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Original Paper

Association analysis of phenotypic and metabolomic changes in Arabidopsis accessions and their F_1 hybrids affected by different photoperiod and sucrose supply

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Abstract Photoperiod and sucrose (Suc) assimilation play important roles in the regulation of plant growth and development. However, it remains unclear how natural variation of plants could contribute to metabolic changes under various growth conditions. Here, we investigated the developmental and metabolomic responses of two natural accessions of *Arabidopsis thaliana*, Columbia (Col) and C24, and their reciprocal F_1 hybrids grown under four carbon source regimens, i.e., two different photoperiods and the presence or absence of exogenous Suc supply. The effect of exogenous Suc clearly appeared in the growth of Col and the F_1 hybrid but not in C24, whereas long-day conditions had significant positive effects on the growth of all lines. Comparative metabolite profiling of Col, C24, and the F_1 hybrid revealed that changes in metabolite levels, particularly sugars, were highly dependent on genotype-specific responses rather than growth conditions. The presence of Suc led to over-accumulation of seven metabolites, including four sugars, a polyamine, and two amino acids in C24, whereas no such accumulation was observed in the profiles of Col and the F_1 hybrid. Thus, the comparative metabolite profiling revealed that the two parental lines of the hybrid show a distinct difference in sugar metabolism.

Key words: Arabidopsis, growth, metabolite, photoperiod, sucrose.

Introduction

Both photoperiod and sucrose (Suc) affect various metabolic processes and growth and development of plants (Fujiwara et al. 2010; Gibson 2005; Kusano et al. 2011a; Van Dingenen et al. 2016). Assays of diurnal cycle changes and exogenous Suc supply can enable investigation of the response of metabolites grown under carbon source-related growth conditions (Badr et al. 2011; Baerenfaller et al. 2015; Kusano et al. 2011a). Photosynthesis in leaves is primarily responsible for providing energy for plant growth and development in the form of sunlight. As a result, photoperiod-responsive changes widely influence proteins, metabolite production, and metabolism (Jeong and Clark 2005; Kusano et al. 2011a; Seaton et al. 2018; Sulpice et al. 2014). In *Populus*, metabolite profiles change in the leaves of hybrid aspen under different day lengths (Kusano et al. 2011a). In *Arabidopsis thaliana* (Arabidopsis), responses to photoperiod length influence flowering time, sugar levels, and protein abundance (Jeong and Clark 2005; Seaton et al. 2018; Sulpice et al. 2014).

The uptake of exogenous Suc, which is known as a soluble carbohydrate source, can supply both carbon and energy for respiration as well as primary and secondary metabolite production to support plant growth and development (Gibson 2005). Sugars not only play a key regulatory role in plant metabolism but also act as signal molecules to regulate gene expressions involving growth, development, anthocyanin biosynthesis, and environmental changes (Kojima et al. 2007; Sulpice et al. 2014; Teng et al. 2005; Van Dingenen et al. 2016). Promotion of cell proliferation and leaf growth stimulation can be induced by exogenous Suc supply in

Abbreviations: Suc, sucrose; SD, short-day condition; LD, long-day condition; DAS, days after sowing; GC-TOF-MS, gas chromatography time-of-flight-mass spectrometry; PCA, principal component analysis; HCA, hierarchical cluster analysis; ANOVA, analysis of variance; TCA, tricarboxylic acid; ZT, zeitgeber time.

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Arabidopsis seedlings, resulting in growth, development, and expansion of the rosette area (Van Dingenen et al. 2016).

Various physiological phenomena have been found to be associated with Suc response among Arabidopsis accessions (Wang et al. 2019; Wuyts et al. 2011). In the presence of Suc, Columbia (Col) exhibited increased elongation zone length and doubling of the elongation rate, whereas C24 did not (Wuyts et al. 2011). There was little effect on the fresh weight of C24 when increasing the exogenous Suc concentration from 1.5 to 2% (Wang et al. 2019). Geographical variation of Col and C24 accessions might have a considerable influence on nutrient uptake or photoperiodic stimulus (Fujimoto et al. 2012; Hu et al. 2017; Ibañez et al. 2017; Miao et al. 2018; Stangeland et al. 2009; Yang et al. 2010).

Metabolomic analysis can aid in the development of standardization methods for understanding natural variation and/or various environmental conditions regarding plant growth (Kusano et al. 2007b; Meyer et al. 2007; Sato and Yanagisawa 2014; Soltis and Kliebenstein 2015; Sotelo-Silveira et al. 2015; Sulpice et al. 2009; Tamura et al. 2018). According to recent studies of plant metabolic processes, metabolic profiling has shown a close link between biomass accumulation and metabolites, as both negative and positive correlations (Meyer et al. 2007; Sulpice et al. 2009). Organic carbon sources such as sucrose, glucose-6-phosphate, fructose-6-phosphate, intermediates of the tricarboxylic acid (TCA) cycle, and amino acids such as glutamine and phenylalanine were negatively correlated with plant growth for recombinant inbred lines of Col and C24 (Meyer et al. 2007). However, it remains unclear how much metabolite change is influenced by genetic variation under various environmental conditions.

In the source cells, Suc can be cleaved into hexose sugars such as glucose or fructose for energy production (Granot et al. 2013). Suc is a main translocator that can be stored in the vacuole or possibly transported in the phloem sap or in the sink tissues by Suc transporters (SUCs) (Durand et al. 2018; Kühn and Grof 2010). In Arabidopsis, nine *AtSUCs* have been studied in terms of their expressions and functions (Durand et al. 2018; Kühn and Grof 2010; Lemoine 2000; Sauer et al. 2004; Sivitz et al. 2008; Srivastava et al. 2008; Williams et al. 2000). Four *AtSUCs*, including *AtSUC1*, *AtSUC2*, *AtSUC3*, and *AtSUC4* are expressed in the root and rosette. *AtSUC1* and *AtSUC2* have higher expression levels than the other genes (Durand et al. 2018). Silencing of *AtSUC2* has been shown to reduce plant growth (Gottwald et al. 2000). *AtSUC1* has been reported as a sugar signaling role in the vegetative tissue (Sivitz et al. 2008).

Most studies have focused on analyzing natural variation under limited growth conditions or on an accession under various growth conditions (Meyer et al. 2007; Sato and Yanagisawa 2014; Seaton et al. 2018; Sotelo-Silveira et al. 2015; Weckwerth et al. 2004). Therefore, expanding research to understand fully functional systems is required (Soltis and Kliebenstein 2015). Here, our comprehensive study investigated the correlation of growth response and metabolite profiling among two Arabidopsis accessions and their F_1 hybrid under short-day conditions (SD) (12 h light, 12 h dark) and long-day conditions (LD) (16 h light, 8 h dark), with or without exogenous Suc. Our data provided experimental evidence concerning the interaction of three factors (genotype, photoperiod, and Suc content) on metabolite changes in the central metabolism. The results provide better insight into the understanding of metabolomic responses to photoperiod length and exogenous Suc in Col, C24, and their F_1 hybrid.

Materials and methods

Plant material and growth conditions

We used Arabidopsis accessions, including Col, C24, and their reciprocal F_1 hybrids. F_1 hybrid seeds were generated by pollinating emasculated flowers of Col/C24 plants with pollen from another accession. Self-pollinated seeds of two ecotypes (Col and C24) were generated by restricting the number of flowers, as described previously (Meyer et al. 2004).

Seeds were sterilized, stored at 4°C for three days, and then sown in square dishes containing half-strength Murashige and Skoog (MS) salts with vitamins, at pH 5.7, with 1% (w/v) agar, with or without 1% (w/v) sucrose (~30 mM). A plate was divided into four compartments consisting of two parental lines and their reciprocal hybrid and then transferred into a growth chamber at 22°C in SD or LD. To equilibrate the total photosynthetic photon flux density, fluxes of 120 μmol photons $m^2 s^{-1}$ and 90 μmol photons $m^2 s^{-1}$ were used for SD and LD, respectively. Seedlings at five days after sowing (5 DAS) were transferred to 150 mm-diameter plates and grown under the same growth conditions. The dishes were rotated daily around the growth chamber to equilibrate lighting conditions.

Measurement of leaf area and fresh weight from seedlings

Each plant was recorded by scanning using an Epson GT-X820 (Epson, Japan). The leaf area of seedlings at 5 to 15 DAS was determined using ImageJ software (Schneider et al. 2012). For leaf number, all visible leaves were manually counted. The fresh weight of seedlings at the 10-leaf number stage of each plant was also measured.

Metabolite profile analyses

Seedlings at the 10-leaf number stage grown under various conditions were harvested as follows; 12 DAS in LD with 1% sucrose (LD suc+), 13 DAS in LD without sucrose (LD suc−), 13 DAS in SD with 1% sucrose (SD suc+), and 15 DAS in

SD without sucrose (SD suc−). We harvested whole rosettes in six replicates per line, each replicate containing two bulked plants in zeitgeber time (ZT)=6 in SD and ZT=8 in LD; then, samples were immediately frozen in liquid nitrogen and stored at −80°C before further analysis. Extraction, derivatization, and injection of metabolites to gas-chromatography-time-of-flight-mass spectrometry (GC-TOF-MS) were conducted as described previously by Kusano et al. (2007a).

Data processing and statistical analysis

Principal component analysis (PCA) was performed using 237 metabolites as variables and four differential growth conditions as observations (SD suc−, SD suc+, LD suc−, and LD suc+). PCA was performed using the pcaMethods package and ggplot2 package in R software (http://R-project.org). Hierarchical cluster analysis (HCA) was visualized through the Gplots package available in R software (http://R-project.org). The data were $\log_2$-transformed to calculate $\log_2$-fold changes. Three-way analysis of variance (ANOVA) was conducted using GraphPad Prism 8 ($p<0.05$) to validate the effects of genotype (Col or C24), photoperiod (SD or LD), and Suc (− or +) on metabolite changes. Statistical analysis was also performed by one-way ANOVA with Tukey's multiple comparisons test using both GraphPad Prism 6 and R software (http://R-project.org), where $p<0.05$ is considered statistical significance. Metabolite changes were visualized on a Venn diagram (Venny 2.1.0) (Oliveros 2007).

Expression analyses

Tissues for RNA extraction were harvested in the same stage and ZT (ZT6 and 8 under SD and LD, respectively) as the samples for metabolite profiling. Three biological replicates were used for each replicate containing the bulk of five root tissue samples. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's manual. The first strand cDNA was synthesized with 1 μg of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, US). Quantitative real-time PCR was performed using a LightCycler 480 II (Roche diagnostics, Germany) with LightCycler 480 SYBR Green I Master (Roche diagnostics, Germany). Gene-specific primer sequences were as follows: *AtSUC1* (AT1G71880) (5′-TGC TGT TCT TGG TAT CCC ATT-3′ and 5′-GTG GTA TCA CAA TCG CCA AA-3′) and *AtSUC2* (AT1G22710) (5′-GAA TGG TCC ACC TCC CAC AG-3′ and 5′-CTT CGC CAT CCT CGG TAT CC-3′). Expressions of these target genes were normalized using *AT5G12240* as a control gene (Czechowski et al. 2005). Primer sequences of *AT5G12240* were described previously (Durand et al. 2018).

Results

Comparison of growth and development patterns among accessions and their F_1 hybrid under the different growth conditions

It is known that delaying flowering can increase vegetative biomass production by allowing prolonged growth before switching to the reproductive phase (Niu et al. 2016). Under experimental conditions, C24 showed late-flowering times compared with Col (Fujimoto et al. 2012). Under LD suc+, the Col accession had almost 80% of plants with the appearance of inflorescence buds 17 DAS, when approximately 14 leaves appeared, whereas 50% of the plants appeared inflorescent under LD suc− at the same 17 DAS (13 leaves). Conversely, Col did not start bolting under SD at the same period (data not shown). Hence, to eliminate any bias concerning plant growth and development ascribed to the delay in flowering transition because of different genetic backgrounds and growth conditions, the time course of experiment was designed to last until 15 DAS for all growth conditions.

To demonstrate in detail how photoperiod length and exogenous Suc supply affect leaf growth for young Arabidopsis seedlings of each accession, we measured leaf area from 5 DAS to 15 DAS under four treatments (Figure 1, Supplementary Table S1). Comparison of SD and LD revealed that both Col and C24 showed significantly increased leaf area over 5 to 15 DAS in LD (Figure 1A, B). In the F_1 hybrid, LD also induced an increase in leaf area compared with plants under SD (Figure 1C, D). These results suggest that an increase in light period positively affected leaf growth in the two accessions and their F_1 hybrid.

Regarding exogenous Suc supply, the rosette leaf area of Col was statistically higher ($p<0.05$) under both SD and LD (Figure 1E). Similar patterns for Col were also observed in two reciprocal F_1 hybrids, which showed increased leaf size in the presence of Suc for both SD and LD (Figure 1G, H). Conversely, when C24 plants were germinated and grown under suc+ conditions, a weak response was observed in each condition (Figure 1F). Hence, there was an inefficient use of exogenous Suc in C24 to enhance leaf area in the early developmental stage. In LD suc+, parental and hybrid lines showed rapid growth, reaching the 10-leaf stage at 12 DAS. SD suc− led to delayed growth at 15 DAS, but then showed the highest biomass of 10 leaf numbers (Figure 1I–K). At this stage, no significant differences between Col and C24 were observed regarding both fresh weight and leaf area (Figure 1J, K). Therefore, samples for further analysis were used that had the same developmental stage (10-leaf stage). It has been reported that F_1 hybrids generated from crosses of Col and C24 show heterosis at an early developmental stage under LD (Groszmann et al. 2014; Meyer et al. 2012). Despite changing growth conditions, F_1 hybrids displayed vigorous characteristics in all experimental conditions. Both fresh weight and leaf area values for reciprocal F_1 hybrids were significantly larger ($p<0.05$) than their parental lines at the 10-leaf stage (Figure 1I–K) under all experimental conditions.

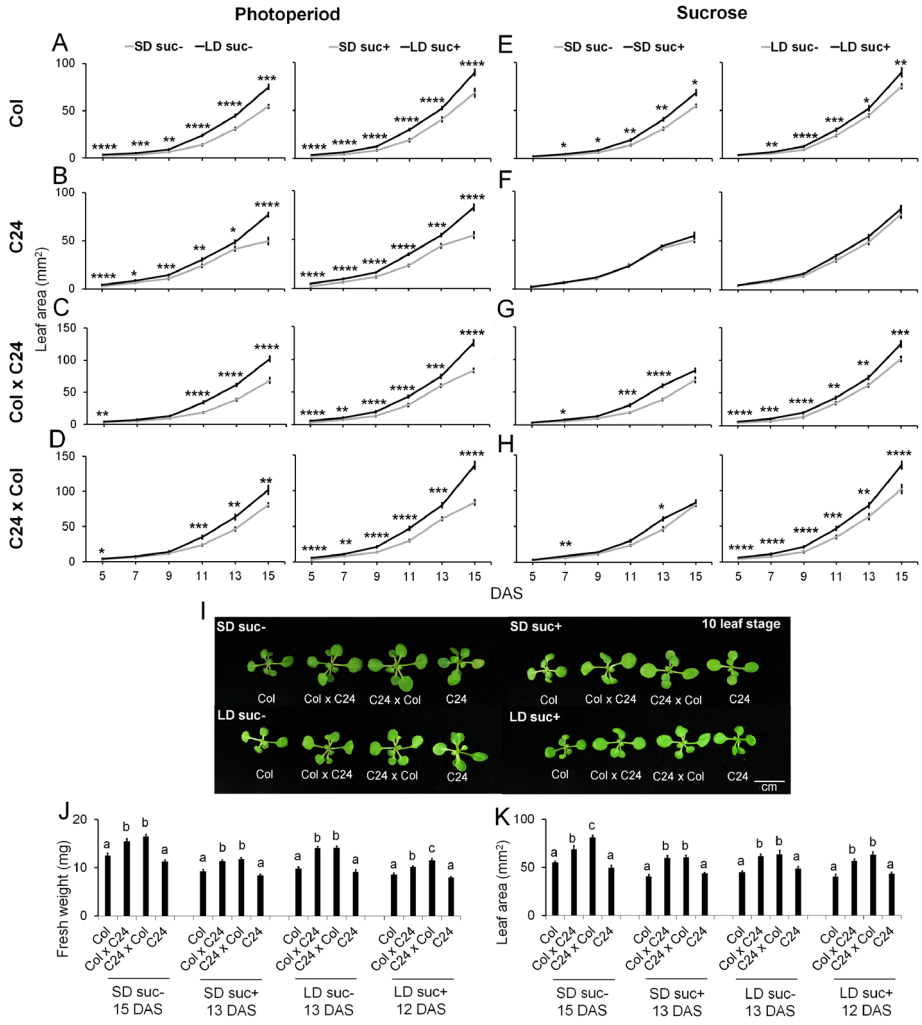

Figure 1. The phenotype of Col, C24 and their F_1 hybrids under different growth conditions. (A to H) Comparison of leaf area from 5 to 15 DAS in response of photoperiods (SDs and LDs) and response of Suc (suc− and suc+) in Col, C24, Col×C24 and C24×Col. *x*-axis and *y*-axis of each graph showed days after sowing (DAS) and leaf area, respectively. Asterisks indicate statistical difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by one-way ANOVA following Tukey's test). (I) Photographs are of representative seedling at 10-leaf stage of Col, C24 and their F_1 hybrids under different conditions. (J) Fresh weight and (K) leaf area of seedling at 10-leaf stage of Col, C24 and their F_1 hybrids under different conditions. Different letters indicate statistical difference ($p<0.05$ comparing among each lines in each growth condition by one-way ANOVA following Tukey's test). Data is average±SE, $n\geq12$.

This result means that the effects of heterosis could be assessed under these conditions in terms of an increase in leaf biomass (Figure 1I–K).

Overall variation of metabolite profiles with respect to photoperiod and exogenous sucrose

The purpose of this experiment was to assess metabolic changes stimulated by various growth conditions, which could enhance knowledge concerning the mechanism whereby different photoperiods and Suc affect the seedling development of various genotypes. A total of 237 metabolites were determined. Of these, 119 metabolites were identified or annotated (Supplementary Table S2).

Principal component analysis (PCA) score plots were applied to the data set for visualization using both annotated and unannotated metabolites (with six biological replicates, for each parent and F_1 hybrid at the 10-leaf stage) (Figure 2). Regarding the PCA score plot, genotypes including inbred lines (Col and C24) and their F_1 hybrids are shown, along with genotype-dependent separation, in principal component 1 (PC1) (Figure 2). This genotype-dependent separation was commonly observed in all four conditions ($n=18$, Figure 2B–E). This result suggests that most metabolite data variation can be attributed to differences in genetic background, rather than growth conditions. Thus, we focused on metabolite differences in Col and C24 because the two genotype-dependent separations can best explain the PC1 distribution.

Next, we conducted HCA to visualize the metabolites (variables) that were highlighted in terms of metabolite changes between the two parental lines (Supplementary Figure S1). The samples were

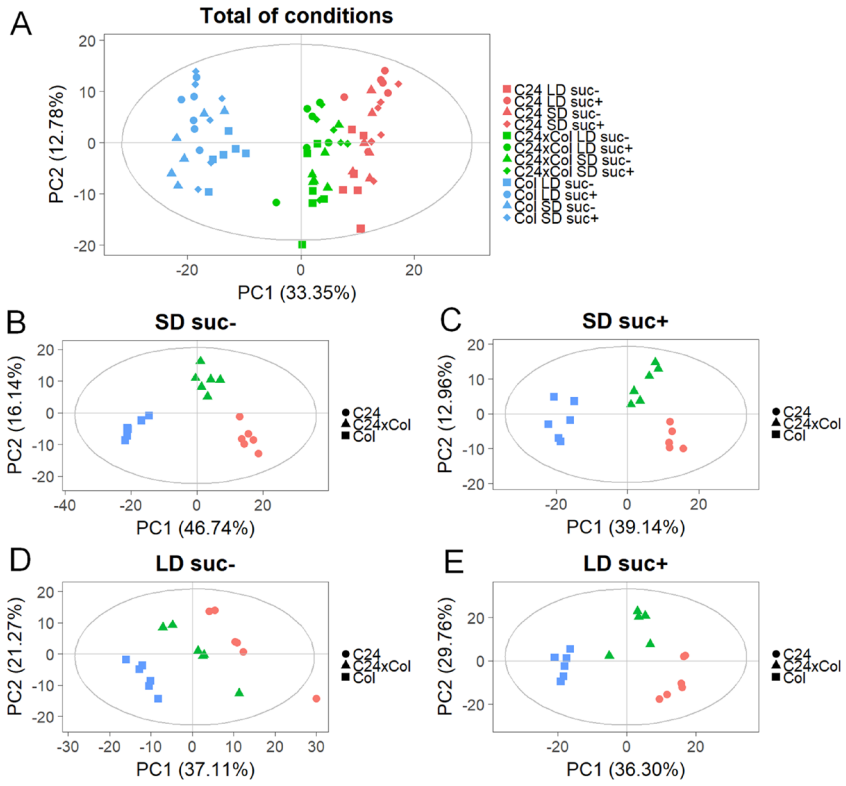

Figure 2. Principal component analysis (PCA) of untargeted metabolome data from leaf in Col, C24 and their F_1 hybrid under different growth conditions. (A) Total of conditions, (B) SD suc−, (C) SD suc+, (D) LD suc−, (E) LD suc+. Principle component 1 (PC1) explained 33.35%, 46.74%, 39.14%, 37.11% and 36.30% of the variability for whole data, SD suc−, SD suc+, LD suc− and LD suc+, respectively. Principle component 2 (PC2) was explained by 12.78% of total variance for the whole data set and 16.14%, 12.96%, 21.27% and 29.76% for SD suc−, SD suc+, LD suc− and LD suc+, respectively.

clustered into two distinct groups by genotype-specific response. The distribution pattern of metabolite changes showed that they were mostly dependent on the genotypes Col and C24, rather than on the photoperiod or presence of Suc (Supplementary Figure S1). In addition, metabolic changes in Col compared with C24 were largely downregulated under all growth conditions (Supplementary Figure S1). To understand the extent to which the metabolite ratio was affected by differences in genetic background (G) and other factors (photoperiod (P) and Suc supply (S)), a three-way ANOVA was conducted (Figure 3). The most prominent factor was genotype (Figure 3). A total of 68.3% (157 out of 230) of metabolites was significantly affected by genotype-dependent differences (Supplementary Table S2). Photoperiod and Suc influenced 37.0% and 43.0% of metabolite levels, respectively (Supplementary Table S2). The contributions of interaction effects between these three factors, i.e., genotype×photoperiod, genotype×Suc, photoperiod×Suc, and genotype×photoperiod×Suc were much smaller than that of genotype (Figure 3, Supplementary Table S2). Recently, many metabolites of three classes, including sugar and starch synthesis, amino acids and nitrogen assimilation, and TCA cycle,

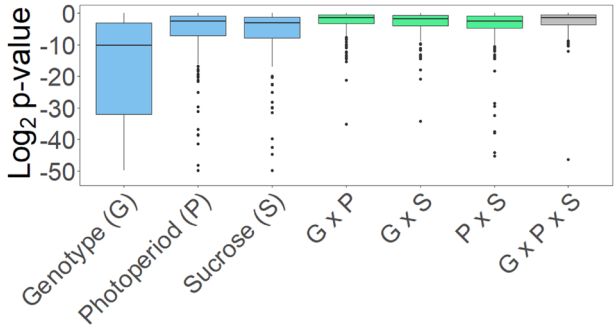

Figure 3. Box plots of the *p*-values from three-way ANOVA performed for the whole metabolites. *p*-value of every metabolite was followed $\log_2$-transformation to construct normal distribution. Genotype (G), photoperiod (P) and sucrose (S) mean metabolites in each category affect one factor (i.e., main effects). G×P, G×S, P×S and G×P×S mean interaction between factors (i.e., interaction effects).

were found to be not only significantly correlated, but also predictive for plant growth and development (Meyer et al. 2007; Sulpice et al. 2009). Hence, from the results of three-way ANOVA analysis, metabolites of these classes were divided into the following groups: group (1), no significant interaction effects but significant main effects; and group (2), significant interaction effects (Wei et al. 2012). Fourteen metabolites were extracted

Table 1. List of metabolites of Arabidopsis accessions under different growth conditions.

Name	Col				C24			
	SD suc−	SD suc+	LD suc−	LD suc+	SD suc−	SD suc+	LD suc−	LD suc+
Sugar and starch synthesis								
1,6-Anhydro-glucose	1 (a)	1.53 (ab)	2.88 (c)	1.74 (b)	13.68 (d)	15.32 (d)	12.49 (d)	11.86 (d)
Galactinol	1 (a)	1.74 (ac)	2.19 (bcd)	1.91 (bc)	1.12 (a)	2.79 (cd)	1.53 (ab)	4.16 (d)
Gluconate	1 (a)	1.51 (ab)	1.43 (ab)	1.08 (a)	1.75 (b)	1.95 (b)	1.58 (ab)	1.98 (b)
Glucose	1 (bc)	1.41 (c)	0.91 (b)	0.61 (a)	1.34 (c)	2.16 (d)	1.35 (c)	2.07 (d)
Maltose	1 (a)	2.87 (b)	1.24 (ab)	1.6 (ab)	2.25 (ab)	2.92 (b)	1.41 (ab)	2.89 (ab)
Raffinose	1 (a)	3.30 (ab)	6.11 (b)	1.74 (ab)	33.34 (c)	41.57 (c)	42.01 (c)	44.74 (c)
Sucrose	1 (ab)	1.39 (b)	0.86 (a)	1.16 (ab)	1.34 (b)	2.97 (d)	1.12 (ab)	1.99 (c)
Trehalose	1 (a)	2.71 (bc)	2.32 (b)	2.35 (bc)	2.63 (bc)	5.95 (d)	3.68 (c)	10.35 (e)
Fructose-6-phosphate	1 (ab)	1.33 (bc)	1.45 (bcd)	0.78 (a)	1.60 (ce)	2.28 (e)	2.00 (ce)	2.02 (de)
Glucose-6-phosphate	1 (ab)	1.38 (bc)	1.63 (bc)	0.68 (a)	1.67 (cd)	2.62 (d)	1.99 (cd)	1.77 (cd)
N assimilation and amino acids								
1,3-diamino-propane	1 (a)	0.96 (a)	1.10 (ab)	0.65 (a)	2.05 (bc)	2.83 (c)	1.98 (bc)	2.77 (c)
4-amino-butyrate	1 (a)	0.88 (a)	1.38 (ab)	0.96 (a)	1.28 (ab)	1.43 (ab)	1.13 (ab)	1.92 (b)
5-hydroxy-tryptamine	1 (cd)	1.62 (d)	0.52 (a)	0.89 (ac)	0.84 (ac)	1.22 (cd)	0.69 (ab)	1.06 (bcd)
Asparagine	1 (bcd)	1.09 (bcd)	0.63 (ab)	0.51 (a)	1.17 (ce)	1.72 (e)	0.86 (ac)	1.47 (de)
Aspartate	1 (ab)	1.65 (e)	1.3 (cd)	1.02 (ab)	1.17 (bc)	1.54 (de)	1.13 (ac)	0.88 (a)
Cystine	1 (ab)	1.10 (ab)	1 (ab)	0.41 (a)	4.11 (bc)	5.95 (bc)	8.06 (c)	2.01 (ab)
Glutamine	1 (bc)	1.09 (c)	0.72 (ab)	0.63 (a)	1.50 (d)	2.23 (e)	1.62 (d)	2.49 (e)
Methionine	1 (ab)	0.59 (a)	0.82 (a)	0.59 (a)	2.04 (c)	1.88 (c)	1.77 (bc)	1.65 (c)
Ornithine	1 (b)	0.55 (a)	0.72 (ab)	0.74 (ab)	4.71 (c)	5.23 (c)	4.06 (c)	3.56 (c)
Phenylalanine	1 (b)	0.45 (a)	0.77 (ab)	0.36 (a)	1.63 (b)	1.69 (b)	1.44 (b)	1.30 (b)
Phosphorate	1 (ac)	1.1 (bc)	1.45 (c)	0.89 (ab)	1.14 (bc)	1.23 (bc)	1.25 (bc)	0.69 (a)
Proline	1 (ab)	4.83 (b)	0.93 (a)	0.72 (a)	0.94 (ab)	5.22 (b)	1.27 (ab)	1.28 (ab)
Putrescine	1 (a)	1.10 (ab)	1.08 (ab)	1.10 (ab)	1.58 (c)	2.14 (d)	1.39 (bc)	2.65 (d)
Pyroglutamate	1 (ab)	1.15 (ac)	0.93 (a)	1.01 (a)	1.21 (bc)	1.89 (d)	1.27 (c)	1.85 (d)
Shikimate	1 (ab)	1.77 (cd)	1.36 (bc)	0.82 (a)	1.50 (c)	2.65 (d)	1.64 (c)	1.53 (c)
Spermidine	1 (b)	1.12 (b)	0.56 (ab)	0.50 (ab)	0.32 (a)	0.39 (a)	0.29 (a)	0.26 (a)
Threonine	1 (b)	0.99 (b)	0.92 (b)	0.54 (a)	1.26 (bc)	1.65 (c)	1.11 (bc)	1.04 (b)
Tryptamine	1 (b)	1.07 (b)	1.12 (b)	0.86 (ab)	0.9 (ab)	1.09 (b)	0.87 (ab)	0.77 (a)
Tryptophan	1 (c)	0.56 (ab)	0.76 (ac)	0.47 (a)	1.73 (c)	1.01 (c)	1.07 (bc)	1.00 (c)
Glycine	1 (b)	1.01 (b)	0.80 (b)	0.52 (a)	1.46 (c)	1.82 (c)	1.60 (c)	1.96 (c)
Serine	1 (ab)	0.76 (a)	0.80 (a)	1.14 (b)	1.57 (c)	1.31 (bc)	1.21 (bc)	1.31 (bc)
TCA cycle								
cis-Aconitate	1 (a)	0.89 (a)	3.50 (c)	1.24 (ab)	1.26 (ab)	1.39 (ab)	1.56 (ac)	2.64 (bc)
Citrate	1 (bc)	1.86 (d)	0.71 (ab)	0.65 (ab)	1.38 (cd)	1.96 (d)	0.52 (a)	0.47 (a)
Fumarate	1 (d)	1.04 (d)	1.03 (d)	0.83 (cd)	0.56 (ab)	0.54 (ab)	0.65 (bc)	0.43 (a)
Malate	1 (cd)	1.06 (d)	0.66 (bc)	0.48 (ab)	0.72 (bd)	0.78 (bd)	0.59 (ab)	0.34 (a)
Succinate	1 (c)	1.41 (de)	0.76 (ab)	0.64 (a)	1.15 (ce)	1.44 (e)	1.07 (cd)	0.90 (bc)

Data included annotated metabolites in group (2) with interaction effects of factors. It represented means of normalized intensity data (relative to Col ecotype in SD suc−). Different letters indicate statistical differences between samples for specific metabolites by one-way ANOVA following Tukey's test ($p<0.05$).

from group (1) (Supplementary Table S3). In group (2), 36 metabolites were identified and extracted by Tukey's multiple comparison tests of treatment means (Table 1). A decrease in the levels of metabolites was observed in Col when compared with C24, except for β-alanine, spermidine, and fumarate (Table 1, Supplementary Table S3). In particular, there is a remarkable increase in sugars in C24 compared with Col (Figure 4A). Raffinose levels showed a greater than 30-fold decrease in Col compared with C24 under SD suc−. Similarly, 1,6-anhydro-glucose levels were greater than 13-fold lower in Col than in C24 under SD suc− (Table 1, Figure 4A). These metabolite levels in the F_1 hybrids were relatively similar to those in C24 (Supplementary Figure S2).

The TCA cycle has emerged as a central mitochondrial hub, necessary to drive ATP production used in photosynthesis optimization (Sweetlove et al. 2010). Analyses of TCA cycle intermediates showed that there was a significant increase in the level of fumarate in Col compared with that in C24. This trend was observed under all experimental growth conditions (Figure 4B, Table 1). This result is well supported by a previous publication regarding a natural promoter polymorphism of *FUM2* between Col and C24 alleles (Riewe et al. 2016). The biomass of Col and C24 grown under LD was significantly increased compared with that for the two genotypes grown under SD (Figure 1A, B). Interestingly, the fumarate/malate ratio also increased by 1.5- and 1.6-

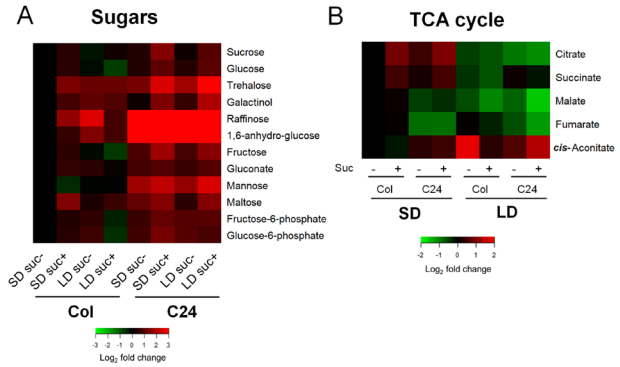

Figure 4. Heat map of metabolites of (A) sugars and (B) intermediates of TCA cycle between Col and C24 under different growth conditions. The heat map values represent a $\log_2$ ratio of Col and C24 under different growth conditions relative to SD suc− in Col (e.g., LD suc−/SD suc−). The red color indicates increased values, green indicates decreased values and black indicates zero; see the color scale.

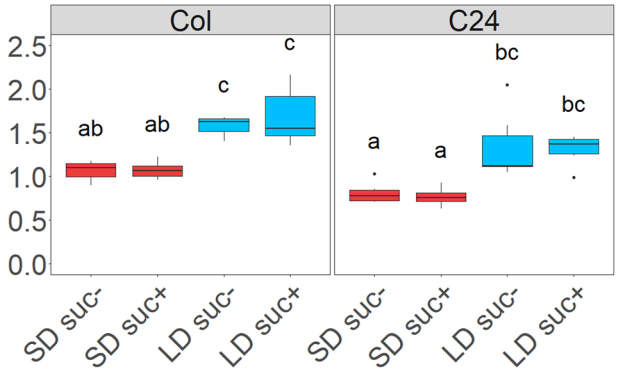

Figure 5. The ratio of fumarate to malate under different growth conditions. Data was visualized using the ratio of normalized intensity between fumarate and malate. Different letters indicate statistical differences ($p<0.05$, comparing among each lines in each growth condition by one-way ANOVA following Tukey's test).

fold under LD suc− and LD suc+ in the profiles of Col and 1.7-fold under both of LD suc− and LD suc+ in C24 when compared with that grown under SD (Figure 5). This observation suggests a tight correlation between the photoperiod-dependent increase in biomass and the high fumarate/malate ratio, but this correlation is not present with respect to the presence or absence of Suc (Figure 5). Moreover, photoperiod-induced changes in citrate, malate, and succinate levels were observed in Col, C24, and their F_1 hybrid (Table 1, Figure 4B, Supplementary Figure S2). The three-way ANOVA also indicated that photoperiod significantly influenced these three metabolites, with $p<0.0001$ (Supplementary Table S4). When Col and C24 were grown under SD with or without Suc, the levels of citrate, malate and succinate increased compared with than LD suc+/− (Table 1, Figure 4B). Our findings clearly suggest that the three metabolites in Col, C24, and their F_1 hybrid show common responses to photoperiod, while the Suc supply had no effect. The TCA cycle may play the key role(s) in regulating an increase in biomass when grown under LD.

As already mentioned, C24 had little response to exogenous Suc supply with respect to enhancement of growth and development (Figure 1F). Hence, to obtain more insight into the metabolite changes associated with exogenous Suc, we chose metabolites that were significantly affected by Suc supply (Table 1, Supplementary Figure S3). The levels of the seven metabolites in the profiles of C24 were significantly higher with Suc supply under both SD and LD, but not in Col (Tukey's test, $p<0.05$) (Table 1, Supplementary Figure S3). In detail, four sugars (sucrose, glucose, galactinol, and trehalose), two amino acids (pyroglutamate and glutamine), and putrescine, which is classified in the polyamine group, were significantly increased in C24 in the presence of exogenous Suc under SD and LD (Supplementary Figure S3B). The high amounts of these metabolic features might be simultaneous effects of genotype, Suc, and their interaction (three-way ANOVA, $p<0.01$) (Supplementary Table S4). Such high levels of sugars were found in C24 under suc+ in comparison with suc− (Figure 4A). The sucrose level increased 2.2- (for SD) and 1.8-fold (for LD) in the presence of Suc when compared with that in suc−. In addition, glucose also showed an increase of 1.6- and 1.5-fold under SD and LD, respectively, with Suc supply. The galactinol level increased 2.5-fold under SD and 2.7-fold under LD with suc+ compared with that in suc− (Table 1, Supplementary Figure S3B). Therefore, sugar accumulation might be a likely explanation for inefficient usage of exogenous Suc supply in C24.

Expression levels of sucrose transporter genes, AtSUCs

In Arabidopsis, nine different SUCs have been identified (Lemoine 2000; Sauer et al. 2004; Williams et al. 2000). Although the well characterized function of SUCs in plants is the uptake of sucrose into the phloem for long-distance transport of photoassimilates (Rottmann et al. 2018), the mechanism of uptake of exogenous Suc from media are less well understood. To examine whether sugar overaccumulation in C24 attributed to the genetic background of Suc uptake activity in root, we investigated the expression patterns of the *AtSUCs* in root of C24. Within nine *AtSUCs*, *AtSUC1* and *AtSUC2* have high expression. *AtSUC3*, *AtSUC4*, and *AtSUC5* were expressed extremely low (below less than 7% of *AtSUC1*), and *SUC6*, *SUC7*, *SUC8* and *SUC9* were not expressed (data not shown). The previous report also showed that *AtSUC1* and *AtSUC2* of Col were expressed high in root (Durand et al. 2018), therefore relative expression of *AtSUC1* and *AtSUC2* in root of Col and C24 with or without Suc was further analyzed (Figure 6).

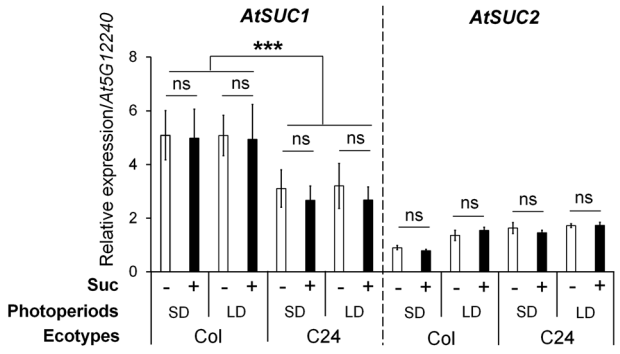

Figure 6. Expression levels of sucrose transporters *AtSUC1* and *AtSUC2* in Arabidopsis root. Transcript levels were measured by qRT-PCR. Each data was shown as normalized expression to the reference gene *At5G12240*. Suc−: white bars; Suc+: black bars. Average and standard errors of three biological replicates were shown. Asterisks and ns indicate statistical difference (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. $p>0.05$, by student's *t*-test).

Expression of both *AtSUC1* and *AtSUC2* genes in root was not induced by exogenous Suc supply in both Col and C24, and each expression levels in C24 were not higher than that of Col (Figure 6). Similar expression pattern was also observed in *AtSUC3*, *AtSUC4*, and *AtSUC5* (data not shown). Thus, overaccumulation of sugars in C24 might not be caused by exogenous Suc nor by genotype-dependent enhancement of Suc uptake ability.

Discussion

In plants, the environment has an effect on growth and development, changes in the molecular basis (Sun et al. 2017; Yanovsky and Kay 2002), and metabolic activity (Kusano et al. 2011a; Sulpice et al. 2013). Recently, metabolic phenotypes have been used to indicate the relationships among accessions with geographical variability (Kleessen et al. 2012). Metabolomic changes in natural variation have been observed among Arabidopsis accessions (Lisec et al. 2009; Sulpice et al. 2009, 2013). We have little knowledge regarding the extent of metabolite changes observed in genotypes under various growth conditions, i.e., photoperiod and the presence or absence of exogenous Suc supply. In PCA and HCA, comparison of metabolite profiles between Col and C24 showed clear divergences (Figure 2A, Supplementary Figure S1). Moreover, we demonstrated that Col and C24 had different metabolic features, with approximately 68.3% of metabolites affected by genetic background differences. These results suggest that natural genetic variation is the key contribution to primary metabolism. Significant effects of photoperiod and Suc factors accounted for 37.0% and 43.0% of the detected metabolites, respectively. This result implies an important role(s) for metabolomic reorganization in adapting to new environmental conditions (Caldana et al. 2011; Kusano et al. 2011b). Additionally, interactive effects of natural multifactorial conditions induce metabolite signatures in plant species (Heyneke et al. 2017; Sato and Yanagisawa 2014). Our data also suggest tight interconnections among genotype, photoperiod, and the presence of exogenous Suc affecting the metabolism of carbohydrates, as well as metabolites belonging to the TCA cycle.

The photoperiod has an effect on plant growth and development (Baerenfaller et al. 2015). Total light intensity and duration were taken into account to determine changes in biomass under the four carbon source regimes regarding Col and C24 ecotype growth (Figure 1A, B). Consistent with phenotyping data, LD induced an increasing ratio of fumarate to malate (Figure 5). Supporting our findings, an enhanced fumarate/malate ratio is associated with enhanced growth traits, including biomass, which has been confirmed by large-scale analysis of an Arabidopsis accession population (Riewe et al. 2016). In addition, we found higher levels of intermediate products in the TCA cycle, citrate, malate, and succinate under SD, as in previous studies (Fukushima et al. 2009; Nakamichi et al. 2009). Our data can provide novel insights to explain the close association of the circadian clock with the TCA cycle, although further analysis is required to uncover a specific hypothesis.

Natural variations of Arabidopsis accessions show different phenotypic responses to the presence of exogenous Suc (Teng et al. 2005; Wang et al. 2019; Wuyts et al. 2011). In our data, Col, C24, and F_1 were observed to have different responses to enhanced growth as well as interaction between genotype and Suc on metabolites (Figure 1E–H, Table 1). Col and the F_1 hybrid can use exogenous Suc to achieve a significant increase in leaf area, while Suc was not effective in stimulating the growth of C24. As previously reported, primary root elongation of C24 showed a non-inductive effect when 1% Suc was added to the medium. However, in contrast to C24, Col roots exhibited significant differences in the presence of Suc (Wuyts et al. 2011). The addition of exogenous auxin or high Suc concentration in the medium has been reported to increase the growth of L*er*. Conversely, C24 has a negative response to exogenous auxin and is only slightly affected by increasing Suc concentration (Wang et al. 2019). Our results provide a broader view of how accessions interact with Suc treatment on the metabolome. C24 generally showed a weak response to exogenous Suc supply, while Col demonstrated efficient usage, and the F_1 hybrid might be influenced by the dominant inheritance patterns of their Col genetic background.

Our present data suggest that overaccumulation of sugars in C24 might not be caused by enhancement of Suc uptake ability, whereas, the mechanisms of uptake

of exogenous Suc from media are less well understood. There is still room for verification of involvement of Suc uptake by different classes of sugar transport proteins.

Meyer et al. (2012) reported the transition of metabolite composition from a maternal pattern to a genetic-dependent pattern in Col, C24, and F_1 hybrids. In their study, Col, C24, and F_1 hybrids were separated as three clusters at 8, 10, and 15 DAS; then, the group of reciprocal hybrids were more similar to C24 at the latest time point under LD. Despite the difference in photoperiod and exogenous Suc supply, we were able to distinguish among Col, C24, and F_1 by their metabolite phenotyping. At the 10-leaf stage, the F_1 hybrid had a closer relationship with C24 (Figure 2A). This result suggests that all 10-leaf stage samples reached a similar developmental stage, even though they were collected under different growth conditions. Moreover, the heterosis phenomenon was exhibited under all growth conditions (Figure 1I–K). Therefore, growth conditions may not affect the heterosis mechanism.

In conclusion, the untargeted metabolite profiling captured metabolomic differences between the three genotypes. The results suggest that metabolomic analysis is a useful approach to understanding how accession functions under various growth conditions in a new dimension. There was a clear association among photoperiod, biomass, and intermediates in the TCA cycle. Regarding exogenous Suc response, we found interaction effects between genotype and Suc on the metabolite profiles, especially on sugar profiles. This may affect the efficient use of Suc at different levels to enhance growth among various accessions. Our finding supplies an excellent example of correlations among phenotype, genotype, and metabolites. The results provide new insights into how plant metabolomic functions respond to environmental conditions.

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Conflicts of interest

No potential conflicts of interest are declared in our study.

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