Synergy between the anthocyanin and RDR6/SGS3/DCL4 siRNA pathways expose hidden features of *Arabidopsis* carbon metabolism

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Anthocyanin pigments furnish a powerful visual output of the stress and metabolic status of *Arabidopsis thaliana* plants. Essential for pigment accumulation is TRANSPARENT TESTA19 (TT19), a glutathione S-transferase proposed to bind and stabilize anthocyanins, participating in their vacuolar sequestration, a function conserved across the flowering plants. Here, we report the identification of genetic suppressors that result in anthocyanin accumulation in the absence of TT19. We show that mutations in *RDR6*, *SGS3*, or *DCL4* suppress the anthocyanin defect of *tt19* by pushing carbon towards flavonoid biosynthesis. This effect is not unique to *tt19* and extends to at least one other anthocyanin pathway gene mutant. This synergy between mutations in components of the RDR6-SGS3-DCL4 siRNA system and the flavonoid pathway reveals genetic/epigenetic mechanisms regulating metabolic fluxes.
The conspicuous colors and non-essential nature of anthocyanin pigments have made their biosynthesis one of the best-studied plant metabolic pathways. Anthocyanins are flavonoid pigments synthesized on the cytoplasmic surface of the endoplasmic reticulum and transported to vacuoles, where the acidic pH bestows upon them characteristic red, purple or bluish coloration. Flavonoids are derived from primary metabolism, through the general phenylpropanoid pathway, from the amino acid phenylalanine or tyrosine. Anthocyanin pathway enzymes can be divided into those involved in making the anthocyanidin core, and those mediating modifications, including glycosylation, acylation, and prenylation. The extensive knowledge on anthocyanin biosynthesis enzymes from many plants contrasts with how little is known about the mechanisms by which these pigments are transported from their site of synthesis to the vacuole. Key players in this process are a group of conserved glutathione S-transferase (GST) proteins and ABC transporters that interact with anthocyanins and are believed to facilitate their transport across the tonoplast. The first GST described as associated with anthocyanin transport was maize BRONZE2 (BZ2), initially believed to conjugate glutathione to cyanidin-3-O-glucoside (C3G). Subsequent studies in maize and other plants, however, failed to identify anthocyanin-glutathione conjugates.

In Arabidopsis, TRANSPARENT TESTA19 (TT19) is the functional homolog of BZ2 and is required for the accumulation of anthocyanins as well as proanthocyanidin (PA) pigments in the seed coat. TT19 shows a broad subcellular distribution and can bind cyanidin as well as C3G. There are eight tt19 mutant alleles that have been characterized to some degree. TT19 and related GST proteins function to promote pigment accumulation remains unclear, but some possible roles for these GSTs include the stabilization of anthocyanins and/or PA precursors, or cooperation with transporters for vacuolar uptake.

TT19 is controlled by the R2R3-MYB transcription factor PRODUCTION OF ANTHOCYANIN1 (PAPI/MYB75) and to a lesser extent, PAP2/MYB90, MYB113 and MYB114. PAPI expression, and therefore anthocyanin accumulation, can be induced by high light, likely through the HY5 pathway, by high-sucrose levels, such as those found during senescence, or in Arabidopsis seedlings grown under anthocyanin inducing conditions (AIC), such as 3% sucrose and strong light. The first TT19-related GST described was maize BRONZE2 (BZ2), initially believed to conjugate glutathione to cyanidin-3-O-glucoside (C3G). Subsequent studies in maize and other plants, however, failed to identify anthocyanin-glutathione conjugates.

To investigate possible connections between anthocyanin pathway regulation, metabolic flux, and product sequestration, we screened for genetic suppressors that allow anthocyanins to accumulate in seedlings in the absence of TT19 function. Here, we show that all six tt19-8 suppressors identified correspond to components of the RDR6-SGS3-DCL4 small RNA system. We demonstrate that PAPI over-expression is insufficient to restore anthocyanin accumulation in the tt19 mutant. Indeed, our metabolic analyses show that, contrary to a pulling effect of PAPI through an increased demand for carbon from the flavonoid pathway, the combination of tt19 and mutations in rdr6, sg3 or dcl4 results in a significant push into the pathway, with three to five times more carbon devoted to flavonoid production. In seedlings, this carbon is derived from sucrose through central metabolism, and from the downstream regulation of other metabolic pathways. By combining rdr6 with tds4 (impacting the expression of leucaanthocyanidin dioxygenase), we demonstrate that the increased carbon influx to the flavonoid pathway is not unique to the combination of tt19 and rdr6 mutations, but more likely a generalized consequence of the inability to properly accumulate flavonoids. Our findings support a model in which the small RNAs derived from the RDR6-SGS3-DCL4 system provide a regulatory layer over central metabolism that is only exposed when combined with specialized metabolism dysfunctions.

**Results**

**Identifying tt19 suppressor mutations.** To investigate the role of TT19 in flavonoid/anthocyanin stabilization and to simultaneously discover components of a possible degradation pathway, we embarked on identifying tt19 genetic suppressors by mutating homozygous tt19-8 (SALK_105779C) seeds with ethyl methanesulfonate (EMS). We germinated the M2 seed pools and scored for restoration of anthocyanin pigmentation in 4 day-old seedlings grown under AIC. We identified eight pigmented seedlings and named them tt19 suppressors S1–S8 (Fig. 1 and Supplementary Fig. 1). Based on the anthocyanin content of seedlings grown in AIC, compared to wild-type (100%) and tt19-8 (11%), the eight tt19 suppressors were grouped as strong (49–58%) (S1, S2, S7, and S8), or weak (27–35%) tt19 suppressors (S3, S4, S5, and S6) (Fig. 1).

The eight tt19 suppressors were back-crossed with tt19-8 and F2 seeds (BC1F2) were grown in AIC. The resulting seedlings showed the expected segregation of ~25% pigmented and ~75% non-pigmented, confirming that the tt19 suppressing mutations were all recessive. Using bulked segregant analysis by whole-genome re-sequencing, we found that the eight causative mutations were located in two regions. The tt19 suppressors S1, S2, S4, and S8 corresponded to allelic mutations in At3g45900 (RDR6), with the S1 mutation replacing Q with a stop codon at position 293 (RDR6\*Q293stop), S2 replacing Q with a stop codon at position 1,055 (RDR6\*Q1055stop), S4 changing G664 for R (RDR6\*G664R), and S8 changing W to a stop codon at position 299 (RDR6\*W299stop) (Fig. 1a and Supplementary Fig. 2). In S3, S5, and S6, the same G-to-A mutation at nucleotide position 2283 of At5g23570 (SG3S) caused a splice defect at the 3' end of intron 3 in which the normal acceptor site was mutated. The alignment of SG3S transcript reads from S5 suggested an alternative splicing site 15 nt downstream of nucleotide 2,283 that resulted in the deletion of five amino acids (Fig. 1b). In S7, a G-to-A change at nucleotide position 6,509 resulted in a splice defect at the 5' end of intron 17 of At5g20320 (DCL4), which was confirmed by RNA-seq (Fig. 1c). In summary, an unbiased screen for phenotypic suppressors of tt19-8 resulted in all the mutations mapping to genes encoding components of the RDR6-SGS3-DCL4 small RNA system (Table 1).

To confirm that the identified mutations were responsible for suppressing the anthocyanin pigmentation deficiency of tt19-8, we crossed tt19-8 to characterized reference mutants for RDR6, SGS3, and DCL4 (rdr6-11 and rdr6-15; sg3-11 and sg3-14; and dcl4-2c and dcl4-2r). We confirmed that homozygous double mutant seedlings for tt19-8 rdr6/sg3/dcl4 accumulated anthocyanins in AIC (upper row, Supplementary Fig. 3). Consistent with PAPI being controlled by TAS4 via the RDR6-SGS3-DCL4 system, the single rdr6, sg3, and dcl4 mutant seedlings accumulated more anthocyanins than the wild-type (Col-0) control, at levels comparable to PAPI-D seedlings, in which PAPI is under the control of four copies of CaMV35S promoter (lower row, Supplementary Fig. 3). Similar restoration of the anthocyanin pigmentation phenotypes were observed in tt19 suppressor plants at different conditions (AIC), such as 3% sucrose and strong light, by high-sucrose levels, such as those found during senescence, or in Arabidopsis seedlings grown under anthocyanin inducing conditions (AIC), such as 3% sucrose and strong light...
developmental stages grown on soil (Supplementary Fig. 4). In contrast to the restoration of anthocyanin accumulation in vegetative organs, none of the eight \textit{tt19} suppressors was able to reinstate the seed coat PA pigmentation characteristic of wild-type and significantly reduced in \textit{tt19-8} plants (Supplementary Fig. 5).

\textbf{PAP1 is insufficient for restoring \textit{tt19} pigmentation.} A possible explanation for why mutations in either \textit{RDR6}, \textit{SGS3}, or \textit{DCL4} overcome anthocyanin pigment deficiency in \textit{tt19-8} plants is that more anthocyanins are produced by preventing \textit{PAP1} transcript degradation via \textit{TAS4-siRNA8124} and the subsequent upregulation of anthocyanin biosynthesis genes\textsuperscript{15}. To test whether over-
expression of PAP1 alone can overcome the need for TT19, we crossed tt19-8 to PAP1-D plants15. PAP1 mRNA levels were about fivefold higher in PAP1-D seedlings in AIC, compared to Col-0 seedlings (Fig. 2a), and PAP1-D seedlings showed ~60% increase in anthocyanin accumulation when compared to Col-0 (Fig. 2b). Although the PAP1 mRNA steady-state level in tt19-8 PAP1-D seedlings was comparable to that of PAP1-D (Fig. 2a), the tt19-8 PAP1-D line failed to accumulate more anthocyanins than the tt19-8 seedlings (Fig. 2b). The tt19 suppressor lines that have mutations in RDR6 resulted in an elevation of PAP1 mRNA levels and an increase in anthocyanin levels relative to tt19-8. However, compared to tt19-8 PAP1-D seedlings, the tt19-8 S4 and S8 lines accumulated larger quantities of anthocyanins, despite lower PAP1 mRNA levels (Fig. 2). These results demonstrate that, although PAP1 upregulation is likely necessary for enhanced anthocyanin accumulation in the tt19 suppressor lines, it is clearly insufficient to overcome the inability of tt19 to accumulate high anthocyanin levels. Therefore, the mechanisms by which the tt19 suppressors restore anthocyanin accumulation involve other factor(s), besides PAP1.

Flavonoid accumulation changes in suppressor lines. Arabidopsis accumulates at least 15 distinct anthocyanins (A1-A15)29,30, most of which are detectable under the metabolic stress conditions imposed by AIC22,31. To compare the anthocyanin composition of the suppressor lines with those present in Col-0 and tt19-8, we grew homozygous seedlings in AIC and analyzed their anthocyanin profiles. Under AIC, the prevalent anthocyanins in Col-0 were A3, A5, and A7 to A11, with A11 being the most abundant (Supplementary Fig. 6a). Despite tt19-8 accumulating only ~10% of the anthocyanin content in Col-0, the relative peak abundance remained unaltered (Supplementary Fig. 6b). The anthocyanin profiles of the suppressor lines were also indistinguishable from those of wild-type and tt19-8, despite accumulating overall different pigment quantities (Fig. 1; Supplementary Fig. 6b).

As previously reported, tt19 mutants accumulate significantly larger quantities of flavonols than wild-type plants8. Thus, we analyzed the flavonol aglycone composition of seedling from the different genotypes grown in AIC. Our results showed that tt19-8 seedlings had an ~25% increase in total flavonol content, primarily represented by quercetin, compared to Col-0. Unexpectedly, S2, S4, S5, S7, and S8 showed 4- to 9-fold increases in

Table 1 Gene mutations underlying the tt19 suppressors.

| Suppressor | Chromosome | Position | Mutation | Reference amino acid | Strand | Accession |
|-----------|------------|----------|----------|----------------------|--------|-----------|
| S1        | 3          | 18352329 | G to A   | Gin (Q) to Stop      | —      | At3g49500 |
| S2        | 3          | 18349621 | G to A   | Gin (Q) to Stop      | —      | At3g49500 |
| S4        | 3          | 18351216 | C to T   | Gly (G) to Arg (R)   | —      | At3g49500 |
| S5        | 3          | 18352310 | C to T   | Trp (W) to Stop      | —      | At3g49500 |
| S3/S5/S6  | 5          | 7945406  | G to A   | Splice defect (Donor loss) | +     | At5g23570 |
| S7        | 5          | 6862758  | C to T   | Splice defect (Acceptor loss) | —     | At5g20320 |

Fig. 1 Molecular characterization of the tt19-8 phenotypic suppressors S1-S8 and corresponding anthocyanin accumulation. a Structure of the RDR6 gene indicating the position and nature of the EMS-induced mutations for the S1, S8, S4, and S2 suppressors. The images show 4-day-old seedlings grown in AIC and accumulating various levels of anthocyanins evaluated by absorbance at 532 nm (A532), and normalized to the dry weight of the seedlings. The levels present in Col-0 were taken as 100%. b Structure of the SG53 gene indicating the mutations for the S3, S5, and S6 suppressors. All three suppressors showed similar levels of anthocyanins in AIC and the same mutation, which abolishes a splicing acceptor site. The genome browser image on the top right shows the reads obtained in RNA-seq experiments for S5, and the region demonstrating the use of an alternate splicing acceptor site in the S5 mutant is shown expanded on the lower image. c Structure of the DCL4 gene indicating the mutation for the S7 suppressor, which abolishes a splicing donor site, as evidenced by the genome browser images comparing the RNA-seq reads of Col-0, tt19-8, and S7. Similar anthocyanin quantification results were obtained from at least three independently repeated experiments. Bar, 400 μm.

Fig. 2 Over-expression of PAP1 is not sufficient to suppress the anthocyanin deficiency in tt19-8. a PAP1 mRNA accumulation levels were evaluated by RT-qPCR and normalized to ACT2 between the various lines of RNA extracted from 4-day-old seedlings grown in AIC. n = 9, 7, 8, 7, 8, 8 biological replicates. b Anthocyanin levels from three independent experiments were measured by A532 (normalized per mg of dry weight). The error bars represent the standard deviation of the average. Different letters indicate significant differences between the lines based on one-way ANOVA with Tukey’s Honest Significant Difference test (P < 0.05). Source data are provided as a Source Data file.
Contrasting features that were mapped to predictive pathways and those involved in late anthocyanin biosynthesis, consistent with the lower accumulation of anthocyanins in tt19-8. However, this displayed as pathway cloud plots (Supplementary Fig. 8a–e). Two compounds, likely corresponding to sucrose ([M + Cl]−, 377.0859) and glucose ([M + Cl]−, 215.0315), were identified as part of dysregulated sucrose and trehalose degradation pathways with a lesser representation in all five suppressor lines than in tt19-8, but not different between Col-0 and tt19-8 (Supplementary Fig. 8). Authentic standards were used to determine the absolute quantities of sucrose and glucose in all the genotypes (Supplementary Fig. 9a, b). The significant decrease in sucrose and glucose levels in the tt19 suppressor lines compared to tt19-8 indicated that more sucrose and glucose was being consumed in the tt19 suppressors, providing the extra carbon necessary for the enhanced flavonoid production.

To determine to what extent the observed increase in sugar (sucrose and glucose) metabolism was a consequence of the high-sucrose levels in the media, we grew Arabidopsis seedlings in water in high-light conditions. Anthocyanin accumulation was still evident, but at about half the levels present in high-sucrose media (Supplementary Fig. 9c). Logically, sucrose levels were considerably lower (>90% reduction) in extracts of water-grown seedlings (Supplementary Fig. 9a). Interestingly, the suppressor lines presented a similar reduction in endogenous sucrose and glucose levels as observed when the seedlings were grown in sucrose-rich media, and this reduction was not observed in the tt19-8 or rdr6-11 single mutants (Supplementary Fig. 9a, b). These results indicate that the additional carbon required for the increased flavonoid biosynthesis in the suppressor lines could be provided by sugar catabolism.

### Enhanced sugar metabolism likely source of extra carbon

Our results show that a significantly larger amount of carbon is devoted to flavonoid biosynthesis in the strong suppressor lines, compared to Col-0 or the single mutants (Fig. 3). To determine the possible source of this extra carbon, we performed untargeted metabolic profiling of the extracts obtained from seedlings corresponding to Col-0, tt19-8, and five suppressor lines under AIC, resulting in the identification of ~7,000 altered m/z differentially accumulated features (Supplementary Data 1). Pairwise analyses between tt19-8 and each suppressor line permitted to identify the contrasting features that were mapped to predictive pathways and displayed as pathway cloud plots (Supplementary Fig. 8a–e). Two compounds, likely corresponding to sucrose ([M + Cl]−, 377.0859) and glucose ([M + Cl]−, 215.0315), were identified as part of dysregulated sucrose and trehalose degradation pathways with a lesser representation in all five suppressor lines than in tt19-8, but not different between Col-0 and tt19-8 (Supplementary Fig. 8). Authentic standards were used to determine the absolute quantities of sucrose and glucose in all the genotypes (Supplementary Fig. 9a, b). The significant decrease in sucrose and glucose levels in the tt19 suppressor lines compared to tt19-8 indicated that more sucrose and glucose was being consumed in the tt19 suppressors, providing the extra carbon necessary for the enhanced flavonoid production.

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### Transcript accumulation changes in suppressor lines

To better understand the cross-talk between the RDR6-SGS3-DCL4 system and the TT19 pathway, we carried out RNA-seq on seedlings grown in AIC for Col-0, tt19-8, and tt19 suppressor lines. The tt19-8 mutant showed differences in mRNA accumulation patterns when compared to Col-0, with 2,642 genes displaying statistically significant (FDR < 0.05) differences (Supplementary Data 2). The genes with ~2-fold reduced expression included those involved in late anthocyanin biosynthesis, consistent with the lower accumulation of anthocyanins in tt19-8. However, this...
reduction in gene expression does not explain why \( tt19-8 \) shows only ~10% of the anthocyanin content of wild-type plants (Fig. 1a).

Gene expression patterns in the \( tt19 \) suppressor lines were rather different from both \( tt19-8 \) and Col-0. Gene ontology enrichment of \( K \)-means clusters showed that a large number of genes (captured in clusters 1, 2, 3, and 5, Fig. 4a) exhibited different expression in \( tt19-8 \) S2 (strong suppressor), compared to \( tt19-8 \) or Col-0. Cluster 2 included late anthocyanin pathway genes that showed decreased mRNA accumulation in \( tt19-8 \) and early anthocyanin pathway genes (e.g., CHS and CHI) that are also required for flavonol accumulation (Fig. 3, Supplementary Data 3).
One of the genes that showed a dramatic increase in mRNA accumulation in the suppressor lines was At1g02940 (AtGSTF5) encoding a phi-type GST (Supplementary Fig. 10a). The Arabidopsis Next-Gen Sequence DB\textsuperscript{32} shows the existence of a single siRNA that potentially targets AtGSTF5 for degradation. AtGSTF5 is a GST similar to TT19 (Supplementary Fig. 10b), and therefore, we surmised that it might compensate for the flavonoid binding function missing in tt19-8. Constructs for wild-type AtGSTF5 CDS (WT) as well as for an optimized version (OPT) harboring synonymous substitutions across the entire CDS that would render it insensitive to siRNAs were transformed into tt19-8. We verified expression of both versions of AtGSTF5 by RT-qPCR in the transgenic plants, yet observed no complementation of the anthocyanin deficiency phenotype. To determine whether AtGSTF5 is necessary for the restoration of anthocyanin synthesis in the tt19-8 suppressor lines, we designed two guide RNAs to edit target sequences in exons 2 and 4 of AtGSTF5 by CRISPR/Cas9 and identified six tt19 suppressor lines with mutated AtGSTF5 as homozygous, harboring various indels in AtGSTF5. However, when we compared the anthocyanin levels between the tt19-8 suppressors with wild-type or mutated AtGSTF5, we did not detect statistically significant differences (Supplementary Fig. 10). This result demonstrates that AtGSTF5 is unlikely to participate in the stabilization/vacular sequestration of anthocyanins, yet its enhanced expression in the suppressor lines, compared to both Col-0 and tt19-8, suggests a possible role in another cellular function normally carried out by TT19.

Whereas all tt19 suppressors had similar effects on anthocyanin accumulation, they showed surprisingly different patterns of gene expression (Fig. 4b), indicating that, while they probably function together as part of the RDR6-SGS3-DCL4 system, each one has separate functions that are independent of the others. The tt19 S4 (RDR6\textsuperscript{G664A}) suppressor is potentially interesting, since it showed an effect on mRNA accumulation that was dramatically different from the effects observed for S2 and S8, both harboring nonsense mutations in RDR6 (Fig. 4c). Only 244 differential expressed genes were shared by all the five suppressor lines analyzed against tt19-8 (Fig. 4b, Supplementary Data 4). From these 244 genes, 197 show changes in mRNA accumulation with the same trend (up or down). We rationalized that these 197 genes contained those that are most likely responsible for the ability of the suppressor lines to accumulate anthocyanins in the absence of TT19 function.

As anticipated, among these 197 genes, there were 22 upregulated genes related to phenylpropanoid/flavonoid biosynthesis or regulation, and an additional 26 repressed genes associated with various metabolic or regulatory activities (Fig. 4b, Supplementary Data 4). Among those 26 genes, six have been functionally characterized (or predicted) to be involved in competitive pathways with phenylpropanoid/flavonoid biosynthesis, including CYP79B3 and MYB34 involved in indole glucosinolate biosynthesis\textsuperscript{35,36}; CYP84A4 involved in arabidopyrone biosynthesis\textsuperscript{35}; CYP90A1/CDP involved in a critical step for brassinosteroid biosynthesis\textsuperscript{36}; UGT77CI involved in the decoration of salicylic acid\textsuperscript{37}; and flavonol—phenylacetyltransferase 1 (FPT1) likely participating in phenylalanine consumption\textsuperscript{38}. Thus, a shared feature of all the suppressor lines is the increased expression of phenylpropanoid/flavonoid-related genes, and decreased accumulation of gene products likely competing for the early precursors of the pathway (e.g., Phe and Tyr).

To explore the possible link between the increased sugar metabolism providing the extra carbon and those genes differentially expressed in tt19 suppressor lines compared to Col-0 and tt19-8, we analyzed GO term enrichment in the six clusters shown in Fig. 4a. We identified 15 genes under the GO term monosaccharide/hexose/glucose metabolic process only in Cluster 2, corresponding to genes highly expressed in tt19-8 S2 (Supplementary Fig. 11). An analysis of previous published RNA-seq data for rdr6-11 and Col-0 from Arabidopsis shoots\textsuperscript{39} confirmed that 13 of these genes are highly expressed in tt19-8 S2, but not in rdr6-11 alone. Thus, the upregulation of these 13 carbohydrate metabolic process-related genes in the tt19 suppressors provides one likely explanation for the increased carbon flux towards flavonoids in the suppressor lines.

We investigated whether the synergistic effect of the tt19 and RDR6-SGS3-DCL4 system mutations on increased flux toward the flavonoid pathway involved small RNAs. We performed a global analysis of small RNAs (20 to 24 nt) between Col-0, tt19-8, and tt19-8 S2 and S7 seedlings, all grown in AIC (Supplementary Fig. 12a and Supplementary Data 5). As anticipated, the suppressor mutations abolished the biogenesis of 21 nt tasiRNAs, which was also evidenced by the increased accumulation of the TAS4 precursor RNA in all the suppressor lines, as well as increased mRNA accumulation of tasiRNA gene targets, including the TAS4 targets PAP1 and PAP2\textsuperscript{40}, and TAS3 target ARF4\textsuperscript{40}. We found that levels of five miRNAs were significantly different between Col-0 and tt19-8 seedlings (Supplementary Fig. 12b). Significantly higher in tt19-8 than in Col-0 were mir158b, mir167a-5p and mir399c-5p, while miR161.2 and miR164b-5p showed the opposite pattern (Supplementary Fig. 12b). The increased expression of mir158b in tt19-8 did not revert to wild-type levels in the S2 and S7 suppressors. Similarly, the low accumulation of miR161.2 in tt19-8 compared to wild-type, was not restored in the suppressor lines, indicating that the accumulation of mir158b and miR161.2 does not involve the RDR6-SGS3-DCL4 system. In contrast, the effect of the tt19-8 mutation on the accumulation of mir399c-5p, mir167a-5p, and miR164b-5p was abolished in the S2 and S7 suppressor lines. These results suggest that these miRNAs (or their targets) participate in the cross-talk between the flavonoid pathway and the RDR6-SGS3-DCL4 system.

Since RNA-seq analysis identified 13 carbohydrate metabolic process-related genes with higher mRNA steady-state
accumulation in tt19-8 S2, we mapped the reads from the small RNA-seq analysis to these differentially expressed genes. One of the genes, At3g43190 (SUS4), encoding SUCROSE SYNTHASE 4, showed increased mRNA levels in tt19-8 S2 (Supplementary Fig. 12c). Our analyses identified a small RNA (known as siR661141 or AtsRR17827842) derived from the PPR (Pentatricopeptide repeat) gene (At1g62930) whose expression is significantly decreased in the suppressor lines (Supplementary Data 5), and which may target SUS4 mRNA for cleavage43 (Supplementary Figure 12c and Supplementary Data 6). Indeed, the formation of siR6611 required miR161.2 and tasiRNAs derived from TAS244-46, providing one mechanism by which the RDR6-SGS3-DCL4 system and the TT19 pathway may converge on the control of sugar metabolism (Supplementary Fig. 12d).

Second flavonoid mutant similarly affected by rdr6 mutation. The results so far cannot distinguish whether the synergistic effect of RDR6-SGS3-DCL4 and tt19-8 on the increase of carbon flux into the flavonoid pathway relates to the absence of TT19 function itself, or to the abnormal flavonoid accumulation that characterizes the tt19-8 mutant (Fig. 3). To distinguish between these two possibilities, we generated a double mutant between tdr-15 and tds4-4. tds4-4 corresponds to a null allele of the anthocyanidin synthase (ANS) gene, which encodes an enzyme that converts leucoanthocyanidins into 3-hydroxyanthocyanidins (hydrolysis converts naringenin chalcone to naringenin), because of the block in the pathway (Supplementary Fig. 13a–d). When we evaluated the absolute values of glucose and sucrose in the tt5-4 rdr6-15 double mutant, they were not significantly different from those in Col-0 or single mutants (Supplementary Fig. 13e, f), indicating that it was not the absence of flavonoids that synergized with the RDR6-SGS3-DCL4 system, but an imbalance between different flavonoids caused by the tt19-8 or tds4-4 mutants.

We then took advantage of the possibility to feed seedlings grown in AIC with various flavonoids to determine whether the accumulation of flavonols in the absence of anthocyanins would have an effect. For this, we added quercetin or kaempferol, but neither was sufficient to trigger an enhanced catabolism of glucose or sucrose (Supplementary Fig. 13e, f), despite flavonols accumulating at high levels in the seedlings (Supplementary Fig. 13a, b). The addition of naringenin (the product of chalcone isomerase) resulted in flavonol and cyanidin formation in ratios slightly different from those present in Col-0, but still not sufficient to trigger enhanced sugar catabolism (Supplementary Fig. 13). The results suggest that what triggers enhanced sugar catabolism in the presence of a disabled RDR6-SGS3-DCL4 system is the absence of anthocyanins (regardless of whether flavonols are present or not), or a pathway dysfunction, likely characterized by the accumulation of a pathway intermediate specific to the anthocyanin branch of the pathway present in both tds4-4 and tt19-8, but absent in tt5-4.

Discussion

Our quest for phenotypic suppressors of tt19 resulted exclusively in the identification of multiple recessive alleles of components of the RDR6-SGS3-DCL4 siRNA pathway, which is involved in the post-transcriptional gene silencing of a large number of genes39. It is notable that the only identified context in which anthocyanins can still accumulate in a tt19 mutant is by disabling a major regulatory mechanism that causes global gene expression changes. Our results demonstrate that a consequence of these gene expression changes is a flux increase into the phenylpropanoid/flavonoid pathway, resulting in greater levels of anthocyanins and other flavonoids (Fig. 6). Thus, while our results cannot rule out a role of TT19 in the stabilization, solubilization, and/or vacuum sequestration of anthocyanins, it is evident that if enough anthocyanins are produced, they can accumulate, even in the absence of TT19. A possibility that we considered is that the gene expression changes promoted by the tt19 suppressors might induce a TT19-like protein. We identified AtGSTF5, which is normally expressed at very low levels in both wild-type and tt19-8, as one of such candidates. However, by combining gain- and loss-of-function approaches, we showed that it is neither necessary nor sufficient to overcome the absence of TT19 function (Supplementary Fig. 10). Another possibility that our experiments ruled out is that enhanced expression of PAP1, as a consequence of disabling the RDR6-SGS3-DCL4 siRNA system24,27, would be sufficient to overcome the need for TT19 (Fig. 2). Indeed, our results show that the augmented anthocyanin accumulation that characterizes rdr6, sgs3, and dcl4 plants48 occurs largely through the impaired tasiRNA-mediated degradation of the PAP1 mRNA.
Thus, while increased \textit{PAP1} expression is necessary, it is clearly insufficient to explain the anthocyanin pigmentation of the \textit{tt19} suppressors.

The \textit{tt19} suppressors are characterized by a three to fivefold increase in carbon flow into the flavonoid pathway (Fig. 6). Increase in \textit{PAP1} mRNA accumulation, either by \textit{PAP1-D} or when the RDR6-SGS3-DCL4 siRNA system is disabled (Fig. 6), results in a modest (~1.7 fold) increase in overall carbon to the pathway, likely caused by the transcriptional activation of late pathway genes by \textit{PAP1}, which results in a flavonoid biosynthesis pull. In contrast, we propose that most of the additional carbon dedicated to flavonoid accumulation in the \textit{tt19} suppressor lines is a consequence of carbon being pushed into the pathway, by a synergistic interaction between the loss RDR6-SGS3-DCL4-controlled siRNAs and a flavonoid metabolic dysfunction. In addition, we show that the cross-talk between the impaired RDR6-SGS3-DCL4 system and flavonoid pathway dysfunction is not specific to the loss of \textit{TT19} as a second anthocyanin pathway mutant (\textit{tds4-4}) shows a similar carbon flow synergistic effect with \textit{rdr6} (Fig. 5).

What is the source of the additional carbon pushed into the flavonoid pathway in the \textit{tt19} suppressors? The RNA-seq results demonstrate that part of the carbon is likely coming from pathways that compete with phenylpropanoid/flavonoid biosynthesis.

Fig. 6 Flavonoid flux maps displaying total carbon input into the different branches of the pathway. The block arrow (black at the top) displays the estimated total input of carbon for flavonoid biosynthesis. Green and purple arrows show the distribution of flavonols and anthocyanins, respectively. The data used for the graphical representation of carbon distribution is provided in Supplementary Fig. 7.
or no changes in the
Fig. 9). Interestingly, the induction of anthocyanin triggered by
DCL4 siRNA system controls the expression of a large number of
fl
RDR6-SGS3-DCL4 system on
miR161.2 is a
RNAs and specialized metabolites are rapidly evolving60,61, pro-
central metabolism and
Methods
the metabolic engineering of desirable phytochemicals that might
providing ample opportunities for emerging synchronization, and for
Methods
Chemicals and instruments. Chemical standards (apigenin, luteolin, naringenin,
eriodictyol, dihydrokaempferol, dihydroquercetin, kaempferol, quercetin,
epicatechin) and HPLC grade solvents were purchased from Sigma (St. Louis, MO). Cyanidin (chloride), pelargonidin (chloride), peonidin (chloride) were purchased from Cayman Chemical (Ann Arbor, MI). Pictures of Arabidopsis seeds and seedlings were taken using a dissecting microscope with a Nikon SMZ1500 camera with a Nikon WD54 HR Plan Apo lens. A BioMate 35 UV-visible spectrophotometer (ThermoFisher Scientific, Waltham, MA) was used to measure absorbance.

Plant materials and growth condition. Arabidopsis thaliana (L.) accession
Integrated DNA Technologies (Skokie, Il). All plants were grown in Suremix
growth medium (Michigan Grower Products Inc, MI) under controlled conditions
(22 °C and 16 h light/8 h dark photoperiod).

Constructs. For generation of the AIGSTS5 overexpression lines, two versions of the AIGSTS5 CDS clones (WT and OPT) were synthesized by ThermoFisher Scientific and subsequently integrated into the pGWB902 binary vector resulting in the pSSS/AIGSTS5 (WT and OPT) constructs via LR reaction. For creation of the AIGSTS5 knockout lines, two CRISPR guide DNAs were designed by CRISPR-Plant (http://www.genome.arizona.edu/crispr/) and cloned into the Cas9 vector pHHE401 via a Golden Gate reaction. The resulting AIGSTS5 overexpression and CRISPR constructs were then introduced into Agrobacterium tumefaciens
and used for transformation of tt19-8 and tt19-8 SSS/S7/S8 by floral dip. For the selection of transformed lines, seeds were surface-sterilized and cultured on Murashige and Skoog (MS) solid media including 5% sucrose with hygromycin (50 mg/L).

EMS mutagenesis to isolate tt99 suppressors. Taking advantage of the very low anthocyanin accumulation of tt19-8 seedlings, we screened 2.5 g (~125,000) of EMS-treated seeds for potential dominant suppressors by scoring restoration of anthocyanin pigmentation of 4 day-old M1 seedlings grown in AIC. We then transferred ~10,000 of the M1 seedlings to soil, grew to maturity and collected into ~300 pools of M2 seeds. The recessive suppressors that restored anthocyanin accumulation were isolated from the M2 seed pools germinated under AIC.

Anthocyanin extraction and composition analysis. Arabidopsis seedlings were grown in AIC for four days. Seedlings were then lypophilized and dry tissue weighted. Total anthocyanins were extracted with 50% methanol containing 3% sucrose and incubated two days at 4 °C for strification. After a cold treatment for two days, the seeds were cultured under continuous cool-white light with 20 xg shaking at 23 °C for four days. For compound feeding assays, Arabidopsis seedlings were grown in AIC for three days and then 100 μL of naringenin/querzetin/ kaempferol were added for 24 h.

Flavonoid quantification. Aglycone flavonoids were prepared by HCl hydrolysis62. Briefly, lypophilized Arabidopsis seedlings (four days in AIC) were soaked with 2 M HCl (1 mg dry weight in 10 μL) and incubated at 100 °C for 20 min. Half volume of isooamylycohol (1 mg dry weight in 5 μL) was then mixed to extract and concentrate aglycone flavonoids (top layer after centrifugation). The amount of flavonoids in each sample was detected by multiple reaction monitoring (MRM) and quantified by comparing with flavonoid standards on high-performance reverse phase liquid chromatography coupled with triple quadrupole mass spectrometer63 using Waters MassLynx v4.1 SCN805. For creating standard curves of flavonoid compounds, the flavonoid standards were dissolved in 80% of methanol with 0.1% formic acid. The UHPLC-MS/MS run was 10 min with a flow of 0.3 μL/min at 40 °C. The gradient was as follows with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile): 0–0.5 min, 90% A and 10% B;
Untargeted metabolite profiling and LC-MS/MS data analysis. For extraction of total metabolites, lyophilized Arabidopsis seedlings (four days in AIC or water) were incubated with methanol/water (1/1, v/v) containing 0.1% formic acid and 1 μM telsmisan (internal standard) at the ratio 1 mg dry weight in 10 μL extraction buffer overnight at room temperature. After centrifugation at 10,000 × g for 5 min, the supernatant was then diluted ten times with 80% of methanol with 0.1% formic acid for LC-MS/MS analysis. All the genotypes were analyzed in triplicate by LC-MS/MS using Acquity UHPLC system with HSS T3 C18 column (100 × 2.1 mm, 1.8 μM, Waters, Milford, MA) linked to the mass spectrometer (Xevo G2-MS QTOF, Waters). At a flow rate of 0.3 mL/min and the column temperature of 40 °C, a 10 min gradient run was applied from solvent A (0.1% formic acid in water) and solvent B (acetonitrile): 0–0.5 min, 100% A; 0.5–6 min, 50% A and 50% B; 6.01–6.5 min, 1% A and 99% B; 6.51–8.5 min, 1% A and 99% B; and 8.51–10 min 99% A and 1% B. Mass spectrum acquisition was performed in negative mode with m/z scan range at 50–2000. All MS raw data files (raw) were converted to mzXML format using Proteowizard version 3.64 and then uploaded to XCMS online35,36 (https://xcmsonline.scripps.edu/) for data processing. For all the samples, peak alignment (feature detection), candidate metabolite identification, MS/MS matching, and statistical analysis were performed. Feature detection and pairwise analysis were performed with the following parameters: centWave method for feature detection (Δm/z = 1 ppm, minimum peak width = 5 s, and maximum peak width = 50 s); obiwamp method for retention time correction (prostep = 1); and settings for alignment (bw = 5, minfrac = 0.5, mzwid = 0.015) and statistics (Welch t-test). The total numbers of aligned features and pairwise cloud plots were exported from XCMS online and reported in Supplementary Data 1 and Supplementary Fig. 8, respectively.

Whole-genome re-sequencing. Total genomic DNA from pooled leaves of 50 Arabidopsis BC1F3 pigmented plants and 50 non-pigmented plants were extracted using QIAgen Genomic DNA Purification Kit (Promega, WI). The pooled gDNA samples were then sent to BGI genomic services (http://www.genomics.cn/en/) for whole-genome re-sequencing data were uploaded to Sequence Read Archive (SRA) under accession numbers GSE136680 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136680], GSE136901 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE136901], and PRJNA562949 [https://www.ncbi.nlm.nih.gov/traces/study?acc=PRJNA562949], respectively. Source data underlying Figs 2, 3, and 5, and Supplementary Figs 6, 7, 9, 10b, c, 12a, b, and 13 are provided as a Source Data file.

Received: 4 October 2019; Accepted: 26 April 2020;
Published online: 15 May 2020

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Small RNA-seq analysis. Total RNA was used to generate small RNA-seq library and sequenced with 50 bp paired-end reads using the BGISEQ-500 platform. Adapter sequences were trimmed-off from the remaining high-quality sequences using Adapter Trimming for Small RNA-sequencing (v0.3.2, http://www.bcgsc.ca/platform/bioinfo/software/adapter-trimming-for-small-RNA-sequencing) and sequences smaller than 15 nt were discarded. Unique small RNA tags were mapped to Arabidopsis thaliana reference genome (TAIR10) using Bowtie (v1.2.2). In order to obtain mirRNA abundance, we used the miRBase 22.1 database (http://www.mirbase.org/) annotations for Arabidopsis thaliana. The siRNA annotation was performed using compiled annotations from Araboport111. In the global quantification analysis of small RNAs derived from miRNAs, TAS loci, transposable elements, and protein-coding genes, only small RNAs unique to single gene categories were included. For the quantification of small RNAs, we used a local Perl script for exact quantification, using loci length and read size restrictions depending of the small RNA category analyzed. For read normalization and differential expression analysis of small RNAs, we used edger (v3.26.5) to apply trimmed means of M-values as a normalization method (TMM) with FDR threshold value less than 0.05.

Statistical analyses. Statistical analyses were conducted by GraphPad Prism 6.0c software (San Diego, California).
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Acknowledgements
This work was supported in part by NSF MCB-1822343. The authors thank Arabidopsis Biological Resource Center (Columbus, OH) for all the seeds and plasmids provided. We also thank Lina Gomez Cano and Tony Schilmiller at Michigan State University for assistance in the quantification of flavonoids and acknowledge the use of instruments at the Michigan State University Mass Spectrometry and Metabolomics core.

Author contributions
N.J. carried out the identification and characterization of suppressors and determination flavonoid quantities; N.J. performed overexpression and CRISPR knockout with the assistance of Y.S.L.; A.G.D. and E.M.M. carried out the computational analyses and data were interpreted by N.J., A.G.D., and Y.S.L.; N.J. and E.G. wrote the article with contributions from all the authors, but particularly from B.C.M. and M.S.O. who also contributed with some of the ideas that resulted in specific experiments being carried out; E.G. coordinated and supervised the research project and agrees to serve as the author responsible for contact and ensures communication.

Competing interests
The authors declare no competing interests.