Flavonoids are a major class of phenolic secondary metabolite in plants that have a C6-C3-C6 carbon structure. Almost 6500 flavonoids have been documented to be produced in plants. Anthocyanins are a pigmented subclass of flavonoid that have been commonly used by scientists to study chemical, genetic, and biochemical aspects of plants for centuries. In the 1660s, Robert Boyle characterized how acids and bases change the color of anthocyanin pigments. In the 1860s, Gregor Mendel used the color they impart to flowers to understand fundamental aspects of genetic inheritance. Recent studies have demonstrated a role of anthocyanins in providing tolerance to oxidative stress. Other flavonoid species may act as signaling molecules as evidenced by their alteration of auxin transport or nuclear localization. The accumulation of anthocyanin pigments is a visual hallmark of plant stress responses. Starving seedlings of nitrogen in conjunction with sucrose supplementation results in a C:N nutrient imbalance stress that particularly enhances anthocyanin biosynthesis compared to a balanced nutrient medium containing sucrose. This nutrient imbalance stress, named AIC, was recently demonstrated to stimulate the biosynthesis of a wide range of anthocyanin species, providing a fingerprint that was distinct from other abiotic stresses. Hormones are major signaling molecules that effect sucrose-mediated induction of anthocyanin biosynthesis. Sucrose induction of the anthocyanin biosynthesis was repressed by the addition of GA, whereas JA or ABA had synergistic positive effects. Increased amounts of sucrose in MS medium caused elevated levels of anthocyanin pigmentation and reduced levels of flavonol staining. Since abiotic stress responses result from the culmination of multiple hormone signals, here we hypothesized that hormone deficient mutants grown in AIC would exhibit distinct flavonoid profiles compared to the WT.

To determine how flavonoid levels changed in response to C:N imbalance stress, we compared the levels of 19 flavonoids from seedlings grown in AIC stress to those grown in half-strength MS 3% sucrose medium, referred to hereafter as the balanced nutrient medium. Overall, 11 flavonoids exhibited a >2-fold change in their levels across three independent experiments (P < 0.05; two-tailed Paired Student’s t test) (Figure 1(a)). Increases were observed for the anthocyanins A5 and A11, the flavonols F1 and F3, and the unknown flavonoid-like metabolites U1, U2, and U5. Decreases were observed for F7, U3, A9, and A10. Thus, not all anthocyanin and flavonol species were increased and decreased, respectively.

Since Arabidopsis seedlings produced distinct proportions of individual anthocyanin species in response to different abiotic stresses, we also analyzed the changes in metabolite proportions (i.e. the relative level of a metabolite compared to the overall flavonoid levels, see Methods). The proportions of all flavonoids changed in response to AIC except for the flavonol F4 (Figure 1(b)). Increases and decreases were observed for anthocyanin and flavonol species. The anthocyanins A10 and A9 exhibited greatest increases, whereas A11 and U1 exhibited the greatest decreases.

Upon careful investigation of the error bars of flavonoid levels and proportions (Figure 1(a,b)), it appeared that flavonoid proportions generally exhibited less variation compared to flavonoid levels. To compare, we calculated the SD of the
levels and proportions of each flavonoid and then normalized all values by expressing them as percentage of their respective flavonoid level or proportion. On average, flavonoid proportions exhibited less than a quarter of the SD than flavonoid levels (9.3% versus 39.6%, respectively) (Figure 1(c)). This difference was found to be highly significant (ANOVA $P = 1.31 \times 10^{-7}$, $\alpha = 0.05$).

To examine whether this phenomenon was observed in seedlings exposed to other abiotic stresses, we compared the average SD of flavonoid proportions to the SD of their levels from seedlings grown under seven other abiotic stress conditions. The stresses were cold (4°C), heat (30°C), osmotic stress (250 mM mannitol), pH 3.3 medium, pH 7.3 medium, 100 mM NaCl, 100 mM MgSO$_4$, and medium without phosphate. Overall, flavonoid proportions exhibited 10% less SD than flavonoid levels (18.8% versus 28.4% SD, respectively) (Figure 1(d)). Again, the difference was highly significant (ANOVA, $P = 0.001$, $\alpha = 0.05$). Thus, in general, the relative proportions of flavonoids are more important for signaling or responding to an abiotic stress than the levels of one or more particular flavonoids.

To determine the potential causation of flavonoid amounts being more variable than their proportions, we measured the variation in the levels of transcription of the early-stage flavonoid biosynthesis genes CHS and CHI that are responsible for determining the overall flux into the flavonoid pathway and the late-stage flavonoid genes SAT, UGT79B1, and BGLU10 that add chemical groups to the flavonoid backbone and thus determine the relative proportions of individual flavonoid species. We used three separate primer pairs for each gene to account for any primer bias. The SD of CHS expressions was 9- to 79-fold greater than those of the late-stage genes SAT, UGT79B1, BGLU10 (Figure 1(e)). CHS expressions were only 10- to 22-fold greater than the late-stage genes, demonstrating that the greater SD of CHS was not simply proportional to its higher level of expression (Figure 1(f)). The SD of the other early-stage gene, CHI was not significantly greater than UGT79B1 and BGLU10. These results suggest that the relatively large variance in the levels of flavonoids is due to large SD in CHS gene expressions,
whereas the lower variance in flavonoid proportions is due to lower SD in late-stage gene expressions.

Since abiotic stress responses result from the culmination of multiple hormone signals, we hypothesized that hormone deficient mutants would exhibit distinct flavonoid proportion fingerprints in AIC. We conducted a cluster analysis on mutants deficient in ABA (aba2 and aba3), JA (jar11 and opc11), GA (ga1 and ga2), and independent transgenic lines undergoing artificial microRNA-based silencing of four or five of the aminocyclopropane-1-carboxylic acid synthase (ACC) genes involved in Eth biosynthesis, namely acs4m and acs5m, respectively. Mutants deficient in JA, GA, and Eth formed distinct clusters (Figure 2(a)). opc11 and jar1 clustered most closely to WT, indicating that JA deficiency conferred the least amount of change in flavonoid proportions compared to mutants that were deficient in the other hormones. aba2 formed a cluster that was distinct from JA mutants and WT. However, aba3 did not cluster with aba2 nor with any other hormone mutants. In contrast to ABA2 that encodes a short-chain dehydrogenase/reductase involved specifically in the conversion of xanthoxin to ABA-aldehyde, ABA3 encodes a Moco sulfatase that supplies cofactor for the last step of ABA biosynthesis, in addition to purine catabolism and protein import into the chloroplast. Thus, its unique flavonoid fingerprint may due to its pleiotropic effects, rather than an ABA deficiency specifically.

To determine whether the fingerprints were merely due to differences in rates of seedling development rather than in stress signaling, we compared flavonoid proportions at various times after germination between WT and aba2, a mutant with reduced dormancy. Cluster analysis demonstrated that aba2 was distinct from the wild-type at all time points measured (Figure 2(b)). In conclusion, our results demonstrate that hormone deficient mutants have distinct flavonoid proportion fingerprints in AIC. Future work will determine whether this can be used as a tool to predict functional involvement of uncharacterized genes in hormone-mediated stress responses.

Methods

Plant lines and growth conditions

All lines of Arabidopsis thaliana were of the Columbia ecotype. jarl-11 (CS67935), opc11 (CS65801), ga1 (SALK_027931C), ga2 (CS878765), acs4m (amiR acs1-1, acs2-1, acs4-1, acs5-2; CS16651) and acs5m (amiR acs1-1, acs2-1, acs4-1, acs5-2, acs6-1; CS16650) were obtained from the Arabidopsis Biological Resource Center. aba2-1 (CS156) and aba3-1 (CS157) were kind gifts from J.C.

Figure 2. Hormone-deficient mutants exhibit distinct flavonoid proportion fingerprints in response to AIC stress. (a) Clustering of ABA and ethylene biosynthesis mutant flavonoid proportions at 4 d after germination in AIC. (b) Cluster analysis of aba2-1 and wild-type flavonoid proportions from seedlings over time (3–6 d) following germination in AIC.
Jiang and Col-0 from Erich Grotewold (Ohio State University). All lines were grown in parallel at 22°C with 16 hours light in 6 inch pots in SunGrow Soil in the WVU greenhouse and were harvested within a 1 month time frame of each other (postharvest age was 8–9 months). Before plating in AIC (liquid 3% sucrose) or balanced nutrient medium [½ MS (Caisson Labs, Smithfield, UT), 1x MS vitamins, 3% sucrose, pH 5.8], seeds were surface sterilized according to6. For AIC, 120 seeds were plated per 35 mm Petri and dried before addition of medium. Plates were wrapped with parafilm and stored in 4°C for 3 days then transferred to a rotary shaker (100 rpm) and grown under 90 μE m⁻² s⁻¹ cool white fluorescent light for the times indicated. Other stress conditions were conducted as indicated in6.

Metabolite and RNA extractions

Seedlings were immediately flash frozen and lyophilized for 3 d. Dry weights were measured and 50 μg μL⁻¹ extraction solvent [50% (v/v) methanol, 3% (v/v) formic acid] was added and incubated in the dark overnight at room temperature on a rotary shaker. Two volumes of dilution solution [3% (v/v) formic acid in water] were added. Tubes were centrifuged at 13,500g for 2 min and the supernatant was passed through 0.2 μM filters (Nanosep ODM02C35), and the filtrate analyzed by UPLC-PDA-MS⁸. For RNA extraction, lyophilized seedlings were pulverized for 3 min using 5 mm stainless steel grinding balls in a MM400 mixer mill (Retsch, Newtown, CT, USA) at frequency of 30/s. RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, MI).

Flavonoid analysis

UPLC-PDA-MS⁸ was conducted using an Accela system (Thermo Scientific, San Jose, CA, USA) consisting of a 1250 pump, Open AS autosampler, and PDA detector connected to a Q-Exactive–Orbitrap MS containing a HESI. Separations were achieved with an Acquity UPLC BEH shield RP18 column (2.1 mm i.d. × 150 mm, 1.7 μm particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH shield RP18 VanGuard precolumn (2.1 mm i.d. × 5 mm, 1.7 μm particle size; Waters) heated to 35°C. Solvent A was 5% formic acid (v/v) in water and B was 5% formic acid (v/v) in acetonitrile. The flow rate was 300 μL min⁻¹ with a gradient of 10% to 25% B over 2 min; 2–9 min, from 25% to 50% B; 9–12 min, 50% B; 12–17 min from 50% to 100% B; 22–24 min 100% to 10% B, and 24–30 min 10% B. MS analysis was performed in Full MS/AIF mode in positive polarity. Full MS properties were: resolution 70,000, AGC target 3e6, maximum IT 200 ms, and scan range 120–1200 m/z. AIF properties were: resolution 70,000, AGC target 3e6, maximum IT 200 ms, NCE 35, and scan range 80–1200 m/z. Nitrogen was used as sheath and auxiliary gas. Five μL of sample was injected and compounds were identified by comparison of parent and daughter ion fragmentation patterns and PDA absorbance scans to the literature (see Supporting Table S1).⁶ Levels of each flavonoid were determined by detecting the area under the UPLC peak using Xcalibre software (Thermo Scientific, San Jose, CA, USA). Flavonols and unknowns were measured at 350 nm and anthocyanins at 530 nm. Flavonoid proportions were calculated as the percentage that a peak area of a flavonoid represented relative to the total peak areas for all 19 flavonoids. For calculation of % SD of a flavonoid level, the SD of the biological replicates was determined and was expressed as a percentage of the average level of the flavonoid. The % SD of proportions were calculated similarly. Fold change in metabolite levels were calculated by dividing the average flavonoid level from seedlings grown in AIC to those obtained from seedlings grown on the balanced nutrient medium. Hierarchical clustering was conducted using Multi-Experiment Viewer Version 4.9.0 with the Euclidean Distance algorithm.

qRT-PCR

cDNA synthesis and qRT-PCR were performed as described.¹⁵ Primers are listed in Supporting Table S2.

Abbreviations

| Abbreviation | Description                      |
|--------------|----------------------------------|
| GA           | gibberellic acid                 |
| JA           | jasmonic acid                    |
| Eth          | ethylene                         |
| MS           | molybdenum cofactor              |
| N            | nitrogen                         |
| AIC          | anthocyanin induction condition  |
| C            | carbon                           |
| Eth          | ethylene                         |
| GA           | gibberellic acid                 |
| JA           | jasmonic acid                    |
| Moco         | molybdenum cofactor              |
| MS           | molybdenum cofactor              |
| N            | nitrogen                         |
| SD           | standard deviation               |
| U1           | unknown flavonoid 1              |
| U2           | unknown flavonoid 2              |
| UPLC-PDA-MS⁸ | ultrahigh performance liquid chromatography-photodiode array-tandem mass spectrometry |

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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