Melatonin Represses Oil and Anthocyanin Accumulation in Seeds[1][OPEN]

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Previous studies have clearly demonstrated that the putative phytohormone melatonin functions directly in many aspects of plant growth and development. In Arabidopsis (*Arabidopsis thaliana*), the role of melatonin in seed oil and anthocyanin accumulation, and corresponding underlying mechanisms, remain unclear. Here, we found that *serotonin N-acetyltransferase1* (*SNAT1*) and *caffeic acid O-methyltransferase* (*COMT*) genes were ubiquitously and highly expressed and essential for melatonin biosynthesis in Arabidopsis 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.

Arabidopsis (*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 (*Homo sapiens*) 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

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D.L., H.M., and M.C. conceived the original screening and research plans; M.C. and S.W.H. supervised the experiments; D.L. performed most of the experiments; D.Z., S.C.H., and J.G. provided technical assistance to D.L.; D.L., Y.G., and M.C. designed the experiments and analyzed the data; M.C. conceived the project and wrote the article with contributions of all the authors; X.G, Z.W., L.J., and X.D. supervised and complemented the writing.

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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 Arabidopsis 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.

N-acetyl-5-methoxytryptamine (Melatonin), a highly conserved bioactive molecule, is ubiquitously present in all plant species (Tan et al., 2012, 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 by caffeic acid *O*-methyltransferase (COMT), which are subsequently metabolized into melatonin by COMT or SNAT, respectively (Lee et al., 2014b). There are two *SNAT* isogenes, *SNAT1* and *SNAT2* (Back et al., 2016; Lee et al., 2019), and a single copy of *COMT* in the Arabidopsis 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., 2017b; Zheng et al., 2017), heavy metals (Cai et al., 2017; Gu et al., 2017; Kobylinska et al., 2017; Lee and Back, 2017b; Zhang et al., 2017b; Luo et al., 2018; Nawaz et al., 2018), high nitrate levels (Zhang et al., 2017c), K^+ deficiency (Chen et al., 2017a), drought (Antoniou et al., 2017; Wang et al., 2017b), high pH (Gong et al., 2017), cold (Bajwa et al., 2014; Li et al., 2017b, 2018c), high temperature (Xu et al., 2016; Zhang et al., 2017a; Qi et al., 2018), and various pathogens (Yin et al., 2013; Lee et al., 2014a; Lee and Back, 2016, 2017a; Wei et al., 2017).

Melatonin has also been demonstrated to be involved in other aspects of plant growth and development, such as root development (Hernández-Ruiz et al., 2005; Arnao and Hernández-Ruiz, 2007; Chen et al., 2009; Wang et al., 2016), cotyledon and seedling growth (Hernández-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 Arabidopsis, 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 (*Glycine max*) seeds (Wei et al., 2015), and increases anthocyanin biosynthesis in cabbage (*Brassica oleracea*) 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 Arabidopsis.

In this study, we demonstrated that melatonin functions as a negative regulatory signal for seed oil and anthocyanin accumulation during the maturation of Arabidopsis 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 (Fig. 1A) and cytoplasm (Fig. 1B), respectively, which is consistent with a previous study (Lee et al., 2014b). We also found that the green fluorescence of COMT-GFP was colocalized with that of the nuclear marker DAPI, indicating that COMT was also localized in the nucleus (Fig. 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 (Fig. 2, A and 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 (Fig. 2, D and E). During seed development, the expression of *SNAT1* and *COMT* exhibited a similar pattern and increased rapidly from 8 d after pollination (DAP) to the maximal level at 10 DAP, and then decreased gradually afterward (Fig. 2, B and 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 (Fig. 2C, C1), cotyledons, rosette and cauline leaves (Fig. 2C, C1–C3), flower buds and open flowers (Fig. 2C, C4), and developing seeds, including embryos and seed coat during seed maturation (Fig. 2C, C5–C8). The *COMT* transcript level was predominant in the tissues of roots (Supplemental Fig. 2F1), cotyledons and just-emerged true leaves (Fig. 2F, F1), flower buds and open flowers (Fig. 2F, F3 and F4), and developing seeds inclusive of embryos and seed coat during seed maturation (Fig. 2F, F5–F8). The expression of *SNAT1* was much higher than that of *COMT* in the seed coat (Fig. 2C, C5–C8, and 2F, F5–F8). However, GUS staining was hardly observed in stems

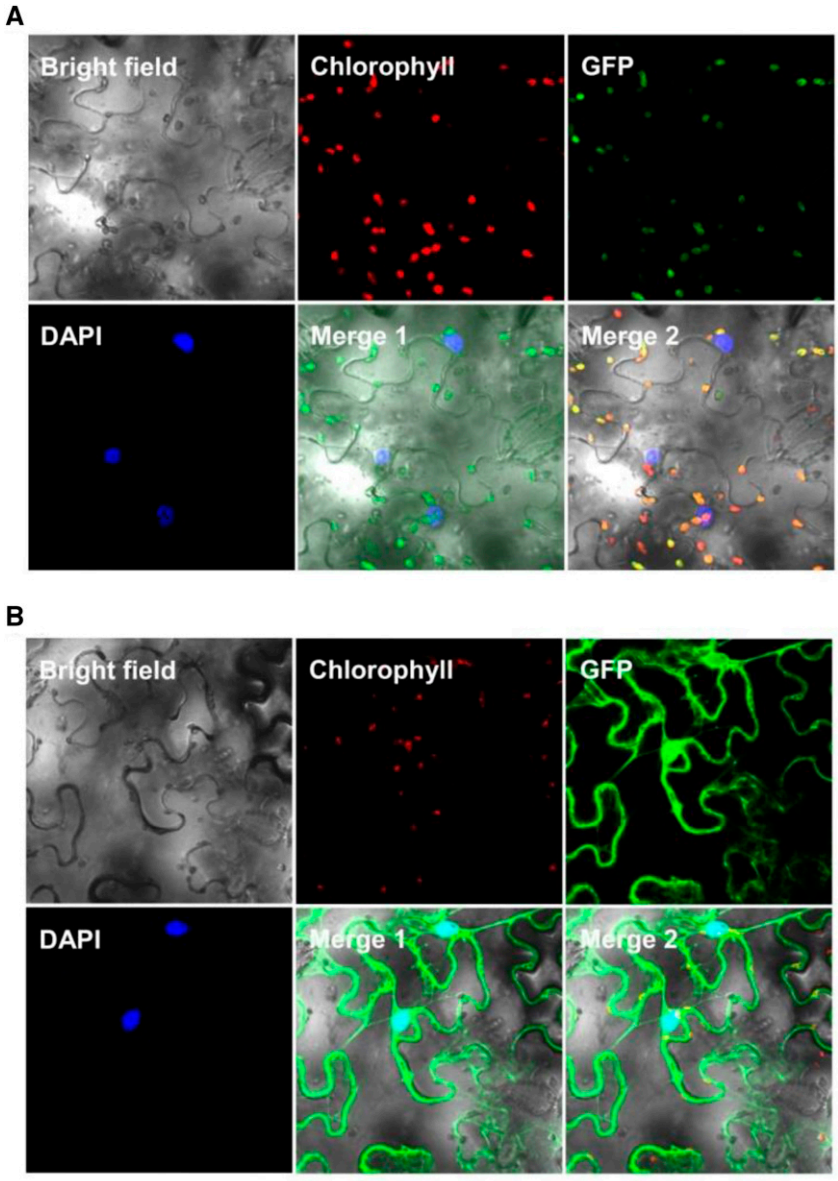

Figure 1. Subcellular localization of the SNAT1 and COMT proteins in *N. benthamiana* leaves. Subcellular distribution of the SNAT1 (A) and COMT (B) proteins fused with GFP (*35S:SNAT1-GFP* or *35S:COMT-GFP*). DAPI, Fluorescence of 4′, 6-diamino-2-phenylindole; Merge 1, merge of GFP, DAPI, and bright-field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

of the *pSNAT1:GUS* line (Fig. 2C, C3), as well as in rosette leaves (Fig. 2F, F2), cauline leaves (Fig. 2F, F3), and stems (Fig. 2F, F3) 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 (Fig. 3C; Supplemental Fig. S1), confirming that they are loss-of-function mutants. The transferred DNA (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 (Fig. 3A). The results of PCR-based genotyping (Fig. 3B) indicated the presence of the homozygous *comt-2* mutant, which completely lacks the *COMT* transcript, as determined by RT-PCR (Fig. 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*

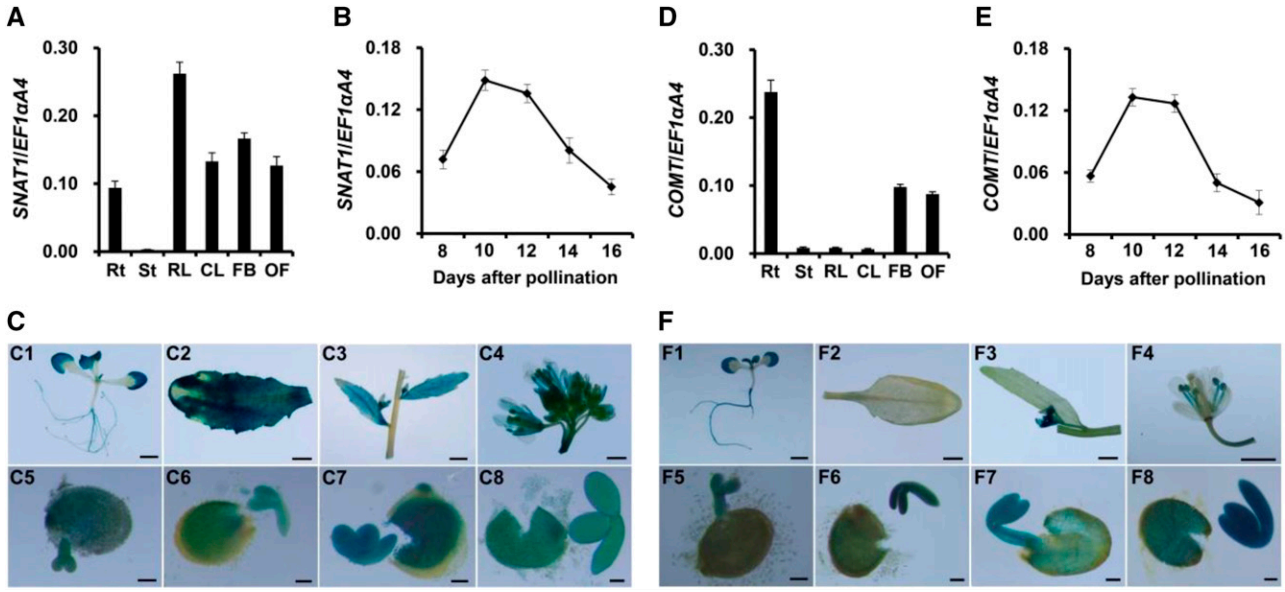

Figure 2. Tissue-specific analyses of *SNAT1* and *COMT* expression patterns. A and D, RT-qPCR analysis of *SNAT1* (A) and *COMT* (D) expression in various tissues of wild-type (Col-0) plants. Rt, Roots; St, stems; RL, rosette leaves; CL, cauline leaves; FB, flower buds; OF, open flowers. Values are 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 photos successively (from left to right) indicate 9-d–old seedlings (C1), rosette leaves (C2), stems and cauline leaves (C3), and flower buds and open flowers (C4), and lower photos successively (from left to right) represent developing seeds at different developmental stages (C5–C8). F, Upper photos successively (from left to right) indicate 8-d–old seedlings (F1), rosette leaves (F2), stems, cauline leaves, and flower buds (F3), and open flowers (F4), and lower photos successively (from left to right) represent developing seeds at different developmental stages (F5–F8). The RT-qPCR results were normalized against the expression of *EF1αA4* as an internal control. Scale bars = 2 mm (C1–C4 and F1–F4), except for seeds, where scale bars = 100 μm (C5–C8 and F5–F8).

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 (Fig. 3D), which is consistent with the fact that another *SNAT* isogene (*SNAT2*) is present in the Arabidopsis genome (Back et al., 2016; Lee et al., 2019). These results suggested that SNAT1 and COMT additively promote melatonin biosynthesis in Arabidopsis 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 Supplemental Table S1, the seed FA contents in all three single mutants of *snat1-1*, *comt-1*, and *comt-2* were ~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 (Fig. 4A; Supplemental Table S1). These results indicated that SNAT1 and COMT have an additive effect in the repression of FA accumulation in Arabidopsis 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 (Fig. 4C; Supplemental 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* (Fig. 4C; Supplemental Table S2). These results suggested that SNAT1 and COMT have a nonadditive 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

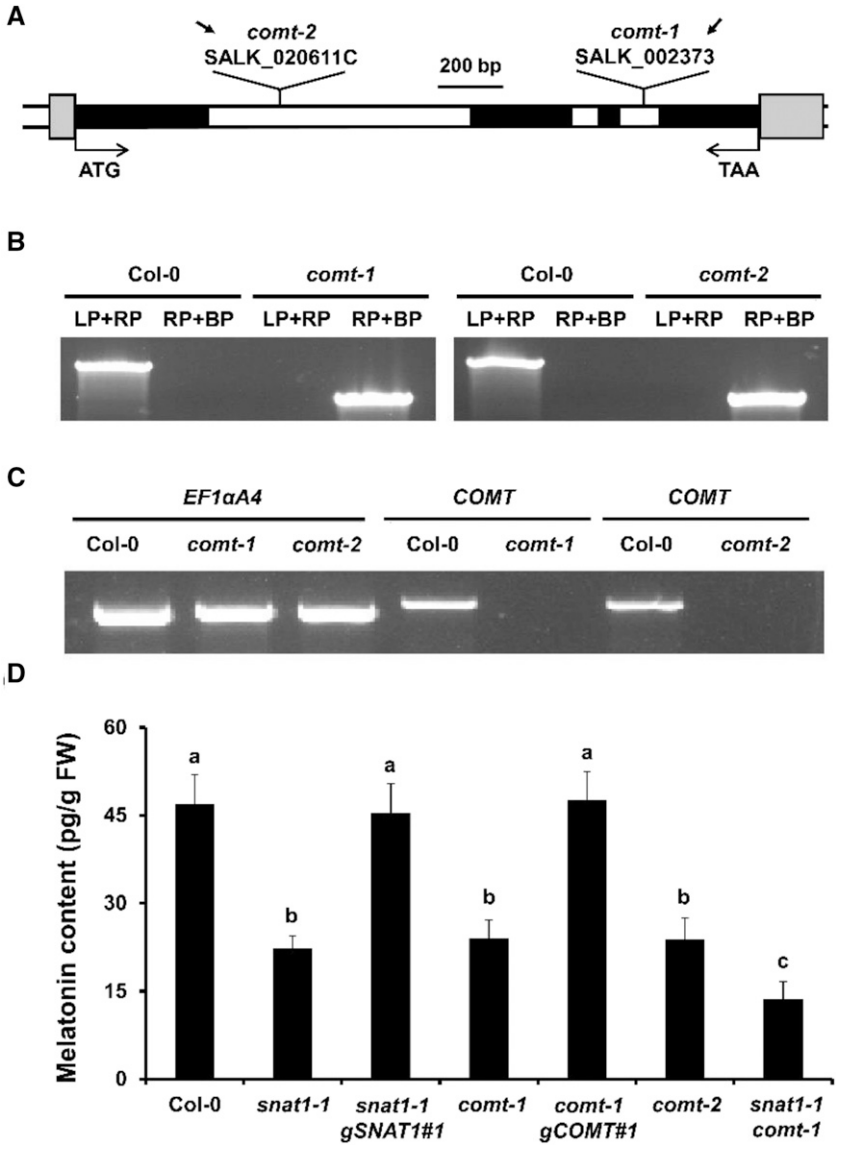

Figure 3. Melatonin quantification in developing siliques from various lines of *SNAT1* and *COMT*. A, Structure of the *COMT* (AT5G54160) gene showing the position of T-DNA insertions in SALK_002373 (*comt-1*) and SALK_020611C (*comt-2*) mutants. The coding and untranslated regions are represented by 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, RT-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 DAP 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 honest significant difference test). FW, Fresh weight.

SNAT1 and *COMT* were restored to wild-type levels (Supplemental Fig. S2), and the lower melatonin content in both *snat1-1* and *comt-1* was also fully rescued to wild-type levels (Fig. 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 (Fig. 4, A and C). These results implied that SNAT1 and COMT indeed inhibit the accumulation of seed oil and anthocyanins in Arabidopsis.

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 (Fig. 4B; Supplemental Table S1) and anthocyanin (Fig. 4D; Supplemental 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 (Fig. 4B; Supplemental 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 (Fig. 4B; Supplemental 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 Arabidopsis seeds.

In addition, under exogenous melatonin treatment, the anthocyanin content in *snat1-1* seeds was the same as that of wild-type plants (Fig. 4D; Supplemental 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 (Fig. 4D; Supplemental

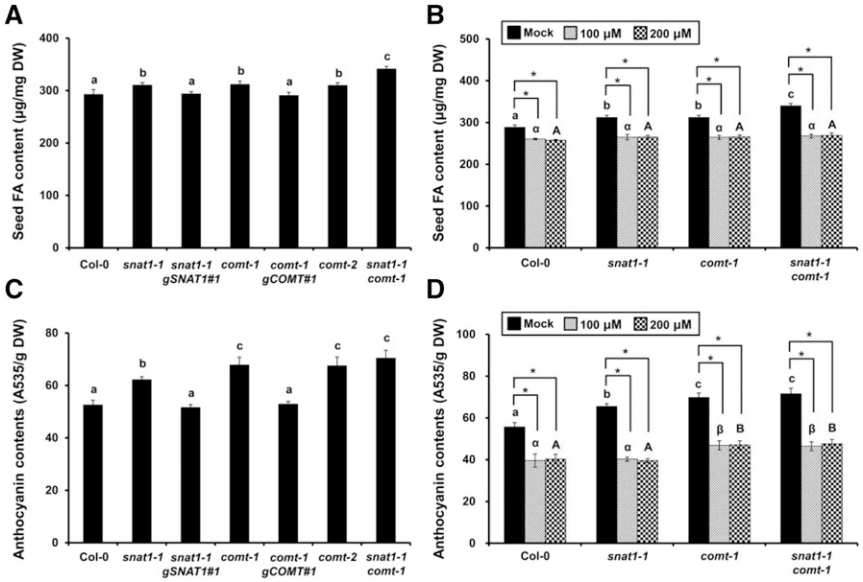

Figure 4. Effect of endogenous deficiency and exogenous application of melatonin on seed FA and anthocyanin accumulation. A and C, Total FA (A) and anthocyanin (C) contents in seeds from wild type (Col-0); the single mutants of *snat1-1*, *comt-1*, and *comt-2*; the double mutant *snat1-1 comt-1*; and 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 honest significant difference test). B and D, Total FA (B) and anthocyanin (D) contents in seeds of wild type (Col-0), and *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 honest 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, $P \leq 0.05$). A to D, Values are means ± SD ($n = 5$). DW, Dry weight.

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 (Supplemental Fig. 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 Arabidopsis seeds.

Genome-Wide Analysis of DEGs in Developing Seeds at 12 DAP between Wild-Type and *snat1-1 comt-1* Plants

In Arabidopsis 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* (Fig. 4A; Supplemental 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 Arabidopsis seeds.

RNA-sequencing (RNA-seq) analysis identified 243 differentially expressed genes (DEGs), among which 119 were upregulated (Supplemental Table S3) and 124 were downregulated (Supplemental 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 (Supplemental 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 (Supplemental Table S5). Up to nine (7.6%) upregulated genes and no downregulated genes were related to carbohydrate metabolism (Supplemental Tables S3 and S4). Multiple upregulated (16; 13.4%) and downregulated (30; 24.2%) genes were involved in general protein

metabolism in *snat1-1 comt-1* seeds (Supplemental Tables S3 and S4). The storage proteins mainly contain legumin-type 12S globulins and napin-type 2S albumins in Arabidopsis 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 (*2S1–2S5*), between wild-type and *snat1-1 comt1-1* seeds (Supplemental Table S5). Consistently, there was no substantive difference in the content of seed storage proteins between wild-type and *snat1-1 comt1-1* plants (Supplemental Fig. 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 (Supplemental 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 upregulated 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 COA-ACP MALONYL-TRANSFERASE* (*MCAMT*), *PLASTID LIPASE1* (*PLIP1*), and *LIPID TRANSFER PROTEIN3* (*LTP3*), in *snat1-1 comt-1* developing seeds at 12 DAP (Fig. 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 (Fig. 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 declined afterward 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*, *UDP-GLUCOSYL-TRANSFERASE73B2* (*UGT73B2*), and *GLC-6-PHOSPHATE/PHOSPHATE TRANSLOCATOR2* (*GPT2*), in *snat1-1 comt-1* developing seeds at 12 DAP (Fig. 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 (Fig. 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 (Fig. 6). The relative expression levels of *4CL1* and *CHI* increased from 12 DAP to the peaks at 14 DAP and then decreased

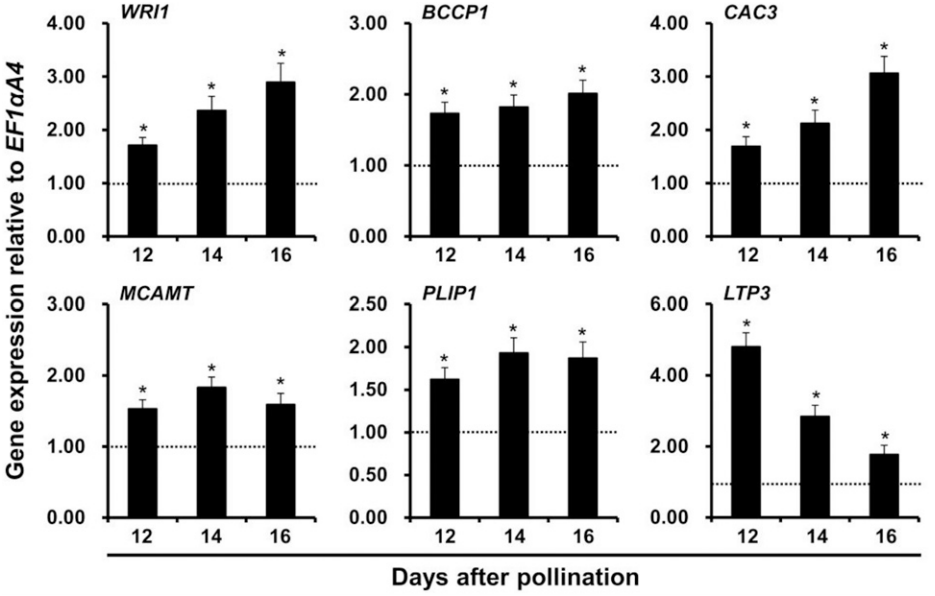

Figure 5. Dynamic expression analysis of genes related to seed oil accumulation in developing seeds of wild-type (Col-0) and *snat1-1 comt-1* plants. Gene expression was normalized against the expression of *EF1αA4* as an internal control, and the expression level in wild type was set to 1 (dotted line). 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, $*P \leq 0.05$).

Table 1. *DEGs important for seed oil accumulation in the developing seeds of* snat1-1 comt-1 *plants at 12 DAP*

DEGs with $|\log_2 \text{ratios}| \geq 0.58$, and only Gene Ontology Slim identifications with false discovery rate ≤ 0.05, are listed here.

DEGs	Log_2 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., 2017a
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	Beisson et al., 2007; Yang et al., 2012; Jayawardhane et al., 2018
LTP3 (AT5G59320)	2.42	Promoting soluble sugar accumulation and enhancing the in vitro transfer of phospholipids between membranes and binding acyl chains with no obvious effect on oil accumulation in the single mutant	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 (AT3G51600)	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

afterward in the *snat1-1 comt-1* mutant compared to wild type (Fig. 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 Arabidopsis seeds (Shi et al., 2012; Liu et al., 2017; Li et al., 2018a). 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 (Fig. 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 (Fig. 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 (Fig. 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 et al., 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 (Supplemental Table S5). On the other hand, exogenous application of melatonin to wild-type plants did not alter the accumulation of seed-coat mucilage (Supplemental Fig. 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 downregulated (Fig. 7B) and upregulated (Fig. 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 (Fig. 7, B and 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 Arabidopsis developing seeds.

SNAT1 and COMT Inhibited Seed Coat Proanthocyanidin Deposition

Flavonoids, as secondary metabolites, are generally classified into three major classes in Arabidopsis—flavonols,

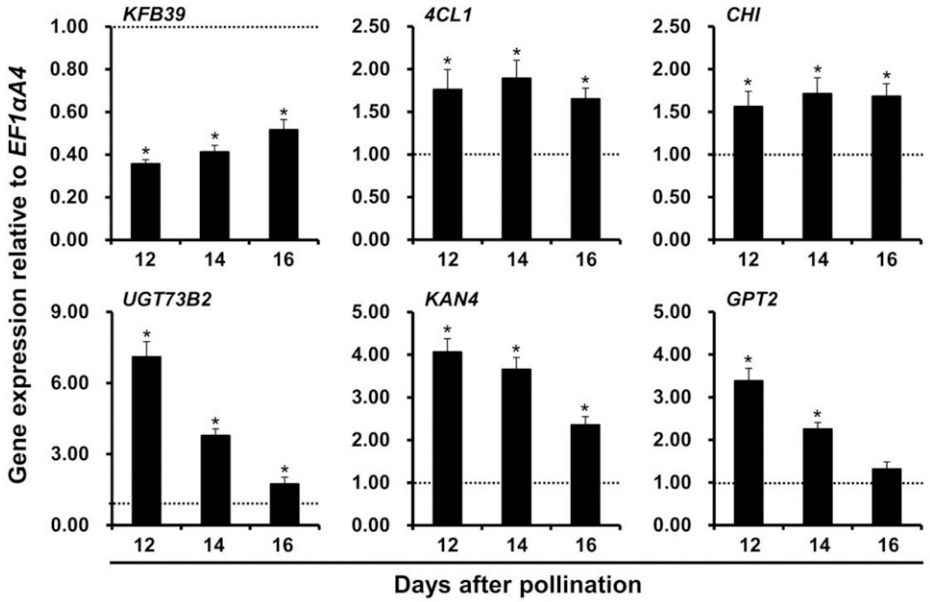

Figure 6. Dynamic expression analysis of genes contributing to seed anthocyanin accumulation in developing seeds of wild-type (Col-0) and *snat1-1 comt-1* plants. Gene expression was normalized against the expression of *EF1αA4* as an internal control, and the expression level in wild type was set to 1 (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, $^*P \leq 0.05$).

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 (Fig. 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 (Fig. 8, B and C). Moreover, the higher amounts of PAs in *snat1-1* and *comt-1* were fully rescued by the introduction of *gSNAT1* and *gCOMT*, respectively (Fig. 8, B and 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 (Fig. 8). These results suggested that SNAT1 and COMT act in a nonadditive manner, and COMT exhibits a greater role than SNAT1 in inhibiting the deposition of PAs in the Arabidopsis 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 Arabidopsis. 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

Table 2. *DEGs contributing to anthocyanin biosynthesis in the developing seeds of* snat1-1 comt-1 *plants at 12 DAP*

DEGs with $|\log_2 \text{ratios}| \geq 0.58$, and only Gene Ontology Slim identifications with false discovery rate ≤ 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 Glc 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

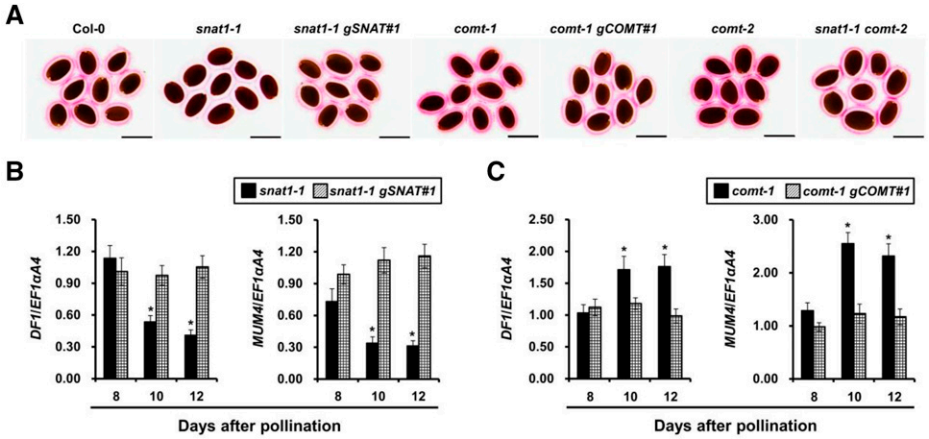

Figure 7. Effect of SNAT1 and COMT on seed-coat mucilage deposition. A, Comparison of the mucilage layer attached to the seed coat among wild type (Col-0); the single mutants of *snat1-1*, *comt-1*, and *comt-2*; the double mutant *snat1-1 comt-1*; and the transgenic plants of *snat1-1 gSNAT1-1* and *comt-1 gCOMT-1*. Scale bars = 500 μm. B, Comparison of the dynamic expression of *DF1* and *MUM4* in developing seeds from 8 to 12 DAP 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 DAP 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, $*P \leq 0.05$).

a dramatic decrease in the levels of total FAs and anthocyanins in mature seeds (Fig. 4; Supplemental 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 (Figs. 5 and 6; Tables 1 and 2; Supplemental Tables S3 and S4). These results, together with the observation of increased expression of *SNAT1* and *COMT* in developing seeds at the seed maturation stage (Fig. 2, B, C, E, and F), suggest that melatonin is an important player in the regulatory network that represses the accumulation of both oil and anthocyanins in Arabidopsis seeds.

Several previous studies showed a negative correlation between the contents of oil and flavonoids in Arabidopsis seeds (Chen et al., 2012b, 2014, 2015; Li et al., 2018a; 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

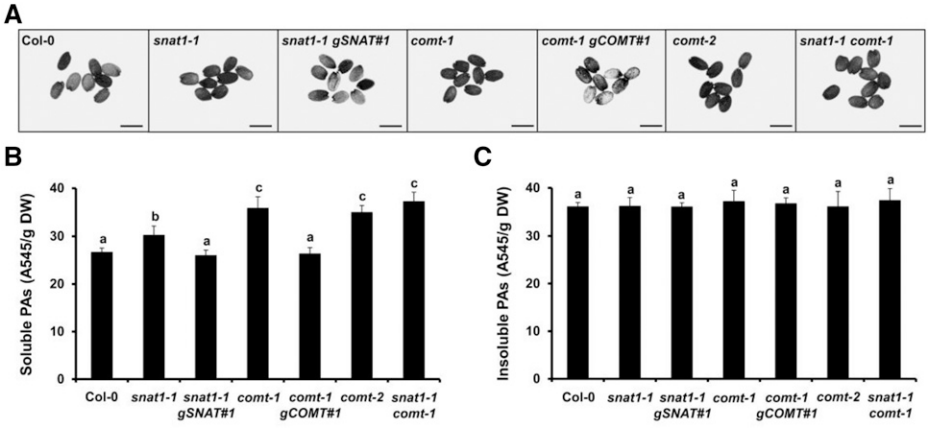

Figure 8. Effect of SNAT1 and COMT on the accumulation of PAs in seeds. A, Seeds stained with DMACA for 16 h among wild type (Col-0); the single mutants of *snat1-1*, *comt-1*, and *comt-2*; the double mutant *snat1-1 comt-1*; and the transgenic plants of *snat1-1 gSNAT1-1* and *comt-1 gCOMT-1*. Scale 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 honest significant difference test). DW, Dry weight.

flavonoids, including anthocyanins and PAs (Figs. 4 and 8; Supplemental Tables S1 and S2). It is known that Suc from photosynthesis is hydrolyzed to Glc, 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 upregulated is much higher than that of downregulated genes in developing seeds of *snat1-1 comt-1* plants at 12 DAP (Supplemental Tables S2 and S3). In addition, LTP3, a member of a family of lipid-transfer proteins that encode 7 to 10 kD 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 Glc 6-phosphate/phosphate translocator, is thought to be involved in the transport of Glc 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 (Supplemental Table S3), together with the upregulation of *LTP3* and *GPT2* genes (Figs. 5 and 6; Tables 1 and 2; Supplemental Table S3), could supply more carbon resources for glycolysis, 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 (Fig. 4; Supplemental Tables S1 and S2). Thus, the genes related to the accumulation of oil (Fig. 5; Table 1; Supplemental Tables S3 and S4) and anthocyanins (Fig. 6; Table 2; Supplemental Tables 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, and α-carboxyltransferase—and one plastid-localized subunit, β-carboxyltransferase, which are encoded by *BCCP1* and *BCCP2*, *CAC2*, *CAC3*, and *ACCD*, respectively, in Arabidopsis (Li et al., 2011). The complete loss of BCCP1 function results in embryo lethality, and reduced BCCP1 activity markedly decreases FA accumulation in Arabidopsis seeds (Li et al., 2011). MCAMT, localized in both chloroplasts and mitochondria, converts malonyl-CoA and *ACYL CARRIER PROTEIN* (*ACP*) into malonyl-ACP and CoA, and significantly promotes oil accumulation in Arabidopsis 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 Arabidopsis seeds (Wang et al., 2017a). The *LTP3* loss-of-function mutant seeds contain oil content similar to wild-type plants (Pagnussat 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-ACYLTRANSFERASE2 (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 Suc and Glc into TAGs during seed maturation (Focks and Benning, 1998) through directly promoting the expression of *ABNORMAL SUSPENSOR2*, *PKp β1*, *BCCP1*, *BCCP2*, *3-KETOACYL-ACYL CARRIER PROTEIN SYNTHASEI*, and *REDUCED OLEATE DESATURATION1*; and by indirectly activating the expression of *ACP1*, *CAC2*, *CAC3*, *BIOTIN AUXOTROPH2*, *PDH E1α*, *3-KETOACYL-ACYL CARRIER PROTEIN SYNTHASEIII*, and *MOSAIC DEATH1*, which are involved in the late glycolysis and plastidial FA biosynthetic pathways during seed development in Arabidopsis (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 upregulated (Fig. 5; Table 1; Supplemental Table S3), and the expression of other genes was not altered (Supplemental Table S5) in *snat1-1 comt-1* developing seeds at 12 DAP, indicating that melatonin controls the expression of *BCCP1* and *CAC3* through the upregulation 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., 2012a), 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 (Supplemental Table S4). Therefore, the increased expression of *BCCP1*, *CAC3*, *MCAMT*, *PLIP1*, *LTPs*, *GPAT2*, and *WRI1* contributing

to oil biosynthesis (Fig. 5; Table 1; Supplemental Table S3) and the decreased expression of the two GDSL-type lipase genes (Supplemental Table S4) together assist in promoting seed oil accumulation (Fig. 4A; Supplemental Table S1) in *snat1-1 comt-1* developing seeds.

Anthocyanin biosynthesis starts from the phenylpropanoid pathway (Lepiniec et al., 2006). KFB39 negatively regulates anthocyanin accumulation by directly controlling the stability and activity of Phe 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 4CL4, 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 (Lepiniec et al., 2006). The isoform 4CL1 accounts for the majority of total 4CL activity and positively regulates the accumulation of anthocyanins in Arabidopsis (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 Glc 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 Golden2, ARR-B, and 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*, *TRANSPARENT TESTA8*, and *TRANSPARENT TESTA GLABRA1*, and the structural genes *CHALCONE SYNTHASE*, *CHI*, *FLAVONOID 3′ HYDROXYLASE*, *DIHYDROFLAVONOL 4-REDUCTASE*, and *ANTHOCYANIDIN SYNTHASE* (Gao et al., 2010). Our results showed that only *CHI* was upregulated (Fig. 6; Table 2; Supplemental Table S3) and the expression of the other genes was not altered in *snat1-1 comt-1* developing seeds (Supplemental Table S5), implying that melatonin controls *CHI* expression through the upregulation of KAN4, and other genes were regulated by a complex upstream regulatory network. The transcription factor MYB56 acts in a Suc-dependent manner to control *GPT2* expression in response to the circadian cycle, thus promoting anthocyanin accumulation (Jeong et al., 2018). Therefore, the downregulation of *KFB39* and upregulation of *4CL1*, *CHI*, *UGT73B2*, *KAN4*, and *GPT2* related to anthocyanin biosynthesis (Fig. 6; Table 2; Supplemental Tables S3 and S4) are helpful for anthocyanin accumulation (Fig. 4C; Supplemental Table S2) in *snat1-1 comt-1* developing seeds.

It is worth mentioning that *snat1-1* seedlings accumulate fewer 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 Arabidopsis 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, 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* (Fig. 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 (Fig. 4; Supplemental 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

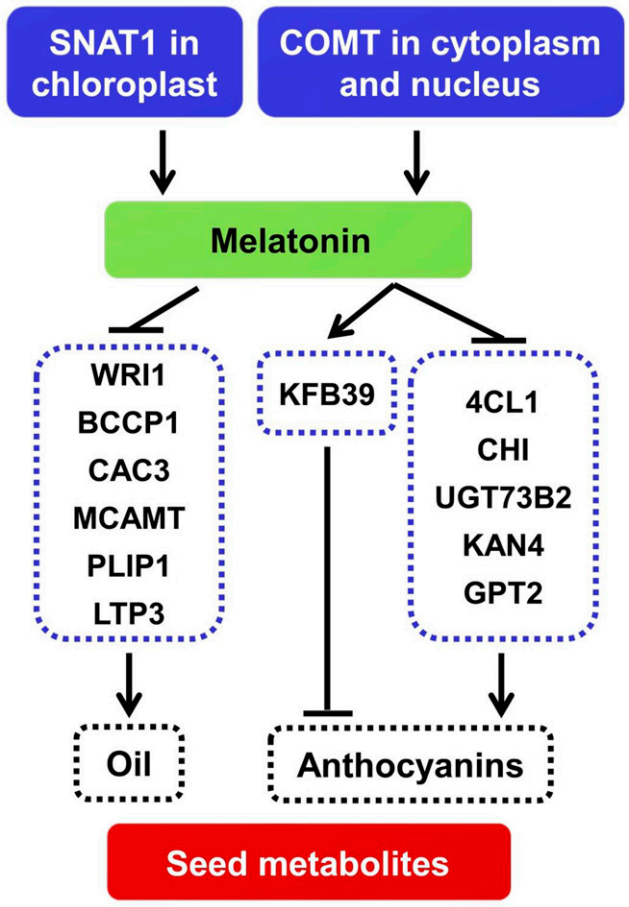

Figure 9. A proposed working model shows that the deficiency of melatonin by knocking out SNAT1 and/or COMT represses the accumulation of oil and anthocyanins by regulating the expression of key genes that control the biosynthesis of oil and anthocyanins, respectively, in Arabidopsis seeds. Arrows and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

multienzyme 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 (Fig. 1A) and the cytoplasm and nucleus (Fig. 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 (Fig. 4, A and B; Supplemental Table S1), flavonoids inclusive of anthocyanins (Fig. 4, C and D; Supplemental Table S2) and PAs (Fig. 8), and mucilage (Fig. 7; Supplemental Fig. 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 (Fig. 4; Supplemental Tables S1 and S2), and SNAT1 and COMT had a common and additive role in melatonin biosynthesis in developing siliques (Fig. 3D; Lee et al., 2014b; Back et al., 2016), we might conclude that melatonin represses the accumulation of oil and anthocyanins in Arabidopsis seeds; an underlying mechanism is proposed in Figure 9.

In summary, this 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 Arabidopsis 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., 2018a). The results presented here indicate that manipulation of this coregulation 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 Arabidopsis (*Arabidopsis 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 Supplemental Table S6. The *comt* mutants were obtained from the Arabidopsis Biological Resources Center at Ohio State University. The growth conditions of all Arabidopsis plants in this study have been reported in detail (Li et al., 2017a, 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 10 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-Aldrich 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 tumefaciens*-mediated floral dip method (Clough and Bent, 1998). The transgenic plants were selected by Basta (Bayer) 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 complementary DNA 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 (*Nicotiana benthamiana*) leaves as described in Yang et al. (2000). Images were obtained with a model no. IX83 confocal microscope (Olympus) 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) 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 Supplemental Table S6.

Microscopic Observation of Arabidopsis Seed Traits

Mature seeds of different Arabidopsis lines were harvested from major inflorescences, specifically from siliques at the basal region, and then randomly selected to be photographed with a SZ61 stereomicroscope (Olympus) for seed traits, including color, size, and seed-coat mucilage and PAs.

The ruthenium red staining of seed-coat mucilage was performed as reported in McFarlane et al. (2014). In brief, dry mature seeds were shaken vigorously in an EDTA (0.05 M at 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 distilled water.

The DMACA staining of seed coat PAs was conducted as described in Abrahams et al. (2002). Dry seeds were stained with the DMACA reagent (2% [w/v] DMACA in 3 cM of 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 in Poirier et al. (1999) and Chen et al. (2012b). The extraction and methylation of FAs on 300 individual intact seeds was performed in a methanol solution containing 2.5% (v/v) H_2SO_4 at 80°C for 2 h. After cooling to room temperature,

the FA methyl esters were extracted with 2 mL of hexane and 2 mL of 0.9% (w/v) NaCl, and the organic phase was analyzed by gas chromatography, using methyl heptadecanoate as an internal standard. The GC-2014 instrument (Shimadzu) was equipped with a flame ionization detector and a 30-m (length) × 0.25-mm (internal diameter.) × 0.5-μm (liquid membrane thickness) column (Supelco wax-10, Cat. no. 24079; Supelco). 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 reported in Chen et al. (2015). Briefly, 1 mg of mature dry seeds was homogenized with 50 μL of extraction buffer (100 mM of Tris-HCl at 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-PAGE.

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 (~100 mg) at 12 DAP were pulverized to powder in a 2-mL Eppendorf tube (Eppendorf North America) filled with liquid nitrogen and thoroughly homogenized in 1 mL of 2:2:1 methanol/acetonitrile/water (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/water (v/v). After 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 Nexera LC-30AD UHPLC system (Shimadzu) with an ACQUITY UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm; Waters) and an QTRAP 5500 mass spectrometer (AB SCIEX). 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^{-1}. 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. The mass transition from mass-to-charge ratio 233.2 to 174.1 was identified as melatonin; the retention time was 3.48 min. The software Analyst (v1.5.2; AB SCIEX) was used for data integration.

Measurement of Seed Anthocyanins and PAs

The anthocyanin content was measured as described in Li et al. (2018b), with some modifications. Briefly, ~5 mg of 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 A_{535} was determined using a model no. DU730 spectrophotometer (Beckman-Coulter), 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 described in Kitamura et al. (2010), with some modifications. Briefly, ~10 mg of mature seeds were frozen in liquid nitrogen and ground in 1.5 mL of 70% (v/v) acetone containing 5.26 mM of $Na_2S_2O_5$, followed by sonication for 20 min at room temperature. After centrifugation at 1,500 rpm for 15 min, the supernatant was dried and resuspended in 0.4 mL of 70% (v/v) acetone containing 5.26 mM of $Na_2S_2O_5$ and 1.6 mL of HCl/butanol (1:5 v/v). The A_{545} of this resuspended solution was determined using an Infinite M200 PRO (Tecan). After hydrolysis at 95°C for 1 h, the A_{545} 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 of 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 A_{545} of the supernatant was measured and then normalized to the weight as the content of insoluble PAs.

RNA-Seq 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-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 DEGs between wild type and *snat1-1 comt-1*. The DEGs with $|\log_2 \text{ratios}| \geq 0.58$ and false discovery rate ≤ 0.05 are listed in Supplemental 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 in Jefferson et al. (1987). Primers used for gene expression analyses are listed in Supplemental Table S1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database (https://www.arabidopsis.org/) 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).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Molecular verification of the *snat1-1* mutation.

Supplemental Figure S2. RT-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 dry weight) in mature seeds of various lines in this study.

Supplemental Table S2. Comparison of anthocyanin content (A535/g dry weight) in mature seeds of various lines in this study.

Supplemental Table S3. List of upregulated genes in developing seeds of *snat1-1 comt-1* plants at 12 DAP.

Supplemental Table S4. List of downregulated genes in developing seeds of *snat1-1 comt-1* plants at 12 DAP.

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 DAP.

Supplemental Table S6. Primers used in this study.

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