Characterization of Metabolic States of Arabidopsis thaliana Under Diverse Carbon and Nitrogen Nutrient Conditions via Targeted Metabolomic Analysis

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Plant growth and metabolism are regulated in response to various environmental factors. To investigate modulations in plant metabolism by the combined action of elevated atmospheric CO2 concentration and other nutritional factors, we performed targeted metabolomic analysis using Arabidopsis thaliana plants grown under 24 different conditions where the CO2 concentration, amounts and species of nitrogen source, and light intensity were modified. Our results indicate that both the biosynthesis of diverse metabolites and growth are promoted in proportion to the CO2 concentration at a wide range of CO2 levels, from ambient concentrations to an extremely high concentration (3,600 p.p.m.) of CO2. This suggests that A. thaliana has the potential to utilize effectively very high concentrations of CO2. On the other hand, ammonium (but not nitrate) supplied as an additional nitrogen source induced drastic alterations in metabolite composition, including increases in the contents of glucose, starch and several amino acids, and reductions in the tricarboxylic acid (TCA) cycle-related organic acid content under any CO2 conditions. Hierarchical clustering analysis using the metabolite profiles revealed that ammonium is a prominent factor determining metabolic status, while the CO2 concentration is not. However, ammonium-induced metabolic alterations were differently modified by high concentrations of CO2. Hence, our results imply that increases in CO2 concentration may differently influence plant metabolism depending on the nitrogen nutrient conditions.

Keywords: Ammonium response • Arabidopsis thaliana • CO2 response • Nitrogen response • Targeted metabolomic analysis.

Abbreviations: CE-MS, capillary electrophoresis-mass spectrometry; Fum, fumarate; G1P, glucose 1-phosphate; IC-PAD, ion chromatography with pulsed amperometry detection; Mal, malate; 2OG, 2-oxoglutarate; RuBP, ribulose 1,5-bisphosphate; RubiscO, ribulose 1,5-bisphosphate carboxylase/oxygenase; TCA, tricarboxylic acid.

Introduction

Plant metabolism, substance production and growth are regulated in response to various environmental factors. These factors include the nutrient and light conditions under which the individual plants grow, because plants synthesize diverse organic compounds using atmospheric carbon dioxide (CO2) and nutrient elements in the soil. Since plants synthesize carbohydrates from atmospheric CO2 via photosynthesis, a process that comprises the heart of plant metabolic processes, the recent increase in the concentration of atmospheric CO2 may affect the rate of photosynthesis and, hence, the rate of organic compound production and plant growth. Accordingly, the effects of elevated concentrations of CO2 on plant metabolism and growth have become a matter of urgent concern. Since atmospheric CO2 is initially assimilated into 3-phosphoglycerate by the carboxylase reaction catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), increases in atmospheric CO2 concentration are expected to promote photosynthesis by providing larger amounts of substrate for the carboxylase reactions as well as by restraining the oxygenase reaction by RubisCO during short-term exposure (Sage et al. 1989, Lawlor and Mitchell 1991, Stitt 1991, Van der Kooij et al. 1999). However, it was also reported that plants decrease their rates of photosynthesis during long-term exposure to high concentrations of CO2 (Sage et al. 1989, Stitt 1991, Stitt and Krapp 1999). Thus, plant responses to high concentrations of CO2 appear to be complicated. Although examining modulations in stomatal density and aperture was expected to help reveal plant acclimation responses to high concentrations of CO2, stomatal responses to high CO2 concentrations were found to differ among plant species (Drake et al. 1997). On the other hand, the transcript levels of photosynthesis-related genes decrease in plants acclimated to high CO2 conditions, implying that elevated levels of sugars under high CO2 conditions may suppress the expression of photosynthesis-related genes, leading to decreases in growth (Drake et al. 1997, Cheng et al. 1998).
Nitrogen (N) assimilation is another vital aspect of plant metabolism, which is absolutely necessary for the biosynthesis of N-involving organic compounds, including amino acids, proteins, nucleotides, nucleic acids and Chl (Lam et al. 1996). N assimilation and photosynthesis are closely interdependent (Huppe and Turpin 1994, Lawlor 2002). Photosynthesis provides carbon (C) skeletons and reducing power for N assimilation. Conversely, a large portion of assimilated N is devoted to maintaining photosynthesis. Actually, approximately 20% of assimilated N is distributed into RubisCO protein in Arabidopsis thaliana leaves (Izumi et al., 2010). Chl, a pigment essential for photosynthesis, is also biosynthesized in response to the N assimilation rate (Foyer et al. 2003). Furthermore, metabolite profiling with tomato plants grown using 8 mM and 0.4 mM nitrate, which were set for the N-sufficient and N-deficient conditions, respectively, revealed that nitrate nutrition has wide-ranging effects on plant metabolism (Urbanczyk-Wochniak and Fernie 2005). N availability is therefore a possible critical environmental factor that strongly influences the effects of elevated CO2 concentrations on plant metabolism. In fact, it has been shown that decreased growth in response to high concentrations of CO2 does not occur under N-abundant conditions, implying that decreases in the protein content and/or N-involving metabolite contents under high CO2 conditions may lead to a reduction in photosynthesis (Geiger et al. 1999, Stitt and Krapp 1999, Seneweera et al. 2011). Differences in N availability are also known to influence enzyme activity and photosynthesis rates under high CO2 conditions (Geiger et al. 1999, Stitt and Krapp 1999, Sun et al. 2002, Tocquin et al. 2006).

In addition to total amounts of available N sources, differences in species of N sources might affect the effects caused by elevated concentrations of CO2. Nitrate and ammonium in the soil are major N sources of plants. Since nitrate acts as a signaling molecule and modulates expression of the genes involved in photosynthesis, glycolysis and the tricarboxylic acid (TCA) cycle, as well as genes involved in nitrate reduction and assimilation (Scheible et al. 1997, Stitt 1999), nitrate uptake may influence C metabolism through modifications in gene expression. Furthermore, a recent study also revealed that ammonium specifically induces the expression of a number of genes that are not up-regulated by nitrate (Patterson et al. 2010). Nitrate and ammonium supplies were also found to lead to distinct phosphorylation patterns of proteins (Engelsberger and Schulze 2012). The metabolite compositions of nitrate-grown plants have been shown to differ from those of ammonium-grown plants (Chaillou et al. 1991, Geiger et al. 1999, Pasqualini et al. 2001, Hachiya et al. 2012, Masakapalli et al. 2013). Thus, differences in species of N sources are a possible factor that might influence the effects of elevated concentrations of CO2 on plant metabolism and growth.

Light intensity is another critical environmental factor involved in plant growth. ATP (which delivers chemical energy) and the reduced form of ferredoxin and NADPH (which deliver reducing power to metabolic reactions) are produced using light energy captured by Chl. Because large amounts of these molecules are consumed for regeneration of ribulose 1,5-bisphosphate (RuBP) in the Calvin cycle and N assimilation in chloroplasts (Huppe and Turpin 1994, Lawlor 2002), light energy is indispensable for the functions of both photosynthesis and N assimilation (Buchanan 1980). In fact, several studies revealed that the sizes of amino acid, organic acid and carbohydrate pools are modulated by light quantity and quality (Bräutigam et al. 2009, Wulff-zottele et al. 2010, Jänkänpää et al. 2012). Furthermore, illumination also affects primary metabolism in different manners. For instance, the carboxylation activity of RubisCO is enhanced by light, because the ADP/ATP ratio and redox potential in chloroplasts regulate the activity of RubisCO activase, which modulates the activation rate of RubisCO (Zhang and Portis 1999, Portis 2003). Hence, light intensity may represent an environmental factor that influences the modulation of plant metabolism in response to elevated CO2 concentrations.

In the present study, to reveal how plant metabolism is modulated by the combined action of elevated CO2 concentrations and other nutritional factors, particularly differences in the N nutrient condition, we examined the metabolic states of A. thaliana plants grown under various growth conditions by profiling their metabolite contents.

Results and Discussion

Growth of A. thaliana plants under various growth conditions

To investigate the combined effects of elevated CO2 concentrations and other nutritional factors on metabolism and growth, we initially assessed the growth of A. thaliana plants under various growth conditions by measuring the fresh and dry weights, C and N contents, and starch and Chl contents of the plants (Figs. 1–3; Supplementary Table S1). We used two light intensities (50 and 200 μEm⁻² s⁻¹ for the low and high light conditions, respectively) and four N nutrient conditions (10 mM nitrate, 10 mM nitrate plus 1 mM ammonium, 10 mM nitrate plus 10 mM ammonium, and 20 mM nitrate). We also used three CO2 concentrations (ambient, 1,200 p.p.m. and 3,600 p.p.m.). The CO2 concentrations in the Silurian and Devonian periods (when land plants emerged), and the Triassic and early Jurassic periods (when angiosperms emerged), are estimated to be approximately 3,600 and 1,200 p.p.m., respectively (Berner and Kothava 2001). We thus employed a total of 24 growth conditions (Figs. 1, 2; Supplementary Table S1).

As shown in Fig. 1, the plants grown under high light conditions exhibited significantly higher fresh weights and starch contents than those grown under low light conditions. These high light effects were markedly increased in proportion to the increase in CO2 concentration. On the other hand, increases in N availability did not produce pronounced effects on fresh weights under high or low light conditions, except for the ‘3600 p.p.m. CO2 and high light’ condition. Under this growth
CO2 concentrations reduced the N content by half under high light conditions (Fig. 3C), the dry weight per shoot in plants under high CO2 conditions was almost double that under low CO2 conditions (Fig. 3A). The N contents of plants grown with 10 and 20 mM nitrate and both 10 mM nitrate and 10 mM ammonium under the high light and ambient CO2 condition were 0.117, 0.119 and 0.160 mg per shoot, respectively, whereas they were 0.155, 0.199 and 0.203 mg per shoot under the high light and high CO2 (3,600 ppm) condition. Hence, high CO2 concentrations greatly elevated the C content per shoot but only slightly increased the N content per shoot. Taken together, these results indicate that the growth of A. thaliana is prompted in proportion to increases in CO2 concentrations due to the plant’s potential to utilize high concentrations of CO2 when light energy and N sources are abundant, whereas differences in the species of N sources influence metabolic balance rather than growth itself. The distinct metabolic balances in response to differences in the species of N sources were further clarified by different correlations between the starch and N contents in the plants grown with nitrate as the sole N source and the plants grown with 10 mM ammonium as an additional N source (Fig. 3E).

**Targeted metabolomic analysis of primary metabolism**

To scrutinize the metabolic states of A. thaliana plants under different growth conditions, we employed targeted metabolomic analysis of major metabolites. Using capillary electrophoresis-mass spectrometry (CE-MS) and ion chromatography with pulsed amperometry detection (IC-PAD), we could analyze the contents of 71 metabolites in the Calvin and TCA cycles and in pathways for sugar and starch synthesis, glycolysis, photorespiration, N assimilation and amino acid biosynthesis. These 71 metabolites and their abbreviations are listed in Table 1; we will use abbreviations to describe these metabolites in principle hereafter. By performing targeted metabolomic analysis, we successfully measured the contents of 57–61 of the 71 metabolites from each shoot sample using a criterion in which the signal-to-noise ratio required to confirm the presence of a metabolite by MS analysis was >3 (Table 2). However, we could not detect eight of the metabolites (E4P, Gal1P, GAP, Gco, Gox, OH-Pyr, CoA and AccoA) in any of the samples and four of the metabolites (DHAP, SBP, Cys and NADH) in most of the samples. As the contents of the other 59 metabolites could be measured in most of the samples, we evaluated the metabolic states of the plants using data sets for 59 metabolites. We note that the RSD% values of these metabolite measurements were between 13.7% and 33.3% in general (Table 2), although the values of several metabolites including monosaccharides (Gluc and Galc), sugar phosphates in the Calvin cycle (RuBP, Ru5P, FBP and S7P), and those associated with redox regulation (AsA and GSH) were >40% (Supplementary Tables S2–S4). The complete metabolite profiles are shown in Supplementary Tables S2–S4.

**Differences in metabolite profiles of A. thaliana plants under different growth conditions**

The metabolite profiles reveal that although the contents of individual metabolites were quite different from one another and ranged from <1 nmol g\(^{-1}\) FW to >1 nmol g\(^{-1}\) FW, the contents of most metabolites were between 10 nmol g\(^{-1}\) FW and several hundred nmol g\(^{-1}\) FW under all of the growth conditions examined (Fig. 4). However, the total metabolite content increased in parallel with the increase in CO2 concentration under high light conditions, independent of the N nutrient condition (Figs. 4, 5), which is consistent with the results of analyses of dry and fresh weights and C and N contents (Figs. 1, 3). On the other hand, ammonium-induced changes in metabolite composition were clearly observed under any
When the 59 analyzed metabolites were classified into seven groups (metabolites related to sugar and starch synthesis, metabolites related to the Calvin cycle, metabolites related to photorespiration, metabolites related to the TCA cycle, amino acids and metabolites related to N assimilation, metabolites related to redox regulation, and nucleotides) (Fig. 5), the use of ammonium (but not nitrate) as an additional N source greatly reduced the contents of organic acids and increased the amino acid and sugar contents, as reported previously (Chaillou et al. 1991, Geiger et al. 1999, Pasqualini et al. 2001, Hachiya et al. 2012, Masakapalli et al. 2013). These ammonium-induced changes in metabolite composition were observed under both light conditions, although they appeared to be more evident under the high light conditions (Fig. 5). Furthermore, the effects were more pronounced under high CO₂ conditions (Fig. 5). Increases in the amount of N source also appeared to have only slightly positive effects on the total metabolite content, except when ammonium was additionally supplied under high light conditions with ambient concentrations of CO₂. Thus, the effects of additionally supplied N sources contrast with the observation that N deficiency exerted wide-ranging effects on metabolism in tomato plants (Urbanczyk-Wochniak and Fernie 2005). We also note that organic acid and sugar pools were enlarged under the high light conditions as described in Jänkänpää et al. (2012) and Urbanczyk-Wochniak and Fernie (2005).

To evaluate the effects of differences in light intensity and the CO₂ concentration on the ammonium-induced changes in metabolite composition in detail, we calculated the ratios of individual metabolites to all metabolites analyzed under each metabolic state (Fig. 6). Previous reports revealed that the presence of ammonium as an N source causes a large reduction in the contents of the TCA cycle-related organic acids, fumarate (Fum) and malate (Mal), and a large increase in the contents of Gln, Asn and Arg (Chaillou et al. 1991, Geiger et al. 1999, Pasqualini et al. 2001, Hachiya et al. 2012, Masakapalli et al. 2013), probably due
to the rapid consumption of absorbed ammonium via assimilation into amino acids that contain organic acids as their carbon skeletons. Our results indicated that the use of ammonium as a supplement fundamentally induces such changes in the metabolite composition independently of light intensity and CO₂ concentration. However, our results also indicated that the metabolite composition of A. thaliana plants grown with N sources containing ammonium was easily modified by other environmental factors (the CO₂ concentration and light intensity). For instance, ammonium as an additional N source reduced the Mal content similarly under any growth condition but the Fum content to different extents under different light and CO₂ conditions (Fig. 6). Interestingly, the metabolite composition of A. thaliana plants grown with an N source comprising nitrate only was robustly maintained under diverse growth conditions (Fig. 6).

**Cluster analysis using the obtained metabolite profiles**

The metabolite profiles indicate that increases in the CO₂ concentration and differences in the N source have different effects on the metabolic state in A. thaliana. To investigate which environmental factor predominantly determines the metabolic status of A. thaliana, we performed cluster analysis using the 24 metabolite profiles that we obtained (Fig. 7). The hierarchical dendrogram indicates that the metabolite profiles under the 24 growth conditions examined could be classified into four groups (C1–C4). The C1 group solely consists of plants grown under all growth conditions employing a 10 mM ammonium supplement. In addition, the C2 and C3 groups consist of plants grown under high light growth conditions, while the C4 group includes only plants grown under low light growth conditions (Fig. 7). Thus, the presence of 10 mM ammonium was identified as the most prominent factor determining the metabolic state of A. thaliana, and the difference in light intensity was the second most important factor. Plants grown in the presence of 1 mM ammonium supplementation were classified into three groups (C2–C4 groups), suggesting that 1 mM ammonium was insufficient to alter the metabolic balance greatly, although 1 and 10 mM ammonium supplements induced similar alterations in metabolite composition to different extents (Fig. 5). On the other hand, we did not identify a cluster reflecting differences in CO₂ concentration, although the C2 and C3 groups appeared to be formed, reflecting differences in CO₂ concentration. Thus, differences in CO₂ concentration may be a weak factor determining the metabolic balance of the plants.

The hierarchical dendrogram also reveals that the levels of some metabolites are modulated in a similar manner. The metabolites that were analyzed can be classified into nine groups, i.e. M1–M9 (Fig. 7). The M1 group consists of metabolites that are present at low levels in the presence of 10 mM ammonium, but at higher levels under high light conditions in the absence of 10 mM ammonium supplementation. Metabolites in the M1 group basically comprise organic acids involved in the TCA

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**Fig. 3** Dry weight (A), C content (B), N content (C) and N/C ratio (D) in shoots of A. thaliana plants grown for 16 d under 12 different growth conditions, and correlation diagram between starch and N contents (E). The 12 different growth conditions are detailed in Supplementary Table S1. Different letters indicate statistical differences detected by Student’s t-test (P < 0.05) in (A–D).
The M2 group contains metabolites that are more abundant in high light than in low light, regardless of the N nutrient condition or the concentration of CO₂. This group includes Glu, which provide amino groups for the synthesis of other amino acids, as well as metabolites in the glutathione–ascorbate cycle that function in the oxidative stress response under strong light (AsA and GSSG), and amino acids that are produced by the shikimic acid pathway (Tyr and Trp). The M3 group of which the characteristics are currently unknown includes Met, Thr and NADPH. The metabolites in the M4 group were present at higher concentrations under the growth conditions described for the C1–C3 groups. The M4 group includes various metabolites, such as those linking the glycolysis pathway to the TCA cycle (3PG and Pyr) and other metabolites including ADPG and 2-oxoglutarate (2OG). The M5 group consists of a Calvin cycle-related sugar phosphate (S7P) as well as amino acids.

Table 1 Metabolites analyzed by our targeted metabolomics

| No. | Compound | Abbreviation | No. | Compound | Abbreviation |
|-----|----------|--------------|-----|----------|--------------|
| 1   | Sucrose  | Sucr         | 36  | 2-Oxoglutarate | 2OG |
| 2   | Galactose| Galc         | 37  | Succinate  | Suc |
| 3   | Glucose  | Gluc         | 38  | Alanine    | Ala |
| 4   | Fructose | Fruc         | 39  | Arginine   | Arg |
| 5   | ADP-glucose | ADPG     | 40  | Asparagine | Asn |
| 6   | UDP-glucose | UDPG   | 41  | Aspartic acid | Asp |
| 7   | Galactose 1-phosphate | Gal1P | 42  | Cysteine | Cys |
| 8   | Glucose 1-phosphate | G1P | 43  | Glutamine | Gln |
| 9   | Mannose 6-phosphate | M6P | 44  | Glutamic acid | Glu |
|     |          |              |     |          |              |
| Group A: sugars and starch syntheses (9) | | | Group E: N assimilation and amino acids (22) | | |
| 10  | Dihydroxyacetonephosphate | DHAP | 45  | Histidine | His |
| 11  | Erythrose 4-phosphate | E4P | 46  | Isoleucine | Ile |
| 12  | Fructose 6-phosphate | F6P | 47  | Leucine | Leu |
| 13  | Fructose 1,6-bisphosphate | FBP | 48  | Lysine | Lys |
| 14  | Glucose 6-phosphate | G6P | 49  | Methionine | Met |
| 15  | Glyceraldehyde 3-phosphate | GAP | 50  | Ornithine | Orn |
| 16  | Lactate | Lac         | 51  | Phenylalanine | Phe |
| 17  | Phosphoenolpyruvate | PEP | 52  | Proline | Pro |
| 18  | 3-phosphoglycerate | 3PG | 53  | Threonine | Thr |
| 19  | Pyruvate | Pyr | 54  | Tryptophan | Trp |
| 20  | Ribose 5-phosphate | R5P | 55  | Tyrosine | Tyr |
| 21  | Ribulose 5-phosphate | Ru5P | 56  | Valine | Val |
| 22  | Ribulose 1,5-bisphosphate | RubP | 57  | o-Acetylserylne | OAS |
| 23  | Sedoheptulose 7-phosphate | S7P | 58  | Citrulline | Ctr |
| 24  | Sedoheptulose 1,7-bisphosphate | SBP | 59  | γ-Aminobutyrate | GABA |

Group B: Calvin cycle and glycolysis (15)

| 25  | Glycerate | Gce | 60  | Ascorbate | AsA |
| 26  | Glycolate | Gco | 61  | Glutathione | GSH |
| 27  | Glyoxylate | Gox | 62  | Glutathione disulfide | GSSG |
| 28  | Hydroxypyruvate | OH-Pyr | 63  | NAD | NAD |
| 29  | Glycine | Gly | 64  | NADH | NADH |
| 30  | Serine | Ser | 65  | NADP | NADP |

Group C: photorespiration (6)

| 31  | cis-Aconitate | cisAco | 66  | NADPH | NADPH |
| 32  | Citrate | Cit | 67  | AMP | AMP |
| 33  | Isocitrate | Isocit | 68  | ADP | ADP |
| 34  | Fumarate | Fum | 69  | ATP | ATP |
| 35  | Malate | Mal | 70  | Coenzyme A | CoA |
| 36  | 2-Oxoglutarate | 2OG | 71  | Acetyl-coenzyme A | AcCoA |

Group D: TCA cycle (7)

Group G: nucleotides and coenzymes (5)

The Metabolic states under high CO₂ conditions.
acids generated by photorespiration (Gly and Ser). Like metabolites in the M4 group, the metabolites in this group appeared to be present at higher concentrations under the growth conditions described for the C1–C3 groups. The M6 group includes GSH, which reduces other compounds and metabolites of carbohydrate metabolism such as glucose 1-phosphate (G1P), UDPG and M6P. The characteristics of the M6 group are currently unknown. The M7 group consists of sugar phosphates, sugars and ATP. The metabolites in this group appeared to increase in abundance under conditions in which both the light intensity and CO2 concentrations were high. The M8 group comprises amino acids whose contents increase in response to supplementation with 10 mM ammonium (i.e. Arg, His, Gln and Asn). The M9 group includes a variety of metabolites (i.e. RuBP, NADP, PEP, Val, Pro and Ala). The increases or decreases in fresh weight and starch content under different growth conditions were similar to the differences observed in the abundance of metabolites of the M7 group (Fig. 7), suggesting a correlation between biomass and the abundance of M7 group metabolites whose synthesis is promoted by high concentrations of CO2. On the other hand, increases or decreases in the Chl content appear to be synchronized with those of the contents of the M8 group metabolites, although 1 mM ammonium appeared to be sufficient to increase the Chl content under some conditions. This observation is consistent with the proposal that the Chl content can serve as a marker for N utilization efficiency (Foyer et al. 2003).

### Table 2 Metabolome data acquired with the CE-MS and IC-PAD systems

| Growth condition | NO3 (mmol l⁻¹) | NH4⁺ (mmol l⁻¹) | CO2 (p.p.m.) | Light intensity (μmol m⁻² s⁻¹) | No. of measured metabolites | Average RSD (%) | Range of RSD (%) |
|------------------|----------------|----------------|--------------|-------------------------------|----------------------------|-----------------|-----------------|
| Ambient          | 50             | 0              | 20           | 20                            | 61                         | 19.2            | 1.7–51.7        |
|                  | 10             | 0              | 10           | 60                            | 60                         | 22.8            | 6.6–50.8        |
|                  | 10             | 1              | 61           |                               |                            | 19.9            | 1.6–50.3        |
|                  | 10             | 10             | 61           |                               |                            | 21.6            | 1.1–74.2        |
| 200              | 20             | 0              | 10           | 60                            | 60                         | 15.7            | 1.7–71.7        |
|                  | 10             | 0              | 61           |                               |                            | 26.3            | 1.9–94.0        |
|                  | 10             | 1              | 58           |                               |                            | 23.0            | 5.9–67.7        |
|                  | 10             | 10             | 59           |                               |                            | 32.3            | 6.3–81.6        |
| 1,200            | 50             | 0              | 20           | 60                            | 59                         | 19.8            | 3.6–55.0        |
|                  | 10             | 0              | 61           |                               |                            | 20.6            | 5.0–49.4        |
|                  | 10             | 1              | 60           |                               |                            | 21.8            | 7.4–72.6        |
|                  | 10             | 10             | 61           |                               |                            | 28.4            | 5.0–115.0       |
| 200              | 20             | 0              | 57           |                               |                            | 13.7            | 1.7–71.2        |
|                  | 10             | 0              | 59           |                               |                            | 28.1            | 4.1–68.8        |
|                  | 10             | 1              | 59           |                               |                            | 28.7            | 6.1–88.0        |
|                  | 10             | 10             | 59           |                               |                            | 30.4            | 5.2–70.0        |
| 3,600            | 50             | 0              | 60           |                               |                            | 17.1            | 2.4–85.3        |
|                  | 10             | 0              | 57           |                               |                            | 25.4            | 3.5–99.6        |
|                  | 10             | 1              | 57           |                               |                            | 19.8            | 3.3–61.5        |
|                  | 10             | 10             | 60           |                               |                            | 26.0            | 2.8–92.7        |
| 200              | 20             | 0              | 60           |                               |                            | 23.0            | 2.8–74.1        |
|                  | 10             | 0              | 59           |                               |                            | 28.1            | 1.8–60.0        |
|                  | 10             | 1              | 58           |                               |                            | 33.3            | 2.6–90.5        |
|                  | 10             | 10             | 60           |                               |                            | 26.1            | 6.3–85.7        |

* RSD (%) = 100×SD/mean.

## Effects of elevated CO2 concentrations on ammonium-induced metabolic alterations in sugar and amino acid synthesis

Although elevated CO2 concentrations appeared to magnify the ammonium effects in general (Fig. 5), we also found that individual ammonium-induced metabolic alterations were differently modified by elevated CO2 concentrations. In the case of sugars, the abundance of sucrose was simply proportional to increases in the CO2 concentration, independent of the N nutrient condition (Fig. 8A), while supplementation with ammonium increased the contents of monosaccharides (glucose, fructose and galactose) under high light conditions. Because the glucose level in tobacco plants grown with 10 mM NH4NO3 was previously reported to be higher than that of tobacco plants grown with 20 mM KNO3 (Geiger et al. 1999), ammonium supplementation may elevate the glucose level in various plants. These ammonium-induced elevations of monosaccharide levels were enhanced by high concentrations of CO2 (Fig. 8B–D), and a similar phenomenon was observed for the starch content (Fig. 2). Thus, sucrose synthesis and monosaccharide and starch synthesis are differently regulated by ammonium supplementation and elevated CO2 concentrations. Both sucrose and starch are synthesized from G1P by UDP-glucose pyrophosphorylase in the cytosol and ADP-glucose pyrophosphorylase in the chloroplast, respectively. Since the G1P content was nearly constant under all growth conditions examined...
Fig. 5 Amounts of metabolites and distributions of metabolite groups in shoots of Arabidopsis plants under different growth conditions. The data set is expressed as the sum of means ± SE and corresponds to the data in Supplementary Tables S2–S4. Metabolites related to sugar and starch synthesis include Sucr, Gluc, Galc, Fruc, M6P, G1P, UDPG and ADPG. Metabolites related to the Calvin cycle include 3PG, R5P, Lac, PEP, Pyr, Ru5P, RuBP, G6P, F6P, FBP and S7P. Metabolites related to photorespiration include Gce, Gly and Ser. Metabolites related to the TCA cycle include Fum, Suc, Mal, 2OG, cisAco, Cit and isoCit. The group of metabolites related to N assimilation and amino acids includes Ala, Pro, Val, Thr, Ile, Leu, Orn, Asn, Asp, Lys, Gln, OAS, Glu, Met, His, Phe, Arg, Tyr, Trp, Ctr and GABA. Metabolites related to redox regulation include AsA, GSH, GSSG, NAD, NADP and NADPH. Nucleotides are AMP, ADP and ATP.

Fig. 4 Distribution of individual metabolite contents in shoots of A. thaliana plants grown for 16 d under different growth conditions. The bottom and top of each blue box indicate the lower and upper quartiles, respectively, and the red bars in blue boxes indicate the medians. The purple bars indicate the mean values of determined metabolites.
(Fig. 8E), the processes catalyzed by these enzymes may be differentially regulated in response to the species of the N source. Alternatively, intracellular distribution of G1P might be modified in response to the species of N sources, as Fettke et al. (2011) recently showed that G1P in the cytosol can pass the plastidial envelope membranes and that its hexosyl residue is converted to starch.

In contrast, elevated CO2 concentrations differently influenced ammonium-induced promotion of amino acid biosynthesis. Supplementation with ammonium induced high accumulation of not only Gln, a direct product of N assimilation (Fig. 8F), and Asn, produced by direct transfer of the amino group of Gln (Fig. 8G), but also Arg, Ctr, His and Gly (Fig. 8H; Supplementary Table S2–S4). Since the ratios of (number of N)/(number of C) of these amino acids were high, modulations of the metabolic balance by ammonium included accumulation of these amino acids, except for Gln, or even reduced the Gly content approximately one-third due to the repression of photorespiration (Fig. 8F–H; Supplementary Table S2–S4).

Hence, our results indicated that elevated CO2 concentrations differently modify individual metabolic modifications caused by ammonium supplementation.

**Effects of elevated CO2 concentrations on N assimilation**

Growth and elementary analyses and metabolite profiling in the current study indicated that the total amino acid content (Fig. 5), the Chl content (Fig. 2B) and the N content per shoot (see the text), which indicates the total amount of inorganic N and organic (assimilated) N, were not noticeably reduced by elevated CO2 concentrations (Fig. 3A, C). Because previous studies showed the dependency of nitrate assimilation on photorespiration and occurrence of a reduction in nitrate assimilation by elevated CO2 concentrations in *A. thaliana* and wheat (Rachmilevitch et al. 2004, Bloom et al. 2010), there is a discrepancy between the previously reported results and those of the present study. However, it would not be suitable to compare our results directly with previously reported studies, because the growth conditions in the previous studies are very different from those in the current study. For instance, 0.2 and 1 mM nitrate was used as the N source in the previous studies, whereas we used 10 and 20 mM nitrate as the N source.
Fig. 7 Hierarchical dendrogram constructed from the metabolite profiles. The contents of 56 metabolites were normalized by Z-score and the hierarchical dendrogram was then constructed from Canberra distances using Ward’s method (Ward 1963). The maximum and minimum values for each heat map are shown on the left side of the heat map. Because the contents of three metabolites (FBP, ADPG and Orn) could not be determined under several growth conditions, their contents were not utilized for the construction of the hierarchical dendrogram. Thus, the heat maps for these metabolites are shown separately, together with the heat maps for fresh weight, starch content and Chl content. In the heat maps, gray blocks indicate that values are missing.
Furthermore, CO₂ concentrations and light intensity applied are also very different. The CO₂ concentrations and light intensity used in the previous studies were 380 and 720 p.p.m. and 350 \( \mu \text{E m}^{-2} \text{s}^{-1} \), respectively. Because we showed that differences in N nutrition and light intensity are prominent factors determining the metabolic state, the appearance of the effects of elevated CO₂ on nitrate assimilation might be influenced by other environmental factors. Furthermore, increases in biomass by elevated CO₂ concentrations also bring about a difficulty in exactly evaluating the effects of elevated CO₂ concentrations on nitrate assimilation. In our case, reductions in the N content per fresh weight by half were equal to 1.3- to 1.7-fold increases.

**Fig. 8** Contents of sucrose (A), glucose (B), fructose (C), galactose (D), G1P (E), Gln (F), Asn (G), Arg (H), Glu (I), and 2OG (J) in shoots of *A. thaliana* plants grown for 16 d under 24 different growth conditions (detailed below the panels). Results are the mean ± SE. The data set is also shown in Supplementary Tables S2–S4. Different letters indicate statistical differences detected by Student’s t-test \((P < 0.05)\).
in the N content per shoot. Although our characterization of metabolic states implied that neither nitrate nor ammonium assimilation is reduced by elevated CO$_2$ concentrations in *A. thaliana*, further careful analyses under a variety of growth conditions would be necessary for a deeper understanding of the effects of elevated CO$_2$ concentrations on N assimilation.

We also note that the contents of Glu and 2OG, key compounds for N assimilation, were almost constant under all growth conditions examined (Fig. 8I, J), indicating that the additional N sources, different CO$_2$ concentrations and different light intensity do not pronouncedly affect the Glu and 2OG contents. In contrast, the levels of precursors of 2OG, Mal and Fum, were greatly modulated in response to the ammonium supplement, high light and elevated CO$_2$ concentrations (Figs. 4, 5). Similarly, the contents of several amino acids, including Gln and Asn, fluctuated sharply in response to environmental factors (Fig. 8F, G). These findings may imply that the abundance of metabolites that have a storage function associated with N assimilation is highly variable and that Glu does not play such a role in *A. thaliana*. The Glu and 2OG concentrations are probably buffered from changes in the environment by predominant modulations in levels of precursors and/or derivatives of Glu and 2OG. Previously, Urbanczyk-Wochniak and Fernie (2005) showed that N deficiency did not lead to reductions in the 2OG content in tomato plants. Our results are therefore consistent with their results.

**Concluding remarks**

By investigating the growth and metabolite profiles of *A. thaliana* plants grown under various conditions, we found that elevated levels of CO$_2$ increase the total metabolite content in plants and promote growth under a wide range of CO$_2$ levels (from ambient levels to 3,600 p.p.m.) when light energy and N sources are sufficient, whereas the presence of ammonium as the N source is a critical nutritional factor that modifies metabolism composition under any CO$_2$ conditions. We also showed that when the light intensity is low, the total metabolite content remains almost constant, independent of the C and N nutrient conditions. This result is consistent with the generally accepted notion that light energy is the fundamental factor for the biogenesis of organic compounds in plants and, therefore, defines the metabolic state of the plant. Significantly, high concentrations of atmospheric CO$_2$ enhanced the ammonium-induced metabolic alterations in general but differently modified the ammonium-induced metabolic alterations of some metabolites. The results of this study therefore suggest that the effects of increased concentrations of atmospheric CO$_2$ may differ among individual plants in the field, reflecting other environmental factors present during the growth of a particular plant. It would be particularly important to focus on the N nutrient effects when evaluating the effects of increased concentrations of CO$_2$ on metabolism of plants in the field, because the N nutrient conditions differ greatly among ecosystems, particularly between agricultural and natural systems.

**Materials and Methods**

**Plant materials and growth conditions**

Seeds of *A. thaliana* (ecotype Columbia-0) were germinated on agar plates containing modified 1/2 MS medium [modified half-strength Murashige–Skoog salt (Murashige and Skoog 1962) and 1 g l$^{-1}$ MES, pH 5.7], and the seedlings were grown on the plates for 5 d. Then, 25 seedlings that were similar in size were transferred to fresh 0.8% agar plates containing 40 ml of modified 1/2 MS medium using a fixed number of seedlings per plastic dish (90 mm diameter × 20 mm depth). The transferred seedlings were grown for 11 d under continuous light (50 or 200 µE m$^{-2}$ s$^{-1}$) at 23°C in a growth chamber in which the CO$_2$ concentration was set at ambient, 1,200 p.p.m. or 3,600 p.p.m. In the modified 1/2 MS media, only the composition of inorganic N compounds was altered from the original composition; the media contained 10 mM KNO$_3$, 20 mM KNO$_3$ or 10 mM KNO$_3$ plus 1 or 10 mM ammonium succinate. We note that supplementation of 10 mM succinate itself did not affect the contents of TCA cycle-related organic acids (T. Fujimori et al. unpublished data), suggesting no apparent effect of the supplementation of succinate on metabolic balance. The same modified medium was used in both the first and second plates used for plant growth. The shoots of the seedlings were harvested, immediately frozen in liquid N$_2$ and stored at −80°C until use.

**Measurement of starch contents**

The starch contents were measured according to the method of Abel et al. (1996) with slight modifications. Frozen tissues (25–50 mg) were homogenized into a fine powder with a Multi-Beads shocker (Yasui Kikai) and incubated with 1 ml of 80% (v/v) ethanol for 30 min at 80°C. Insoluble material was recovered, rinsed with 1 ml of ultrapure water (Milli-Q water) twice, suspended in 0.5 ml of 0.2 M KOH, and incubated for 60 min at 95°C. After neutralization with 0.2 ml of 1 M acetic acid, the sample was centrifuged. The supernatant (30 µl) was mixed with 30 µl of reaction mixture containing 1 U of amyloglucosidase and 1 U of α-amylase in 50 mM sodium acetate (pH 4.5) and incubated for 2 h at 55°C. After 60 µl of Milli-Q water containing 500 µM 2-deoxy-α-glucose as an internal standard was added, the sample was filtered with a 3 kDa cut-off membrane (Japan PALL Corporation). The content of starch that was enzymatically decomposed was determined by measuring the glucose content by ion chromatography using the AEC-PAD system (Japan Dionex Corporation).

**Measurement of Chl contents**

Chl contents were measured according to Moran (1982).

**Measurement of C and N contents**

Analyses of C and N contents of plant materials were outsourced to Sumika Chemical Analysis Service, Ltd.
**Measurement of metabolites using CE-MS and ion chromatography**

Metabolites were extracted by the method described in Sato and Yanagisawa (2010), but with slight modifications. Frozen shoots (50–100 mg) were ground into a fine powder with a Multi-Beads shocker. Immediately, 0.2 ml of ice-cold methanol was added to the sample and mixed vigorously using a vortex mixer to dissolve phospholipids in the membranes and to inactivate metabolic enzymes. Then, 0.2 ml of ice-cold Milli-Q water containing 200 μM PIPES, 200 μM methionine sulfone and 200 μM 2-deoxy-D-glucose as internal standards was added and the sample was mixed again. The sample was centrifuged at 14,000 × g for 30 min, and the supernatant was recovered and filtered with a 3 kDa cut-off membrane (Japan PALL Co.). The filtrate was lyophilized to an appropriate volume using a lyophilizer (Christ Freeze Dryers, Kubota Corporation) to condense the metabolites. The samples were stored at −80°C until analysis. Just before analysis, samples were dissolved with appropriate volumes of Milli-Q water at 20°C.

Amino acids, organic acids, sugar phosphates, nucleotides and coenzymes were analyzed using a CE-MS system composed of an Agilent apparatus for CE, an Agilent 1100-series MSD single quadrupole mass spectrometer, an Agilent 1200-series isocratic HPLC pump, a G1603A Agilent CE–MS adaptor kit and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies). The contents of the cationic metabolites were determined according to Takahashi et al. (2006). The anionic metabolites were determined by the method described previously (Sato and Yanagisawa 2010). The high-resolution mode and the high-speed mode described in Sato and Yanagisawa (2010) were employed for the analysis of DHAP, GAP, R5P, Ru5P and five hexose phosphates and for the analysis of the other metabolites, respectively. The cationic and anionic metabolites were divided into two groups based on their abundance and were analyzed separately by CE-MS to obtain more accurate measurements. As sugars could not be analyzed by the current CE-MS method, their contents were determined using a Dionex ion chromatography ICS-3000 AEC-PAD system (Japan Dionex Co.), which was performed according to the model analytical method provided by the supplier (Application Note 92).

**Statistical and hierarchical clustering analyses**

All statistical analyses and hierarchical clustering analysis were conducted using R software (the R Project, www.r-project.org/). The contents of 56 metabolites were normalized by Z-score and the hierarchical dendrogram was constructed from Canberra distances using Ward’s method (Ward 1963). The results of statistical analysis of fresh and dry weight, starch and Chl contents, C and N contents are shown in Supplementary Fig. S1. The results of statistical analysis of metabolite contents are shown in Supplementary Fig. S2.

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**

Abel, G.J.W., Springer, F. and Willmitzer, L. (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (Solanum tuberosum L.). Plant J. 10: 981–991.

Berner, R.A. and Kothavala, Z. (2001) Geocarb III: a revised model of atmospheric CO2 over phanerozoic time. Amer. J. Sci. 301: 182–204.

Bloom, A.J., Burger, M., Asensio, J.S.R. and Consins, A.B. (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science 328: 899–903.

Bräutigam, K., Dietzel, L., Kleine, T., Ströhér, E., Wormuth, D., Dietz, K.-J. et al. (2009) Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in arabidopsis. Plant Cell 21: 2715–2732.

Buchanan, B.B. (1980) Functions of light in the regulation of chloroplast enzymes. Annu. Rev. Plant Physiol. 31: 341–374.

Cheng, S., Moore, B.D. and Seemann, J.R. (1998) Effects of short- and long-term elevated CO2 on the expression of rubulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of Arabidopsis thaliana (L.) Heynh. Plant Physiol. 116: 715–723.

Chaillou, S., Vessey, J.K., Morot-gaudry, J.F., Raper, C.D. Jr, Henry., l., T., Brautigam, K., Dietzel, L., Kleine, T., Ströhér, E., Wormuth, D., Dietz, K.-J. et al. (2009) Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in arabidopsis. Plant Cell 21: 2715–2732.

Cheng, S., Moore, B.D. and Seemann, J.R. (1998) Effects of short- and long-term elevated CO2 on the expression of rubulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of Arabidopsis thaliana (L.) Heynh. Plant Physiol. 116: 715–723.

Drake, B.G., González-Meler, M.A. and Long, S.P. (1997) More efficient plants: a consequence of rising atmospheric CO2? Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 609–639.

Engelsberger, W.R. and Schulze, W.X. (2012) Nitrate and ammonium lead to distinct global dynamic phosphorylation patterns when resupplied to nitrogen-starved Arabidopsis seedlings. Plant J. 69: 978–995.
Portis, A.R. Jr (2003) Rubisco activase—Rubisco’s catalytic chaperone. *Photosynth. Res.* 75: 11–27.

Rachmilevitch, S., Cousins, A.B. and Bloom, A.J. (2004) Nitrate assimilation in plant shoots depends on photorespiration. *Proc. Natl Acad. Sci. USA* 101: 11506–11510.

Sage, R.F., Sharkey, T.D. and Seemann, J.R. (1989) Acclimation of photosynthesis to elevated CO\(_2\) in five C3 species. *Plant Physiol.* 89: 590–596.

Sato, S. and Yanagisawa, S. (2010) Capillary electrophoresis—electrospray ionization-mass spectrometry using fused-silica capillaries to profile anionic metabolites. *Metabolomics* 6: 529–540.

Scheible, W.-R., Gonzales-Fontes, A., Lauerer, M., Muller-Rober, B., Caboche, M. and Stitt, M. (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9: 783–798.

Seneweera, S., Makino, A., Hirotsu, N., Norton, R. and Suzuki, Y. (2011) New insight into photosynthetic acclimation to elevated CO\(_2\): the role of leaf nitrogen and ribulose-1,5-bisphosphate carboxylase/oxygenase in rice leaves. *Environ. Exp. Bot.* 71: 128–136.

Stitt, M. (1991) Rising CO\(_2\) levels and their potential significance for carbon flow in photosynthetic cells. *Plant Cell Environ.* 14: 741–762.

Stitt, M. (1999) Nitrate regulation of metabolism and growth. *Curr. Opin. Plant Biol.* 2: 178–186.

Stitt, M. and Krapp, A. (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. *Plant Cell Environ.* 22: 583–621.

Sun, J., Gibson, K.M., Kiirats, O., Okita, T.W. and Edwards, G.E. (2002) Interactions of nitrate and CO\(_2\) enrichment on growth, carbohydrates, and rubisco in Arabidopsis starch mutants. Significance of starch and hexose. *Plant Physiol.* 130: 1573–1583.

Takahashi, H., Hayashi, M., Goto, F., Sato, S., Soga, T., Nishikoa, T. et al. (2006) Evaluation of metabolic alteration in transgenic rice over-expressing dihydroflavonol-4-reductase. *Ann. Bot.* 98: 819–825.

Tocquin, P., Ormenese, S., Pieltain, A., Detry, N., Bernier, G. and Perilleux, C. (2006) *Acclimation of Arabidopsis thaliana* to long-term CO\(_2\) enrichment and nitrogen supply is basically a matter of growth rate adjustment. *Physiol. Plant.* 128: 677–688.

Urbanczyk-Wochniak, E. and Fernie, A.R. (2005) Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically grown tomato (*Solanum lycopersicum*) plants. *J. Exp. Bot.* 56: 309–321.

Van der Kooij, T.A.W., De Kok, L.J. and Stulen, I. (1999) Biomass production and carbohydrate content of *Arabidopsis thaliana* at atmospheric CO\(_2\) concentrations from 390 to 1680 \(\mu\)l. *Plant Biol.* 1: 482–486.

Ward, J.H. (1963) Hierarchical grouping to optimize an objective function. *J. Amer. Stat. Assoc.* 58: 236–245.

Wulff-zottele, C., Gatze, N., Kopka, J., Orellana, A., Hoefgen, R., Fisahn, J. et al. (2010) Photosynthesis and metabolism interact during acclimation of Arabidopsis thaliana to high irradiance and sulphur depletion. *Plant Cell Environ.* 33: 1974–1988.

Zhang, N. and Portis, A.R. Jr (1999) Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proc. Natl Acad. Sci. USA* 96: 9438–9443.