction between SlphyB2 and SlPIF4 in *Nicotiana benthamiana* leaves. SlphyB2 interacts with the SlPIF4

protein, but the interaction disappears under red light. (c) SlphyB2 can ubiquitously degrade SlPIF4, and MG132 prevents the degradation. (d) Western blot detection of ubiquitination degradation of SlPIF4 mediated by SlphyB2. The addition of MG132 will inhibit the degradation of SlPIF4 by red light in WT, while in the interference strains of *SlphyB2*, the bands of SlPIF4 are not different. GFP act as an actin ensures consistent protein levels. (e) Gene expression of *SlCOMT2* in the *SlphyB2* interference lines. Samples were collected at Br+3. (f) Silence of *SlPHYB2* makes the plant no longer be induced by the red light to produce more melatonin. The content of melatonin in wild tomato fruit was induced and accumulated by red light, but decreased in RNAi-*SlphyB2* lines, and was no longer induced by red light. Samples were collected at Br+3. Data in (e) and (f) are represented as Mean ± SEM (n=3). In which 10-12 fruit collected from the same seedling were pooled as one biological replicate. For (a), (e) and (f), the P values indicate the results from pairwise comparisons of one-way ANOVA tests. Different letters represent a significant difference at $P < 0.05$.



914

915 **Fig. 6 Schematic representation of the molecular mechanism of**

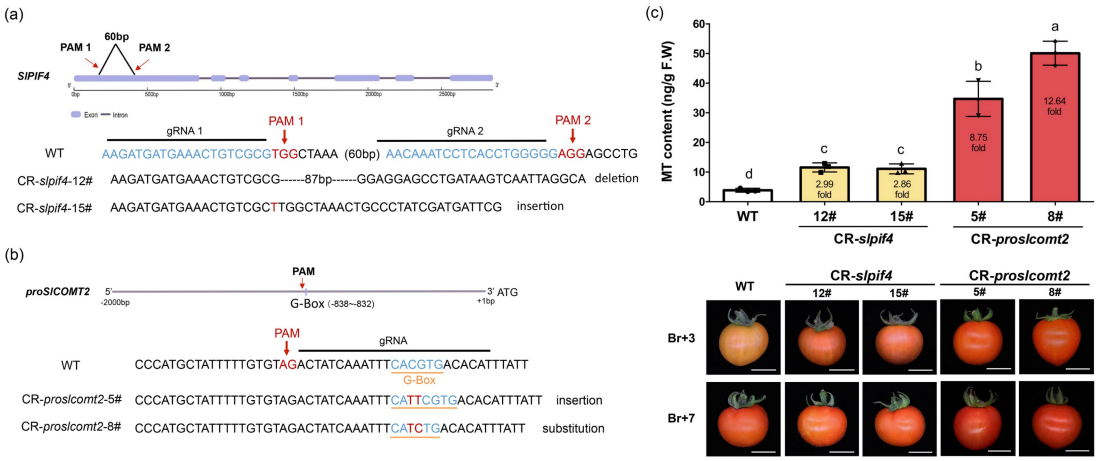
916 **red light-induced melatonin biosynthesis in tomato fruit.**

917 SlphyB2 is activated under the red light supplement and can facilitate the

918 degradation of SlPIF4 through the 26S proteasome pathway, thus removing the

919 inhibition of *SlCOMT2* by SlPIF4, leading to the accumulation of melatonin.

920



921

922 **Fig. 7 Engineering of new tomato germplasm with high**
923 **melatonin content.**

924 (a) CRISPR/Cas9 target site design and sequencing results of gene editing for
925 *SlPIF4*. (b) CRISPR/Cas9 target site design and sequencing results of gene editing
926 for the promoter of *SlCOMT2*. (c) Melatonin content of WT and T2 CR fruits at the
927 Br+3 stage. Data is represented as Mean ± SEM (n=3). 10-12 tomato fruits from
928 the same seedling were pooled as one biological replicate. The P values indicate
929 the results from pairwise comparisons of one-way ANOVA tests. Different letters
930 represent a significant difference at $P < 0.05$. Fruit phenotype of WT and CR fruits
931 at the Br+3 and the Br+7 stage were also presented.

932

933

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.xlsx
- checklist.pdf
- reportsummary.pdf
- reportsummary.pdf
- SupplementaryFigures.pdf