Melatonin represses oil and anthocyanin accumulation in seeds

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Short title
Melatonin limits fatty acid & anthocyanin content

ONE SENTENCE SUMMARY
Melatonin functions as a negative regulatory signal of seed oil and anthocyanin accumulation during the maturation of A. thaliana seeds.
ABSTRACT

Previous studies have clearly demonstrated that the putative phytohormone melatonin functions directly in many aspects of plant growth and development. In *Arabidopsis thaliana*, the role of melatonin in seed oil and anthocyanin accumulation, and corresponding underlying mechanisms, remain unclear. Here, we found that *serotonin N-acetyltransferase*1 (*SNAT1*) and *caffeic acid O-methyltransferase* (*COMT*) genes were ubiquitously and highly expressed and essential for melatonin biosynthesis in *A. thaliana* developing seeds. We demonstrated that blocking endogenous melatonin biosynthesis by knocking out *SNAT1* and/or *COMT* significantly increased oil and anthocyanin content of mature seeds. In contrast, enhancement of melatonin signaling by exogenous application of melatonin led to a significant decrease in levels of seed oil and anthocyanins. Further gene expression analysis through RNA-sequencing and reverse transcription quantitative PCR demonstrated that the expression of a series of important genes involved in fatty acid and anthocyanin accumulation was significantly altered in *snat1-1 comt-1* developing seeds during seed maturation. We also discovered that SNAT1 and COMT significantly regulated the accumulation of both mucilage and proanthocyanidins in mature seeds. These results not only help us understand the function of melatonin and provide valuable insights into the complicated regulatory network controlling oil and anthocyanin accumulation in seeds, but also divulge promising gene targets for improvement of both oil and flavonoids in seeds of oil-producing crops and plants.

**Key words:** oil, anthocyanins, SNAT1, COMT, melatonin, *Arabidopsis thaliana*
INTRODUCTION

*Arabidopsis thaliana* is a popular model system for the study of primary and secondary metabolites, including oil and anthocyanins, in seeds of angiosperms. Seed oil, stored as triacylglycerols (TAGs), not only represents the major form of carbon storage, thus providing nutrients for humans and livestock and energy for seed germination and seedling establishment (Graham, 2008; Baud and Lepiniec, 2009; Li et al., 2016), but also serves as a raw material for various industries and biofuel production (Durrett et al., 2008; Lu et al., 2011; Keneni and Marchetti, 2017; Rodionova et al., 2017). In the plant cell, fatty acids (FAs) are biosynthesized in plastids and to a large extent transported to the endoplasmic reticulum for further elongation, modification, and TAG assembly (Baud and Lepiniec, 2009; Chapman and Ohlrogge, 2012; Li et al., 2016). Anthocyanins are natural water-soluble pigments that belong to the flavonoid class of secondary metabolites (Castaneda-Ovando et al., 2009; Kovinich et al., 2014). Anthocyanins exhibit antioxidant properties and are implicated in protection against abiotic and biotic stresses in plants (Winkel-Shirley, 2002; Petrussa et al., 2013). As signaling molecules in animal cells, anthocyanins participate in protection against cardiovascular illness, diabetes, and certain cancers (Toufektsian et al., 2008; Pan et al., 2010; Pojer et al., 2013; Fang, 2015).

Over the last few decades, major efforts have been undertaken to uncover specific roles of different factors in the accumulation of FAs and anthocyanins. However, few factors have been identified to synergistically improve their contents in seeds. Molecular breeding, a highly effective approach, is
increasingly being utilized to improve the quantity of useful metabolites in crop
seeds. Therefore, investigating the roles of essential factors in the
accumulation of seed oil and anthocyanins in *A. thaliana* would provide useful
information and potential targets for breeders to elevate the contents of these
metabolites in many crops, which is of great economic and social significance.

Melatonin (N-acetyl-5-methoxytryptamine), a highly conserved bioactive
molecule, is ubiquitously present in all plant species (Tan et al., 2012; Tan et
al., 2013). It is produced from serotonin through two consecutive enzymatic
steps. Serotonin is converted into either N-acetylserotonin by serotonin
N-acetyltransferase (SNAT) or into 5-methoxytryptamine (5-MT) by caffeic acid
O-methyltransferase (COMT), which are subsequently metabolized into
melatonin by COMT or SNAT, respectively (Lee et al., 2014a). There are two
*SNAT* isogenes, *SNAT1* and *SNAT2* (Back et al., 2016; Lee et al., 2019), and a
single copy of *COMT* in the *A. thaliana* genome (Nakatsubo et al., 2008).

Melatonin is a potent antioxidant (Tan et al., 2015; Reiter et al., 2016) that
functions directly against a wide range of abiotic and biotic stresses, including
high salt concentrations (Chen et al., 2017a; Zheng et al., 2017), heavy metals
(Cai et al., 2017; Gu et al., 2017; Kobylinska et al., 2017; Lee and Back, 2017 a;
Zhang et al., 2017a; Luo et al., 2018; Nawaz et al., 2018), high nitrate levels
(Zhang et al., 2017b), K⁺ deficiency (Chen et al., 2017b), drought (Antoniou et
al., 2017; Wang et al., 2017a), high pH (Gong et al., 2017), cold (Bajwa et al.,
2014; Li et al., 2017a; Li et al., 2018a), high temperature (Xu et al., 2016; Zhang
et al., 2017c; Qi et al., 2018), and various pathogens (Yin et al., 2013; Lee et al.,
2014b; Lee and Back, 2016, 2017b; Wei et al., 2017).

Melatonin has also been demonstrated to be involved in other aspects of
plant growth and development, such as root development (Hernandez-Ruiz et
al., 2005; Arnau and Hernandez-Ruiz, 2007; Chen et al., 2009; Wang et al.,
2016), cotyledon and seedling growth (Hernandez-Ruiz et al., 2005; Byeon and
Back, 2014; Wei et al., 2015), flowering time (Byeon and Back, 2014; Shi et al., 2016), and seed yield (Byeon and Back, 2014; Wei et al., 2015). As a putative phytohormone, the first identified melatonin receptor, CAND2/PMTR1, was found in *A. thaliana*, and the regulation of stomatal closure by melatonin is dependent on this receptor (Wei et al., 2018). In addition, exogenous application of melatonin enhances the accumulation of FAs in soybean seeds (Wei et al., 2015), and increases anthocyanin biosynthesis in cabbage seedlings (Zhang et al., 2016). However, the effect of melatonin on seed oil and anthocyanin accumulation and the corresponding mechanisms behind it remain unclear in *A. thaliana*.

In this study, we demonstrated that melatonin functions as a negative regulatory signal for seed oil and anthocyanin accumulation during the maturation of *A. thaliana* seeds.

**RESULTS**

**SNAT1 and COMT are expressed abundantly in developing seeds**

We investigated the subcellular localization of SNAT1 and COMT in *Nicotiana benthamiana* leaves using the GFP fusion constructs, 35S:SNAT1-GFP and 35S:COMT-GFP. We observed that SNAT1-GFP and COMT-GFP were localized in the chloroplast (Figure 1A) and cytoplasm (Figure 1B), respectively, which is consistent with a previous study (Lee et al., 2014a). We also found that the green fluorescence of COMT-GFP was co-localized with that of the nuclear marker DAPI, indicating that COMT was also localized in the nucleus (Figure 1B).

To determine the temporal and spatial mRNA distributions of *SNAT1* and *COMT* essential for melatonin biosynthesis, reverse transcription quantitative PCR (RT-qPCR) was conducted to investigate their expressions in various
tissues of wild-type plants. *SNAT1* was highly expressed in various tissues except for stems (Figures 2A, B). *COMT* was widely distributed in different tissues, and its transcript level was much higher in roots, flower buds, open flowers, and developing seeds than in stems, rosette leaves, and cauline leaves (Figures 2D, E). During seed development, the expression of *SNAT1* and *COMT* exhibited a similar pattern and increased rapidly from 8 days after pollination (DAP) to the maximal level at 10 DAP, and then decreased gradually afterwards (Figures 2B, E).

To better investigate the expression patterns of *SNAT1* and *COMT*, we generated at least 15 independent transgenic lines for each of the *pSNAT1*:GUS and *pCOMT*:GUS constructs in a wild-type background. Most transgenic lines of each construct showed similar GUS staining patterns and then one representative line was selected for GUS staining analysis. The results indicated that *SNAT1* was highly expressed in various tissues, including roots (Figure 2C1), cotyledons, rosette and cauline leaves (Figures 2C1-3), flower buds and open flowers (Figure 2C4), and developing seeds, including embryos and seed coat during seed maturation (Figures 2C5-8). The *COMT* transcript level was predominant in the tissues of roots (Figure 2F1), cotyledons and just emerged true leaves (Figure 2F1), flower buds and open flowers (Figures 2F3, 4), and developing seeds inclusive of embryos and seed coat during seed maturation (Figures 2F5-8). The expression of *SNAT1* was much higher than that of *COMT* in the seed coat (Figures 2C5-8 and 2F5-8).

However, GUS staining was hardly observed in stems of the *pSNAT1*:GUS line (Figure 2C3), as well as in rosette leaves (Figure 2F2), cauline leaves (Figure 2F3), and stems (Figure 2F3) of the *pCOMT*:GUS line.

To summarize, gene expression results from GUS staining and RT-qPCR were highly consistent, and both *SNAT1* and *COMT* were abundantly expressed during seed maturation. These results implied that the dynamic...
regulation of the two genes or of melatonin was relevant to the accumulation of seed metabolites occurring mainly at the seed maturation stage.

**Melatonin represses seed oil and anthocyanin accumulation**

To clarify the biological functions of endogenous melatonin on the accumulation of oil and anthocyanins in seeds, we obtained single mutants – snat1-1 of the **SNAT1** gene and comt-1 and comt-2 of the **COMT** gene – and created the double mutant snat1-1 comt-1 through artificial hybridization. No **SNAT1** and **COMT** transcripts were respectively detected in the homozygous snat1-1 and comt-1 plants (Figures 3C and S1), confirming that they are loss-of-function mutants. The T-DNA element in the comt-2 mutant, a previously unfamiliar allele in this study, was inserted in the second intron of the **COMT** gene (Figure 3A). The results of PCR-based genotyping (Figure 3B) indicated the presence of the homozygous comt-2 mutant, which completely lacks the **COMT** transcript, as determined by RT-PCR (Figure 3C).

We determined the melatonin levels in developing siliques at 12 DAP between wild type plants and various single and double mutants of **SNAT1** and **COMT** genes. As illustrated in Figure 3D, the three single mutants of snat1-1, comt-1, and comt-2 contained much less melatonin than wild-type plants, and the double mutant of snat1-1 comt-1 accumulated much less than their corresponding single mutants. The snat1-1 comt-1 mutant still produced melatonin (Figure 3D), which is consistent with the fact that another **SNAT** isogene (**SNAT2**) is present in the *A. thaliana* genome (Back et al., 2016; Lee et al., 2019). These results suggested that SNAT1 and COMT additively promote melatonin biosynthesis in *A. thaliana* siliques.

We measured the quantities of the major FA compositions and total FAs per microgram of mature seeds between wild-type plants and the single and double mutants of **SNAT1** and **COMT** genes. As shown in Figure 4A and Table
S1, the seed FA contents in all three single mutants of snat1-1, comt-1, and comt-2 were about 6% higher than that of wild type plants, and the significant increase of FA contents was accompanied by an increase in all detected FA compositions. The FA content of snat1-1 comt-1 seeds was much higher than that of their corresponding single mutants and was 17% higher than that of wild-type plants (Figure 4A; Table S1). These results indicated that SNAT1 and COMT have an additive effect in the repression of FA accumulation in A. thaliana seeds.

We also analyzed the contents of anthocyanins in seeds of wild-type plants and various single and double mutants of SNAT1 and COMT genes. The loss of function of either SNAT1 or COMT resulted in a significant increase in the accumulation of anthocyanins in seeds, and the comt mutation accumulated more anthocyanins than the snat1-1 mutation (Figure 4C; Table S2). However, no obvious difference was observed in the seed anthocyanin content between the comt mutants and the double mutant snat1-1 comt-1 (Figure 4C; Table S2). These results suggested that SNAT1 and COMT have a non-additive effect on the accumulation of anthocyanins in seeds, and COMT is more important than SNAT1 for seed anthocyanin biosynthesis.

To further confirm the function of SNAT1 and COMT on the accumulation of FAs and anthocyanins, we transformed snat1-1 and comt-1 mutants with the genomic constructs of gSNAT1 and gCOMT, respectively. Among more than 15 independent lines regenerated for each construct, at least three homozygous progenies for each construct containing a single transgene were selected based on a 3:1 Mendelian segregation ratio on glufosinate-ammonium-containing medium. Examination of the representative lines, snat1-1 gSNAT1#1 and comt-1 gCOMT#1, showed that the expression levels of SNAT1 and COMT were restored to wild-type levels (Figure S2), and the lower melatonin content in both snat1-1 and comt-1 was also fully rescued.
to wild-type levels (Figure 3D) in their corresponding rescued lines. Thus, the representative transformants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1 were utilized for further experiments. We found that the higher contents of both FAs and anthocyanins in snat1-1 and comt-1 seeds were fully restored to wild-type levels by introducing gSNAT1 and gCOMT, respectively (Figures 4A, C). These results implied that SNAT1 and COMT indeed inhibit the accumulation of seed oil and anthocyanins in *A. thaliana*.

Meanwhile, we investigated the effect of exogenous application of melatonin on the accumulation of FAs and anthocyanins of wild-type plants, single mutants of snat1-1 and comt-1, and the double mutant snat1-1 comt-1. The results showed that exogenous application of melatonin on wild-type plants led to a significant decrease of both oil (Figure 4B; Table S1) and anthocyanin (Figure 4D; Table S2) levels in seeds. Under exogenous melatonin treatment, the seed oil content of the single and double mutants was almost the same as that of wild-type plants (Figure 4B; Table S1), whereas the seed oil content of the single mutants was slightly lower than that of the double mutant, and slightly higher than that of wild-type plants (Figure 4B; Table S1). These findings showed that SNAT1 and COMT repress FA accumulation in an independent and additive manner, but mainly by influencing melatonin biosynthesis, in *A. thaliana* seeds.

In addition, under exogenous melatonin treatment, the anthocyanin content in snat1-1 seeds was the same as that of wild-type plants (Figure 4D; Table S2), whereas the seed anthocyanin contents of comt1-1 and snat1-1 comt-1 mutants were the same, and higher than that of wild-type plants (Figure 4D; Table S2). These findings indicated that SNAT1 inhibits seed anthocyanin deposition only by affecting melatonin biosynthesis, whereas COMT represses seed anthocyanin accumulation not only by itself, but also by influencing melatonin biosynthesis.
No obvious differences in seed coat color, size, and weight were observed among the single and double mutants of SNAT1 and COMT, the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1, wild-type plants applied with exogenous melatonin, or their corresponding controls (Figure S3). Overall, we demonstrated that, through blocking endogenous melatonin biosynthesis by knocking out SNAT1 and/or COMT and by exogenous application of melatonin, melatonin represses the accumulation of both oil and anthocyanins. In addition, SNAT1 and COMT, independent of melatonin, exhibit distinct roles in the inhibition of oil and anthocyanin biosynthesis in A. thaliana seeds.

**Genome-wide analysis of DEGs in developing seeds at 12 DAP between wild type and snat1-1 comt-1 plants**

In A. thaliana developing seeds, FAs start to accumulate at 6 DAP, and increase linearly from 8 to 18 DAP during seed maturation (Baud and Lepiniec, 2009, 2010). The double mutant snat1-1 comt-1 accumulated much more seed FAs than wild type and single mutants of SNAT1 and COMT (Figure 4A; Table S1). In addition, 12 DAP is the key stage for the biosynthesis of seed flavonoids, including anthocyanins, during seed maturation (Routaboul et al., 2012). Therefore, we utilized developing seeds at 12 DAP to compare the expression profiles at a genome-wide level between wild type and snat1-1 comt-1 plants. These profiles would provide information on the downstream targets of melatonin that contribute to FA and anthocyanin accumulation, as well as facilitate a better understanding of the regulatory network underlying melatonin-mediated metabolites biosynthesis in A. thaliana seeds.

RNA-seq analysis identified 243 differentially expressed genes (DEGs), among which 119 were up-regulated (Table S3) and 124 were down-regulated (Table S4) in snat1-1 comt-1 developing seeds at 12 DAP. Functional analysis
discovered that 12 (4.9%) and six (2.5%) of the DEGs were related to oil and anthocyanin metabolisms, respectively (Tables S2 and S3). However, the expression of other genes that play major roles in oil and anthocyanin accumulation was not altered in snat1-1 comt-1 seeds compared to wild-type seeds (Table S5). Up to nine (7.6%) up-regulated genes and no down-regulated genes were related to carbohydrate metabolism (Tables S3 and S4). Multiple up-regulated (16, 13.4%) and down-regulated (30, 24.2%) genes were involved in general protein metabolism in snat1-1 comt-1 seeds (Tables S3 and S4). The storage proteins mainly contain legumin-type 12S globulins and napin-type 2S albumins in A. thaliana seeds (Heath et al., 1986; Baud et al., 2008). However, no obvious differences were observed in the expression levels of key genes encoding 12S precursors, including CRUCIFERINA1 (CRU1), CRU2, and CRU3, and five genes encoding 2S precursors (2S1 to 2S5), between wild-type and snat1-1 comt1-1 seeds (Table S5). Consistently, there was no substantive difference in the content of seed storage proteins between wild type and snat1-1 comt1-1 plants (Figure S4). It is worth mentioning that the number of DEGs involved in the stress/defense response and other biological processes accounts for the largest proportion of all the DEGs in snat1-1 comt-1 seeds (Tables S3 and S4).

Therefore, simultaneous knockout of SNAT1 and COMT, essential for melatonin biosynthesis, regulates a series of genes important for oil and anthocyanin accumulation and many genes involved in other biological processes during seed maturation.

**Verification of regulated genes involved in oil and anthocyanin biosynthesis at different developmental stages in snat1-1 comt-1 seeds**

To confirm the regulation of DEGs involved in oil and anthocyanin biosynthesis in snat1-1 comt-1 developing seeds at 12 DAP, and to extensively
explore expression alterations of these genes, we performed RT-qPCR to
compare their expression patterns at the seed maturation stages (12-16 DAP)
between wild type and snat1-1 comt-1 plants.

For the highly up-regulated genes related to oil accumulation, we chose
one regulatory gene, WRINKLED1 (WRI1), and five structural genes, Biotin
Carboxyl-Carrier Protein1 (BCCP1), Acetyl Co-Enzyme A
Carboxylase Carboxyltransferase Alpha Subunit (CAC3),
Malonyl Co-ACP Malonyltransferase (MCAMT), Plastid
Lipase1 (PLIP1), and Lipid Transfer Protein3 (LTP3), in snat1-1
comt-1 developing seeds at 12 DAP (Figure 5; Table 1). The expression levels
of all six genes from 12 to 16 DAP were always significantly higher in the
snat1-1 comt-1 mutant than in wild type (Figure 5). As detailed in Figure 5, the
relative expression of WRI1 gradually increased, whereas the relative
expression of LTP3 dramatically decreased from 12 to 16 DAP in the snat1-1
comt-1 mutant compared to wild type. Moreover, both BCCP1 and CAC3
exhibited an expression pattern like that of WRI1. The relative expression
levels of MCAMT and PLIP1 grew from 12 DAP to peaks at 14 DAP and then
decreased afterwards in the snat1-1 comt-1 mutant in comparison with wild type.

For the highly regulated genes contributing to anthocyanin biosynthesis, we
selected two regulatory genes, Kelch-Domain-Containing F-Box
Protein39 (KFB39) and Kanadi4 (KAN4), and four structural genes,
4-Coumarate:CoA Ligase1 (4CL1), Chalcone Isomerase (CHI),
UDP-Glucosyltransferase 73B2 (UGT73B2), and
Glucose-6-Phosphate/Phosphate Translocator2 (GPT2), in
snat1-1 comt-1 developing seeds at 12 DAP (Figure 6; Table 2). Except for
GPT2 expression at 16 DAP, from 12 to 16 DAP the expression levels of all six
genes were dramatically altered in the snat1-1 comt-1 mutant compared to wild
type (Figure 6). Compared to wild type, the relative expression of KFB39 was
always significantly lower, and the relative expression levels of *UGT73B2*,
*KAN4*, and *GPT2* gradually declined in *snat1-1 comt-1* developing seeds from
12 to 16 DAP (Figure 6). The relative expression levels of *4CL1* and *CHI*
increased from 12 DAP to the peaks at 14 DAP and then decreased afterwards
in the *snat1-1 comt-1* mutant compared to wild type (Figure 6).

Taken together, simultaneous knockout of SNAT1 and COMT, essential for
melatonin biosynthesis, inhibits seed oil and anthocyanin accumulation by
regulating a range of genes contributing to oil and anthocyanin biosynthesis,
respectively, during seed maturation.

**SNAT1 and COMT antagonistically affect seed coat mucilage production**

Previous studies showed that seed coat mucilage competes with FAs for
photosynthates in *A. thaliana* seeds (Shi et al., 2012; Liu et al., 2017; Li et al.,
2018b). Therefore, we explored whether melatonin affects the production of
seed coat mucilage. Surprisingly, the *snat1-1* mutant produced less mucilage,
whereas the *comt* mutation accumulated more mucilage in the seed coat in
comparison with wild type (Figure 7A). The altered seed coat mucilage in the
*snat1-1* and *comt-1* mutants was fully restored by the introduction of *gSNAT1*
and *gCOMT*, respectively (Figure 7A). Furthermore, the double mutant *snat1-1*
*comt-1* contained moderate mucilage in comparison with their corresponding
single mutants and had mucilage comparable with wild type in the seed coat
(Figure 7A). Consistently, RNA-seq analysis only detected two regulatory
genes *DE1 BINDING FACTOR1 (DF1*, Kaplan-Levy et al., 2012; Vasilevski et
al., 2012) and *MUCILAGE-MODIFIED4 (MUM4*, Western et al., 2004; Oka and
Nemoto, 2007; Francoz et al., 2015) that positively regulate seed coat mucilage
production, and their expression levels were not altered in *snat1-1 comt-1*
developing seeds (Table S5). On the other hand, exogenous application of
melatonin to wild-type plants did not alter the accumulation of seed coat
mucilage (Figure S5). The results suggested that melatonin has no effect on seed coat mucilage biosynthesis, although SNAT1 and COMT antagonistically affect its production.

To investigate how SNAT1 and COMT separately regulate seed coat mucilage production, we carried out RT-qPCR to compare the expression of DF1 and MUM4 from 8 to 12 DAP, which are the key stages for seed mucilage deposition (Francoz et al., 2015), among wild type, single mutants of snat1-1 and comt-1, and the transgenic plants of snat1-1 gSNAT#1 and comt-1 gCOMT#1. We found that the expression levels of both DF1 and MUM4 were significantly down-regulated (Figure 7B) and up-regulated (Figure 7C) in developing seeds of snat1-1 and comt-1, respectively, at both 10 and 12 DAP, compared with wild type. As expected, the altered expressions of DF1 and MUM4 in snat1-1 and comt-1 developing seeds were fully restored to wild-type levels by the introduction of gSNAT1 and gCOMT, respectively (Figures 7B, C).

These results suggested that SNAT1 and COMT antagonistically affect the production of seed coat mucilage not by influencing melatonin biosynthesis, but instead by regulating the expression of DF1 and MUM4, in A. thaliana developing seeds.

**SNAT1 and COMT inhibited seed coat proanthocyanidin deposition**

Flavonoids, as secondary metabolites, are generally classified into three major classes in A. thaliana—flavonols, anthocyanins, and proanthocyanidins (PAs, Lepiniec et al., 2006). Considering that biosynthesized flavonols are converted into both anthocyanins and PAs in the flavonoid biosynthetic pathway (Lepiniec et al., 2006), we speculated that anthocyanins and PAs compete against each other for flavonols during flavonoid biosynthesis. To test this hypothesis, we investigated the effect of SNAT1 and COMT on the accumulation of PAs that are mainly deposited in the seed coat (Lepiniec et al.,
2006). Dimethylaminocinnamaldehyde (DMACA) staining analysis showed that levels of PAs in the seed coat were markedly higher in the single and double mutants of SNAT1 and COMT genes compared with wild-type plants (Figure 8A). Consistently, acidic hydrolysis of PAs indicated that the single and double mutants of SNAT1 and COMT genes possessed more solvent-soluble PAs in their seeds than wild-type plants (Figures 8B, C). Moreover, the higher amounts of PAs in snat1-1 and comt-1 were fully rescued by the introduction of gSNAT1 and gCOMT, respectively (Figures 8B, C). It is worthy to note that levels of both total and solvent-soluble PAs in the comt seeds were higher than those of snat1-1 seeds, and comparable with snat1-1 comt-1 seeds (Figure 8). These results suggested that SNAT1 and COMT act in a non-additive manner, and COMT exhibits a greater role than SNAT1 in inhibiting the deposition of PAs in the A. thaliana seed coat.

DISCUSSION

In seeds of angiosperms, accumulation of both oil and anthocyanins is coordinately regulated at multiple levels by intricate regulatory networks of various environmental and developmental signals. The mechanisms underlying how phytohormones control the overall amounts of oil and anthocyanins stored in plant seeds are still largely unknown. Previous studies have extensively demonstrated that the putative phytohormone melatonin functions directly in many aspects of plant growth and development. However, the role of melatonin in seed oil and anthocyanin accumulation remains unclear in A. thaliana. In this study, we showed that blocking the biosynthesis of endogenous melatonin through knock-out of two essential genes in the melatonin biosynthetic pathway, SNAT1 and COMT, significantly increased the contents of total FAs.
and anthocyanins, while enhancement of melatonin signaling by exogenous application of melatonin led to a dramatic decrease in the levels of total FAs and anthocyanins in mature seeds (Figure 4; Tables S1 and S2). Furthermore, the expression of a series of important genes involved in FA and anthocyanin accumulation was significantly altered in snat1-1 comt-1 developing seeds (Figures 5 and 6; Tables 1, 2, S3, and S4). These results, together with the observation of increased expression of SNAT1 and COMT in developing seeds at the seed maturation stage (Figures 2B, C and 2E, F), suggest that melatonin is an important player in the regulatory network that represses the accumulation of both oil and anthocyanins in A. thaliana seeds.

Several previous studies showed a negative correlation between the contents of oil and flavonoids in A. thaliana seeds (Chen et al., 2012a; Chen et al., 2014; Chen et al., 2015; Li et al., 2018b; Xuan et al., 2018). Thus, it is generally considered difficult for breeders to synergistically improve both seed oil and flavonoid contents. Interestingly, we demonstrated that the deficiency of endogenous melatonin in the snat1-1 comt-1 mutant resulted in a significant increase of both oil and flavonoids, including anthocyanins and PAs (Figures 4 and 8; Tables S1 and S2). It is known that sucrose from photosynthesis is hydrolyzed to glucose, which is then used for acetyl-coenzyme A (CoA) biosynthesis through glycolysis that can be further converted into malonyl-CoA. Both acetyl-CoA and malonyl-CoA are essential substrates for FA biosynthesis (Baud et al., 2008), while malonyl-CoA also serves as a key substrate for flavonoid production (Lepiniec et al., 2006) in the plant cell. Some studies propose that starch serves as a carbon source for seed compound accumulation during seed maturation (Norton and Harris, 1975; da Silva et al., 1997; Periappuram et al., 2000). The number of genes related to carbohydrate metabolism that are up-regulated is much higher than that of down-regulated genes in developing seeds of snat1-1 comt-1 plants at 12 DAP (Tables S2 and
S3). In addition, LTP3, a member of a family of lipid-transfer proteins that encode 7–10 kDa peptides and are widely distributed among plants (Kader, 1996; Arondel et al., 2000; Wong et al., 2017), promotes the accumulation of soluble sugars (Guo et al., 2013). Previous studies have indicated that GPT2, a glucose 6-phosphate/phosphate translocator, is thought to be involved in the transport of glucose 6-phosphate from the cytosol to plastids, leading to starch biosynthesis (Kammerer et al., 1998; Knappe et al., 2003; Kunz et al., 2010).

Thus, these regulated carbohydrate metabolism genes (Table S3) together with the up-regulation of LTP3 and GPT2 genes (Figures 5 and 6; Tables 1, 2, and S3) could supply more carbon resources for glycolysis, thus promoting acetyl-CoA and malonyl-CoA production and further accelerating FA and flavonoid biosynthesis in snat1-1 comt-1 developing seeds.

Transcriptional regulation is a major means of controlling the accumulation of seed oil and anthocyanins. In angiosperms, this process requires the coordinated expression of genes involved in the biosynthetic pathways of these metabolites. Our results showed that SNAT1 and COMT repress the accumulation of seed oil and anthocyanins mainly by affecting melatonin biosynthesis (Figure 4; Tables S1 and S2). Thus, the genes related to the accumulation of oil (Figure 5; Tables 1, S3, and S4) and anthocyanins (Figure 6; Tables 2, S3, and S4) in snat1-1 comt-1 developing seeds should be predominantly regulated by the deficiency of endogenous melatonin. Of the enzymes involved in oil accumulation, acetyl-CoA carboxylase (ACCase), localized in both plastids and cytosol, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA (Sasaki et al., 1995). The formation of malonyl-CoA is the rate-limiting step for FA biosynthesis (Ohlrogge et al., 1995), and ACCase may serve as a sensor or a gating system to monitor the overall flux of FA biosynthesis (Mu et al., 2008). ACCase contains three nuclear-localized subunits—BCCP, biotin carboxylase (BC), and α-carboxyltransferase.
(α-CT)—and one plastid-localized subunit, β-carboxyltransferase (β-CT), which are encoded by BCCP1 and BCCP2, CAC2, CAC3, and ACCD, respectively, in *A. thaliana* (Li et al., 2011). The complete loss of BCCP1 function results in embryo lethality, and reduced BCCP1 activity markedly decreases FA accumulation in *A. thaliana* seeds (Li et al., 2011). MCAMT, localized in both chloroplasts and mitochondria, converts malonyl-CoA and ACP into malonyl-ACP and CoA, and significantly promotes oil accumulation in *A. thaliana* seeds (Jung et al., 2019). PLIP1, as a chloroplast thylakoid-associated protein, functions in the export of FAs from the chloroplast and the incorporation of FAs derived from the thylakoid membrane lipid pool into TAG, and positively regulates FA accumulation in *A. thaliana* seeds (Wang et al., 2017b). The LTP3 loss-of-function mutant seeds contain oil content similar to wild-type plants (Pignussat et al., 2015). However, LTP3, LTP4 and LTP5, can enhance the *in vitro* transfer of phospholipids between membranes and can bind acyl chains (Kader, 1996; Arondel et al., 2000; Wong et al., 2017); thus they probably have a redundant function in seed oil accumulation. GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE 2 (GPAT2), localized in mitochondria, exhibits *sn*-1 and *sn*-2 acyltransferase activities and utilizes dicarboxylic acyl-CoA as substrate for the biosynthesis of extracellular lipids (Beisson et al., 2007; Yang et al., 2012; Jayawardhane et al., 2018). The transcription factor WRI1, an APETALA2/ethylene-responsive element-binding transcription factor, acts as a master positive regulator in seed oil accumulation by incorporating sucrose and glucose into TAGs during seed maturation (Focks and Benning, 1998); through directly promoting the expression of *ABNORMAL SUSPENSOR2* (*SUS2*), *PKp β1*, *BCCP1*, *BCCP2*, 3-KETOACYL-ACYL CARRIER PROTEIN SYNTHASE I (*KASI*), and *REDUCED OLEATE DESATURATION1* (*ROD1*); and by indirectly activating the expression of *ACYL CARRIER PROTEIN1* (*ACP1*), CAC2, CAC3, BIOTIN AUXOTROPH.
(BIO2), PDH E1α, KASIII, and MOSAIC DEATH1 (MOD1), which are involved in the late glycolysis and plastidial FA biosynthetic pathways during seed development in *A. thaliana* (Cernac and Benning, 2004; Masaki et al., 2005; Baud et al., 2007; Maeo et al., 2009; To et al., 2012). We found that BCCP1 and CAC3 were significantly up-regulated (Figure 5; Tables 1 and S3), and the expression of other genes was not altered (Table S5) in *snat1-1 comt-1* developing seeds at 12 DAP, indicating that melatonin controls the expression of *BCCP1* and *CAC3* through the up-regulation of WRI1, and that other genes were regulated by a complex upstream regulatory network. A previous study showed that five GDSL-type lipase genes, *SEED FATTY ACID REDUCERS*, inhibit seed FA biosynthesis by affecting FA degradation (Chen et al., 2012b), thus the much lower expression of the two GDSL-type lipase genes (AT2G30310 and AT5G45670) observed in our study is helpful for understanding the higher oil content in *snat1-1 comt-1* seeds (Table S4).

Therefore, the increased expression of *BCCP1*, *CAC3*, *MCAMT*, *PLIP1*, *LTPs*, *GPAT2*, and *WRI1* contributing to oil biosynthesis (Figure 5; Tables 1 and S3) and the decreased expression of the two GDSL-type lipase genes (Table S4) together assist in promoting seed oil accumulation (Figure 4A; Table S1) in *snat1-1 comt-1* developing seeds.

Anthocyanin biosynthesis starts from the phenylpropanoid pathway (Lepiniec et al., 2006). KFB39, a member of Kelch domain-containing F-box proteins, negatively regulates anthocyanin accumulation by directly controlling the stability and activity of phenylalanine ammonia-lyase, which is the first rate-limiting enzyme in the phenylpropanoid biosynthetic pathway (Zhang et al., 2015). There are four isoforms of 4CL, namely, 4CL1 to 4, which are essential for the activation of *p*-coumarate to form *p*-coumaroyl CoA in the last step of this pathway. *p*-coumaroyl CoA and malonyl-CoA are ultimately used for the biosynthesis of naringenin chalcone in the anthocyanin biosynthetic pathway.
4CL1 accounts for the majority of total 4CL activity and positively regulates the accumulation of anthocyanins in *A. thaliana* (Li et al., 2015). The CHI enzyme converts tetrahydroxychalcone to naringenin as the second step in the anthocyanin biosynthetic pathway, and its mutation fails to accumulate anthocyanins (Shirley et al., 1992; Pourcel et al., 2013). UGT73B2, a member of group D URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASEs, encodes a flavonol 7-O-glucosyltransferase that preferentially transfers a glucose group to the 3-hydroxyl group of flavonoids *in vitro* (Kim et al., 2006; Lim et al., 2006). KAN4, a member of the MYB-related GARP (Golden2, ARR-B, Psr1) superfamily of type-B response regulators promotes the accumulation of flavonoids by directly activating the expression of flavonoid biosynthetic genes, such as the regulatory genes *TRANSPARENT TESTA2* (*TT2*), *TT8* and *TRANSPARENT TESTA GLABRA1* (*TTG1*), and the structural genes *CHALCONE SYNTHASE* (*CHS*), *CHI*, *FLAVONOID 3′ HYDROXYLASE* (*F3’H*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), and *ANTHOCYANIDIN SYNTHASE* (*ANS*) (Gao et al., 2010). Our results showed that only *CHI* was up-regulated (Figure 6; Tables 2 and S3) and the expression of the other genes was not altered in *snat1-1 comt-1* developing seeds (Table S5), implying that melatonin controls *CHI* expression through the up-regulation of KAN4, and other genes were regulated by a complex upstream regulatory network. The transcription factor MYB56 acts in a sucrose-dependent manner to control *GPT2* expression in response to the circadian cycle, thus promoting anthocyanin accumulation (Jeong et al., 2018). Therefore, the down-regulation of *KFB39* and up-regulation of 4CL1, *CHI*, *UGT73B2*, *KAN4*, and *GPT2* related to anthocyanin biosynthesis (Figure 6; Tables 2, S3, and S4) are helpful for anthocyanin accumulation (Figure 4C; Table S2) in *snat1-1 comt-1* developing seeds.
It is worth mentioning that snat1-1 seedlings accumulate less anthocyanins than wild-type seedlings under cold stress (Zhang et al., 2016), and exogenous application of melatonin increases anthocyanin biosynthesis in cabbage seedlings (Zhang et al., 2016) and enhances FA accumulation in soybean seeds (Wei et al., 2015). For melatonin contents in A. thaliana leaves, no significant differences were found between wild type and the single mutants of SNAT1, SNAT2, and COMT (Byeon et al., 2014; Lee et al., 2015; Lee et al., 2019). In contrast, both flowers of the snat2 mutant (Lee et al., 2019) and developing siliques at 12 DAP of the single and double mutants of SNAT1 and COMT (Figure 3D) contained much less melatonin than their corresponding wild-type tissues. Considering our results showing that both endogenous and exogenous melatonin inhibited seed oil and anthocyanin accumulation (Figure 4; Tables S1 and S2), it could be speculated that the effect of melatonin on seed oil or anthocyanin biosynthesis is plant species- or tissue-specific. In the plant cell, oil biosynthesis occurs in both the plastid and the endoplasmic reticulum (Baud and Lepiniec, 2009; Chapman and Ohlrogge, 2012; Li et al., 2016). Anthocyanins and PAs are biosynthesized in multi-enzyme complexes that are localized at the cytoplasmic surface of the endoplasmic reticulum (Winkel-Shirley, 2002; Winkel, 2004). The seed coat mucilage is mainly composed of pectins, which are largely acidic polysaccharides biosynthesized from Golgi stacks in the secretory cell (Western et al., 2000). Our results showed that SNAT1 and COMT were localized in the chloroplast (Figure 1A) and the cytoplasm and nucleus (Figure 1B), respectively. Thus, the different subcellular localizations of SNAT1 and COMT together with the different biosynthetic sites of oil, flavonoids, and mucilage in the plant cell could explain why SNAT1 and COMT, independent of melatonin, had distinct effects on the biosynthesis of different metabolites in seeds, including oil (Figures 4A, B; Table S1), flavonoids inclusive of
anthocyanins (Figures 4C, D; Table S2) and PAs (Figure 8), and mucilage (Figures 7 and S1). These interesting questions need further investigation. Even so, as exogenous application of melatonin and loss of function of SNAT1 and COMT exhibited opposite effects on seed oil and anthocyanin accumulation (Figure 4; Tables S1 and S2), and SNAT1 and COMT had a common and additive role in melatonin biosynthesis in developing siliques (Figure 3D; Lee et al., 2014a; Back et al., 2016), we might conclude that melatonin represses the accumulation of oil and anthocyanins in *A. thaliana* seeds; an underlying mechanism is proposed in Figure 9.

In summary, the present study provides significant and fresh information in several ways. First, this study demonstrates that melatonin represses seed oil and anthocyanin accumulation during seed maturation by inhibiting the expression of important genes involved in oil and anthocyanin biosynthesis, respectively. Second, in *A. thaliana* seeds, the two essential melatonin biosynthetic genes *SNAT1* and *COMT*, independent of melatonin, have distinct functions on different metabolites, including oil, flavonoids inclusive of anthocyanins and PAs, and mucilage, which might be due to their differential distribution among subcellular fractions. Third, seed metabolite accumulation is controlled by a coordinated regulatory network, which is not only pertinent to major steps of their metabolic pathways but also requires the partitioning of photosynthates (Mu et al., 2008; Li et al., 2018b). The results presented here indicate that manipulation of this co-regulation network is feasible by blocking melatonin biosynthesis through knocking out SNAT1 and/or COMT. In this regard, the genes *SNAT1* and *COMT* are noteworthy genetic resources for genetic modification of oil-producing crops and plants to synergistically improve both oil and flavonoids in seeds.

**MATERIALS AND METHODS**
Plant material and growth conditions

The *A. thaliana* ecotype Columbia (Col-0) was utilized as wild type control. The mutants of *snat1-1* (SALK_032239), *comt-1* (SALK_002373), and *comt-2* (SALK_020611C) were in the Col-0 background, and their genotyping primers are listed in Table S6. The *comt* mutants were obtained from the Arabidopsis Biological Resources Center at Ohio State University, USA. The growth conditions of all *A. thaliana* plants in this study have been reported in detail previously (Li et al., 2017b).

Exogenous application of melatonin to plants

Distilled water as the control was set as Level-1 (0 µM), and melatonin solution concentrations of 100, 200, and 500 µM were set as Level-2, Level-3, and Level-4, respectively. The different levels of melatonin solutions were applied to ten individual plants (Col-0, *snat1-1*, *comt-1*, and *snat1-1 comt-1*) at the bolting stage in one of three randomly arranged blocks every other day until the first silique was harvested. Melatonin from Sigma (St. Louis, MO, USA) was used in this exogenous application experiment.

Plasmid construction and plant transformation

To construct *pSNAT1:GUS* and *pCOMT:GUS*, their 5' regulatory regions upstream of the ATG start codon were amplified and then cloned into pHY107 (Liu et al., 2007), separately. To construct *gSNAT1* and *gCOMT*, a 2.799 kb genomic fragment of *SNAT1* harboring the 1.128 kb 5' upstream sequence, the entire 1.439 kb coding sequence, and the 0.232 kb 3’ downstream sequence, and a 4.956 kb *COMT* genomic region including the 2.631 kb 5’ upstream sequence, the entire 2.093 kb coding sequence, and 0.232 kb 3’ downstream sequence were amplified, digested, and then separately cloned into pHY105 (Liu et al., 2007). The *pSNAT1:GUS* and *pCOMT:GUS* constructs were
introduced into wild-type (Col-0) plants, whereas the gSNAT1 and gCOMT constructs were transformed into snat1-1 and comt-1 plants, respectively, using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998). The transgenic plants were selected by Basta on soil or on culture medium containing glufosinate-ammonium and verified by DNA analysis until T3 homozygous transgenic progeny was generated.

To construct 35S:SNAT1-GFP and 35S:COMT-GFP, the cDNA fragments of SNAT1 and COMT were amplified and then cloned into pGreen-35S-GFP to obtain the fusions of SNAT1-GFP and COMT-GFP under the control of 35S promoter, respectively. The 35S:SNAT1-GFP or 35S:COMT-GFP construct was transiently expressed in tobacco leaves (Nicotiana benthamiana) as previously described (Yang et al., 2000). Images were obtained with an Olympus IX83 confocal microscope (Japan) 72 h after agroinfiltration. GFP was excited with a 488 nm wavelength laser, and emitted light was collected between 500 and 540 nm. Chloroplasts were excited with a 488 nm wavelength laser, and emitted light was collected from 660 to 731 nm. The fluorescence of DAPI (Sangon, Shanghai, China) was excited with a 405 nm wavelength laser, and emitted light was collected from 390 to 465 nm. Plasmid construction primers including restriction sites are listed in Table S6.

Microscopic observation of A. thaliana seed traits

Mature seeds of different A. thaliana lines were harvested from major inflorescences, specifically from siliques at the basal region, and then randomly selected to be photographed with an OLYMPUSSZ61 stereomicroscope (Tokyo, Japan) for seed traits, including color, size, and seed coat mucilage and PAs.

The ruthenium red staining of seed coat mucilage was performed as reported before (McFarlane et al., 2014). In brief, dry mature seeds were shaken vigorously in an Ethylene Diamine Tetraacetic Acid (EDTA, 0.05 M, pH
8.5) solution for 1 h and then stained in a 0.01% (w/v) ruthenium red solution for 1 h at room temperature. Subsequently, the ruthenium red solution was removed and replaced with dH₂O.

The DMACA staining of seed coat PAs was conducted as previously described (Abrahams et al., 2002). Dry seeds were stained with the DMACA reagent (2% (w/v) DMACA in 3 M HCl and 50% (w/v) methanol) at room temperature under dark conditions for 16 h, and then washed three times with 70% (v/v) ethanol.

**Determination of seed FAs and storage proteins**

Seeds for FA determination were collected from the basal region of the major inflorescences of 20 individual plants grown in different pots arranged randomly within one of three blocks. Seed FA determination was performed as described previously (Poirier et al., 1999; Chen et al., 2012a). The extraction and methylation of FAs on 300 individual intact seeds were performed in a methanol solution containing 2.5% (v/v) H₂SO₄ at 80 °C for 2 h. After cooling to room temperature, the FA methyl esters were extracted with 2 mL hexane and 2 mL 0.9% (w/v) NaCl, and the organic phase was analyzed by gas chromatography (GC), using methyl heptadecanoate as an internal standard.

The GC-2014 instrument (Shimadzu, Kyoto, Japan) was equipped with a flame ionization detector and a 30 m (length) × 0.25 mm (inner diameter) × 0.5 µm (liquid membrane thickness) column (Supelco wax-10, Supelco, Cat. no. 24079, Schnelldorf, Germany). The initial column temperature was maintained at 160 °C for 1 min, then increased by 4 °C min⁻¹ to 240 °C and held for 16 min at the final temperature. The peaks of each FA composition were identified by their unique retention times, and their concentrations were calculated against the internal control.
Analysis of seed storage proteins was conducted as previously reported (Chen et al., 2015). Briefly, 1 mg of mature dry seeds was homogenized with 50 μL of extraction buffer (100 mM Tris-HCl, pH 8.0, 0.5% (w/v) sodium salt (SDS), 10% (v/v) glycerol, and 2% (v/v) 2-mercaptoethanol) using a microglass pestle and mortar. After boiling for 5 min, the extract was centrifuged at 13,000 rpm for 10 min and then 15 μL of each extract was used for SDS-polyacrylamide gel electrophoresis.

**Quantification of melatonin**

The quantification of melatonin was performed by Shanghai Bioprofile (http://www.bioprofile.cn) and the detailed analysis procedure was provided as follows. The developing siliques (about 100 mg) at 12 DAP were pulverized to powder in a 2 mL Eppendorf tube filled with liquid nitrogen and thoroughly homogenized in 1 mL of 2:2:1 methanol/acetonitrile/H₂O (v/v/v), followed by sonication for 1 h in an ice-water bath. Subsequently, the mixture was incubated at -20 °C for 1 h and centrifuged at 12,000 rpm for 20 min at 4 °C. Then the supernatant was dried under vacuum and resuspended in 100 μL of 1:1 methanol / H₂O (v/v). Following centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant was collected, and 10 μL aliquots were used for melatonin analysis. Analysis was performed using a Shimadzu Nexera LC-30AD UHPLC system with a Waters® ACQUITY UPLC® BEH Amide column (1.7 μm, 2.1 mm x 100 mm) and an AB SCIEX QTRAP 5500 mass spectrometer. The mobile phase consisted of aqueous formic acid (0.1% v/v, solvent A) and acetonitrile (solvent B). Gradient elution started at 20% solvent B. Within 5 min solvent B was increased linearly to 65%, and then increased linearly to 100% over 2 min with a 3 min hold before returning to the starting mixture during 0.1 min and re-equilibrating the column for 2.9 min. In all experiments, the column was heated to 40 °C under a flow rate of 300 μL/min. The instrument mass parameters were set as follows: Source Temperature 550 °C, Ion Source Gas1:
40, Ion Source Gas2: 50, Curtain Gas: 35, Ion Spray Voltage Floating 5500 V, 
scan type: selected reaction monitoring/multiple reaction monitoring
(SRM/MRM). The mass transition from m/z 233.2 to m/z 174.1 was identified as 
melatonin; the retention time was 3.48 min. AB SCIEX Analyst software
(version 1.5.2) was used for data integration.

**Measurement of seed anthocyanins and PAs**

The anthocyanin content was measured as previously described (Li et al.,
2018c), with some modifications. Briefly, about 5 mg mature seeds were frozen 
in liquid nitrogen and ground in 3 mL buffer consisting of 1% (v/v) HCl in 
methanol. The mixtures were centrifuged at 12,000 rpm for 5 min after 
incubation at 70 °C for 1 h. Then the supernatant was taken and extracted with 
an equal volume of chloroform after adding 2 mL of distilled water. After 
centrifuging at 12,000 rpm for 5 min, the absorbance at 535 nm was determined 
using a Beckman-Coulter DU730 spectrophotometer, and then normalized to 
the total weight of dry seeds for each sample, which is regarded as the 
anthocyanin amount.

Extraction of PAs and acid hydrolysis were performed as previously 
described (Kitamura et al., 2010), with some modifications. Briefly, about 10 mg 
mature seeds were frozen in liquid nitrogen and ground in 1.5 mL 70% (v/v) 
acetone containing 5.26 mM Na$_2$S$_2$O$_5$, followed by sonication for 20 min at 
room temperature. Following centrifugation at 1,500 rpm for 15 min, the 
supernatant was dried and resuspended in 0.4 mL 70% (v/v) acetone 
containing 5.26 mM Na$_2$S$_2$O$_5$ and 1.6 mL HCl/butanol (1:5 v/v). The 
absorbance at 545 nm of this resuspended solution was determined using 
Tecan’s Infinite® M200 PRO. After hydrolysis at 95 °C for 1 h, the absorbance 
at 545 nm was once again determined. Subtraction of the first absorbance 
value from the second followed by weight normalization was defined as the 
content of soluble PAs. The residues were dried by evaporation, and then 2 mL
2:10:3 of HCl:butanol:70% (v/v) acetone was added. After heating at 95 °C for 1 h, the extract was centrifuged for 15 min. The absorbance at 545 nm of the supernatant was measured and then normalized to the weight as the content of insoluble PAs.

RNA-sequencing and data analyses

The flowers of wild type (Col-0) and snat1-1 comt-1 plants tagged with different colored threads indicate DAP. Developing seeds at 12 DAP were taken from the basal region of the major inflorescences of 50 individual plants for each genotype in one biological replicate. These seeds were grown in different pots arranged randomly and were used for the RNA-sequencing (RNA-seq) experiments. Three independent biological replicates from three different plantings were carried out for wild type and snat-1 comt-1 in the RNA-seq experiment. The following analysis was performed using the services of Gene Denovo (http://www.genedenovo.com/) following the standard protocol (http://www.genedenovo.com/product/41.html). The Excel add-in for significance analysis of RNA-seq was utilized to identify differentially expressed genes (DEGs) between wild type and snat1-1 comt-1. The DEGs with $|\log_2$ ratios| ≥ 0.58 and false discovery rate (FDR) ≤ 0.05 are listed in Tables S3 and S4.

Analysis of gene expression

The sampling of developing seeds used for gene expression was the same as that described for the RNA-seq experiment. Other tissues were harvested from at least eight individual plants grown in different pots arranged randomly, and three independent biological replicates from three different plantings were conducted for the expression analysis. Total RNA from various tissues was extracted using the MiniBEST Plant RNA Extraction Kit (TaKaRa) and reverse transcribed using PrimeScript RT (TaKaRa). RT-qPCR was performed in three biological replicates using SYBR Green Master Mix (TaKaRa). GUS staining
was performed as described previously (Jefferson et al., 1987). Primers used for gene expression analyses are listed in Table S1.

**ACCESSION NUMBERS**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: SNAT1 (AT1G32070), COMT (AT5G54160), WRI1 (AT3G54320), BCCP1 (AT5G16390), CAC3 (AT2G38040), MCAMT (AT2G30200), PLIP1 (AT3G61680), LTP3 (AT5G59320), KFB39 (AT2G44130), 4CL1 (AT1G51680), CHI (AT3G55120), UGT73B2 (AT4G34135), KAN4 (AT5G42630), and GPT2 (AT1G61800).

**LIST OF SUPPLEMENTAL DATA**

- **Supplemental Figure S1** Molecular verification of the *snat1-1* mutation.
- **Supplemental Figure S2** Reverse transcription PCR identification of rescued lines of *snat1-1 gSNAT1#1* and *comt-1 gCOMT#1*.
- **Supplemental Figure S3** Comparison of seed traits in various lines.
- **Supplemental Figure S4** Analysis of storage proteins between wild-type (Col-0) and *snat1-1 comt-1* mature seeds.
- **Supplemental Figure S5** Analysis of seed coat mucilage layer in wild type (Col-0) mature seeds exogenously treated with different concentrations of melatonin solutions (0, 100, 200, and 500 μM).
- **Supplemental Table S1** Comparison of FA composition and total FA content (μg/mg seed DW) in mature seeds of various lines in this study.
- **Supplemental Table S2** Comparison of anthocyanin content (A535/g DW) in mature seeds of various lines in this study.
Supplemental Table S3 List of up-regulated genes in developing seeds of snat1-1 comt-1 plants at 12 days after pollination.

Supplemental Table S4 List of down-regulated genes in developing seeds of snat1-1 comt-1 plants at 12 days after pollination.

Supplemental Table S5 List of transcription factors and structural genes contributing to the accumulation of oil, flavonoids, mucilage, and storage proteins whose expressions were not altered in developing seeds of snat1-1 comt-1 plants at 12 days after pollination.

Supplemental Table S6 Primers used in this study.

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Table 1. Differentially expressed genes (DEGs) important for seed oil accumulation in the developing seeds of snat1-1 comt-1 plants at 12 days after pollination (DAP). DEGs with $|\log_2 \text{ratios}| \geq 0.58$, and only GO Slim IDs with FDR $\leq 0.05$, are listed here.

| DEGs     | log2 ratios | Functions                                                                 | References                                                                                       |
|----------|-------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| *WRI1* (AT3G54320) | 0.69        | Promoting oil accumulation                                               | (Focks and Benning, 1998; Cernac and Benning, 2004; Masaki et al., 2005; Baud et al., 2007; Maeo et al., 2009; To et al., 2012) |
| *BCCP1* (AT2G38040) | 0.63        | Promoting oil accumulation                                               | (Ohlrogge et al., 1995; Sasaki et al., 1995; Mu et al., 2008; Li et al., 2011)                 |
| *CAC3* (AT2G38040) | 0.66        | Promoting oil accumulation                                               | (Ohlrogge et al., 1995; Sasaki et al., 1995; Mu et al., 2008; Li et al., 2011)                 |
| *MCAMT* (AT2G30200) | 0.60        | Promoting oil accumulation                                               | (Jung et al., 2019; Mu et al., 2008)                                                          |
| *PLIP1* (AT3G61680) | 0.65        | Promoting oil accumulation                                               | (Wang et al., 2017b)                                                                           |
| *GPAT2* (AT1G02390) | 0.96        | Exhibiting sn-1 and sn-2 acyltransferase activities and utilizing dicarboxylic acyl-CoA as the substrate for the biosynthesis of the extracellular lipids; Promoting soluble sugar accumulation; Enhancing the *in vitro* transfer of phospholipids between membranes and binding acyl chains; No obvious effect on oil accumulation in the single mutant | (Beisson et al., 2007; Yang et al., 2012; Jayawardhane et al., 2018)                          |
| *LTP3* (AT5G59320) | 2.42        | Enhancing the *in vitro* transfer of phospholipids between membranes and binding acyl chains | (Kader, 1996; Arondel et al., 2000; Pagnussat et al., 2015; Wong et al., 2017)              |
| *LTP4* (AT5G59310) | 1.19        | Enhancing the *in vitro* transfer of phospholipids between membranes and binding acyl chains | (Kader, 1996; Arondel et al., 2000; Wong et al., 2017)                                       |
| *LTP5* | 1.06        | Enhancing the *in vitro* transfer of phospholipids between membranes and binding acyl chains | (Kader, 1996; Arondel et al., 2000; Wong et al., 2017)                                       |
Table 2. Differentially expressed genes (DEGs) contributing to anthocyanin biosynthesis in the developing seeds of *snat1-1 comt-1* plants at 12 days after pollination (DAP). DEGs with \(|\log_2\text{ratios}| \geq 0.58\), and only GO Slim IDs with FDR \( \leq 0.05\), are listed here.

| DEGs         | log\(_2\) ratios | Functions                                                      | References                                      |
|--------------|-------------------|----------------------------------------------------------------|-------------------------------------------------|
| *KFB39* (AT2G44130) | -1.90             | Repressing anthocyanin accumulation                             | (Zhang et al., 2015)                            |
| *4CL1* (AT1G51680)     | 0.65              | Promoting anthocyanin accumulation                              | (Li et al., 2015)                               |
| *CHI* (AT3G55120)       | 0.62              | Promoting anthocyanin accumulation                              | (Shirley et al., 1992; Pourcel et al., 2013)    |
| *UGT73B2* (AT4G34135)   | 2.72              | Transferring a glucose group to the 3-hydroxyl group of flavonoids | (Kim et al., 2006; Lim et al., 2006)             |
| *KAN4* (AT5G42630)      | 1.56              | Promoting flavonoid accumulation                                | (Gao et al., 2010)                              |
| GPT2 (AT1G61800) | 1.76 | Promoting anthocyanin accumulation | (Jeong et al., 2018) |
FIGURE LEGENDS

**Figure 1** Subcellular localization of the SNAT1 and COMT proteins in *N. benthamiana* leaves. Subcellular distribution of the SNAT1 (A) and COMT (B) proteins fused with GFP (*35S:SNAT1-GFP* or *35S:COMT-GFP*). DAPI, fluorescence of 4', 6-diamino-2-phenylindole; Merge 1, merge of GFP, DAPI, and bright-field images; Merge 2, merge of chlorophyll, GFP, DAPI, and bright-field images. GFP, green fluorescent protein.

**Figure 2** Tissue-specific analyses of *SNAT1* and *COMT* expression patterns. A and D, RT-qPCR analysis of *SNAT1* (A) and *COMT* (D) expression in various tissues of wild-type (Col-0) plants. Rt, Roots; St, stems; RL, rosette leaves; CL, cauline leaves; FB, flower buds; OF, open flowers. Values are means ± SD (n = 3). B and E, RT-qPCR analysis of *SNAT1* (B) and *COMT* (E) expressions in developing seeds of wild type (Col-0) plants. Values are means ± SD (n = 3). C and F, Representative GUS staining of *pSNAT1:GUS* (C) and *pCOMT:GUS* (F) transgenic plants show *SNAT1* and *COMT* expression levels, respectively, in vegetative and reproductive tissues in wild-type (Col-0) plants. C, Upper panel photos successively (from left to right) indicate 9-day-old seedlings (C1), rosette leaves (C2), stems and cauline leaves (C3), and flower buds and open flowers (C4). F, Upper panel photos successively (from left to right) indicate 8-day-old seedlings (F1), rosette leaves (F2), stems, cauline leaves, and flower buds (F3), and open flowers (F4). Bottom panel photos successively (C and F, from left to right) represent developing seeds at different developmental stages (C5-8 and F5-8). The RT-qPCR results were normalized against the expression of *EF1αA4* as an internal control. Bars = 2 mm, except for seeds, where the bars represent 100 μm. RT-qPCR, reverse transcription quantitative PCR.

**Figure 3** Melatonin quantification in developing siliques from various lines of *SNAT1* and *COMT*. A, Structure of the *COMT* (AT5G54160) gene showing the position of T-DNA insertions in SALK_002373 (*comt-1*) and SALK_020611C
(comt-2) mutants. The coding and untranslated regions are represented by black and gray boxes, respectively, and introns and other genomic regions are represented by open boxes. Translation start site (ATG) and stop codon (TAA) are indicated. The arrow indicates the left border of the T-DNA. B, PCR-based DNA genotyping of the homozygous mutants of the COMT gene. LP and RP refer to the gene specific primers and BP refers to T-DNA right-border primer. Three independent biological replicates were carried out. C, Reverse transcription PCR analysis of COMT transcript in wild type (Col-0) and their corresponding mutants. EF1aA4 was used as an internal control. Three independent biological replicates were conducted. D, Melatonin levels in the developing siliques at 12 days after pollination from wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Values are means ± SD (n = 3). Different lowercase letters within various lines of the SNAT1 and COMT genes indicate significant differences at P ≤ 0.05 (Tukey’s highly significant difference test). FW, fresh weight. **Figure 4** Effect of endogenous deficiency and exogenous application of melatonin on seed FA and anthocyanin accumulation. A and C, Total FA (A) and anthocyanin (C) contents in seeds from wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Different lowercase letters within various lines of the SNAT1 and COMT genes indicate significant differences at P ≤ 0.05 (Tukey’s highly significant difference test). B and D, Total FA (B) and anthocyanin (D) contents in seeds of wild type (Col-0), snat1-1, comt-1, and snat1-1 comt-1 exogenously applied with different concentrations of melatonin solutions (0, 100, and 200 μM). Different letters within each treatment indicate significant differences at P ≤ 0.05 (Tukey’s highly significant difference test); lowercase letters compare with each other, capital
letters compare with each other, and Greek letters compare with each other.

Asterisks denote statistically significant differences between the indicated samples (Student’s $t$-test, $P \leq 0.05$). In A–D: Values are means ± SD ($n = 5$).

FA, fatty acid. DW, dry weight. A535, absorbance at 535 nm.

**Figure 5** Dynamic expression analysis of genes related to seed oil accumulation in developing seeds of wild-type (Col-0) and snat1-1 comt-1 plants. Gene expression was normalized against the expression of $EF1\alpha A4$ as an internal control, and the expression level in wild type was set to 1 (dotted line). Values are means ± SD ($n = 3$). Asterisks indicate significant differences in gene expression levels in snat1 comt-1 plants compared with those in wild-type plants (two-tailed paired Student’s $t$-test, $P \leq 0.05$).

**Figure 6** Dynamic expression analysis of genes contributing to seed anthocyanin accumulation in developing seeds of wild-type (Col-0) and snat1-1 comt-1 plants. Gene expression was normalized against the expression of $EF1\alpha A4$ as an internal control, and the expression level in wild type was set to 1 (dotted line). Values are means ± SD ($n = 3$). Asterisks indicate significant differences in gene expression levels in snat1-1 comt-1 plants compared with those in wild-type plants (two-tailed paired Student’s $t$-test, $P \leq 0.05$).

**Figure 7** Effect of SNAT1 and COMT on seed coat mucilage deposition. A, Comparison of the mucilage layer attached to the seed coat among wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Bars = 500 μm. B, Comparison of the dynamic expression of DF1 and MUM4 in developing seeds from 8 to 12 days after pollination among wild type (Col-0), the single mutant snat1-1, and the transgenic plant snat1-1 gSNAT1#1. C, Comparison of the dynamic expression of DF1 and MUM4 in developing seeds from 8 to 12 days after pollination among wild type (Col-0), the single mutant comt-1, and the transgenic plant comt-1 gCOMT#1. Gene
expression was normalized against the expression of EF1αA4 as an internal control, and the expression level in wild type was set to 1. In B and C: Values are means ± SD (n = 3). Asterisks indicate significant differences in gene expression levels in snat1-1 or comt-1 plants compared with those in wild-type plants (two-tailed paired Student’s t-test, \( P \leq 0.05 \)).

**Figure 8** Effect of SNAT1 and COMT on the accumulation of PAs in seeds. A, Seeds stained with DMACA for 16 h among wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Bars = 500 μm. B and C, Analysis of soluble (B) and insoluble (C) PAs by acidic hydrolysis among wild type (Col-0), single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. In B and C: Values are means ± SD (n = 5). Different letters within various lines represent significant differences at \( P \leq 0.05 \) (Tukey’s highly significant difference test). PAs, proanthocyanidins. DW, dry weight.

**Figure 9** A proposed working model shows that the deficiency of melatonin by knocking out SNAT1 and/or COMT represses the accumulation of oil and anthocyanins by regulating the expression of key genes that control the biosynthesis of oil and anthocyanins, respectively, in *A. thaliana* seeds. Arrows and T bars indicate promoting and inhibitory effects, respectively.

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Figure 1 Subcellular localization of the SNAT1 and COMT proteins in *N. benthamiana* leaves. Subcellular distribution of the SNAT1 (A) and COMT (B) proteins fused with GFP (*35S:SNAT1-GFP* or *35S:COMT-GFP*). DAPI, fluorescence of 4’, 6-diamino-2-phenylindole; Merge 1, merge of GFP, DAPI, and bright-field images; Merge 2, merge of chlorophyll, GFP, DAPI, and bright-field images. GFP, green fluorescent protein.
Figure 2 Tissue-specific analyses of SNAT1 and COMT expression patterns. A and D, RT-qPCR analysis of SNAT1 (A) and COMT (D) expression in various tissues of wild-type (Col-0) plants. Rt, Roots; St, stems; RL, rosette leaves; CL, cauline leaves; FB, flower buds; OF, open flowers. Values are means ± SD (n = 3). B and E, RT-qPCR analysis of SNAT1 (B) and COMT (E) expressions in developing seeds of wild type (Col-0) plants. Values are means ± SD (n = 3). C and F, Representative GUS staining of pSNAT1:GUS (C) and pCOMT:GUS (F) transgenic plants show SNAT1 and COMT expression levels, respectively, in vegetative and reproductive tissues in wild-type (Col-0) plants. C, Upper panel photos successively (from left to right) indicate 9-day-old seedlings (C1), rosette leaves (C2), stems and cauline leaves (C3), and flower buds and open flowers (C4). F, Upper panel photos successively (from left to right) indicate 8-day-old seedlings (F1), rosette leaves (F2), stems, cauline leaves, and flower buds (F3), and open flowers (F4). Bottom panel photos successively (C and F, from left to right) represent developing seeds at different developmental stages (C5-8 and F5-8). The RT-qPCR results were normalized against the expression of EF1aA4 as an internal control. Bars = 2 mm, except for seeds, where the bars represent 100 μm. RT-qPCR, reverse transcription quantitative PCR.
Figure 3 Melatonin quantification in developing siliques from various lines of SNAT1 and COMT. A, Structure of the COMT (AT5G54160) gene showing the position of T-DNA insertions in SALK_002373 (comt-1) and SALK_020611C (comt-2) mutants. The coding and untranslated regions are represented by black and gray boxes, respectively, and introns and other genomic regions are represented by open boxes. Translation start site (ATG) and stop codon (TAA) are indicated. The arrow indicates the left border of the T-DNA. B, PCR-based DNA genotyping of the homozygous mutants of the COMT gene. LP and RP refer to the gene specific primers and BP refers to T-DNA right-border primer. Three independent biological replicates were carried out. C, Reverse transcription PCR analysis of COMT transcript in wild type (Col-0) and their corresponding mutants. EF1αA4 was used as an internal control. Three independent biological replicates were conducted. D, Melatonin levels in the developing siliques at 12 days after pollination from wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and
the transgenic plants of *snat1-1 gSNAT1#1* and *comt-1 gCOMT#1*. Values are means ± SD (n = 3). Different lowercase letters within various lines of the *SNAT1* and *COMT* genes indicate significant differences at $P \leq 0.05$ (Tukey’s highly significant difference test). FW, fresh weight.
Figure 4 Effect of endogenous deficiency and exogenous application of melatonin on seed FA and anthocyanin accumulation. A and C, Total FA (A) and anthocyanin (C) contents in seeds from wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Different lowercase letters within various lines of the SNAT1 and COMT genes indicate significant differences at $P \leq 0.05$ (Tukey’s highly significant difference test). B and D, Total FA (B) and anthocyanin (D) contents in seeds of wild type (Col-0), snat1-1, comt-1, and snat1-1 comt-1 exogenously applied with different concentrations of melatonin solutions (0, 100, and 200 μM). Different letters within each treatment indicate significant differences at $P \leq 0.05$ (Tukey’s highly significant difference test); lowercase letters compare with each other, capital letters compare with each other, and Greek letters compare with each other. Asterisks denote statistically significant differences between the indicated samples (Student’s $t$-test, $P \leq 0.05$). Values are means ± SD (n = 5). FA, fatty acid. DW, dry weight. A535, absorbance at 535 nm.
Figure 5 Dynamic expression analysis of genes related to seed oil accumulation in developing seeds of wild-type (Col-0) and snat1-1 comt-1 plants. Gene expression was normalized against the expression of EF1αA4 as an internal control, and the expression level in wild type was set to 1. Values are means ± SD (n = 3). Asterisks indicate significant differences in gene expression levels in snat1 comt-1 plants compared with those in wild-type plants (two-tailed paired Student's t-test, $P \leq 0.05$).
Figure 6 Dynamic expression analysis of genes contributing to seed anthocyanin accumulation in developing seeds of wild-type (Col-0) and snat1-1 comt-1 plants. Gene expression was normalized against the expression of EF1αA4 as an internal control, and the expression level in wild type was set to 1. Values are means ± SD (n = 3). Asterisks indicate significant differences in gene expression levels in snat1-1 comt-1 plants compared with those in wild-type plants (two-tailed paired Student’s t-test, \( P \leq 0.05 \)).
Figure 7 Effect of SNAT1 and COMT on seed coat mucilage deposition. A, Comparison of the mucilage layer attached to the seed coat among wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Bar = 500 μm. B, Comparison of the dynamic expression of DF1 and MUM4 in developing seeds from 8 to 12 days after pollination among wild type (Col-0), the single mutant snat1-1, and the transgenic plant snat1-1 gSNAT1#1. C, Comparison of the dynamic expression of DF1 and MUM4 in developing seeds from 8 to 12 days after pollination among wild type (Col-0), the single mutant comt-1, and the transgenic plant comt-1 gCOMT#1. Gene expression was normalized against the expression of EF1αA4 as an internal control, and the expression level in wild type was set to 1. Values are means ± SD (n = 3). Asterisks indicate significant differences in gene expression levels in snat1-1 or comt-1 plants compared with those in wild-type plants (two-tailed paired Student’s t-test, P ≤ 0.05).
Figure 8 Effect of SNAT1 and COMT on the accumulation of PAs in seeds. A, Seeds stained with DMACA for 16 h among wild type (Col-0), the single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Bar = 500 μm. B and C, Analysis of soluble (B) and insoluble (C) PAs by acidic hydrolysis among wild type (Col-0), single mutants of snat1-1, comt-1, and comt-2, the double mutant snat1-1 comt-1, and the transgenic plants of snat1-1 gSNAT1#1 and comt-1 gCOMT#1. Values are means ± SD (n = 5). Different letters within various lines represent significant differences at $P \leq 0.05$ (Tukey’s highly significant difference test). PAs, proanthocyanidins. DW, dry weight.
Figure 9 A proposed working model shows that the deficiency of melatonin by knocking out SNAT1 and/or COMT represses the accumulation of oil and anthocyanins by regulating the expression of key genes that control the biosynthesis of oil and anthocyanins, respectively, in A. thaliana seeds. Arrows and T bars indicate promoting and inhibitory effects, respectively.
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