Effects of Elevated Atmospheric CO2 on Primary Metabolite Levels in Arabidopsis thaliana Col-0 Leaves: An Examination of Metabolome Data

Ko Noguchi1,2,*, Chihiro K. Watanabe1 and Ichiro Terashima1

1Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7 3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
2School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
*Corresponding author: E-mail, knoguchi@bs.s.u-tokyo.ac.jp; Fax, +81-5841-4465.
(Received June 2, 2015; Accepted August 25, 2015)

Elevated atmospheric CO2 concentrations ([CO2]) affect primary metabolite levels because CO2 is a direct substrate for photosynthesis. In several studies, the responses of primary metabolite levels have been examined using Arabidopsis thaliana leaves, but these results have not been comprehensively discussed. Here, we examined metabolome data for A. thaliana accession Col-0 leaves that were grown at elevated [CO2] with sufficient nitrogen (N) nutrition. At elevated [CO2], starch, monosaccharides and several major amino acids accumulated in leaves. The degree of accumulation depended on whether the rooting medium contained NH4+ or only NO3−. Because low N conditions induce an increase in carbohydrates similar to that of elevated [CO2], we compared the responses of primary metabolite levels between elevated [CO2] and low N conditions. Levels of the tricarboxylic acid (TCA) cycle-associated organic acids and major amino acids decreased with low N, but not with elevated [CO2]. Even at elevated [CO2], the low N induced the decreases in the levels of organic acids and major amino acids. A small sink size also affects the primary metabolite response patterns in leaves under elevated [CO2] conditions. Thus, care is necessary when interpreting primary metabolite changes in leaves of field-grown plants.

Keywords: Arabidopsis thaliana • Elevated [CO2] • Low nitrogen condition • Primary metabolites.

Abbreviations: [CO2], atmospheric CO2 concentrations; DHAP, dihydroxyacetone phosphate; GABA, 4-aminobutanolic acid; GAP, glycolaldehyde 3-phosphate; GLO, glyoxyl; G1P, glucose 1-phosphate; GSH, glutathione; HI, harvest index; HL, high light; N, nitrogen; MG, methylglyoxyl; 2OG, 2-oxoglutarate; PRK, phosphoribulokinase; RC, reactive carbonyl; RSP, ribose 5-phosphate; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; Ru5P, ribulose 5-phosphate; TCA, tricarboxylic acid.

Introduction

The atmospheric CO2 concentration ([CO2]) has been increasing since the industrial revolution. Because CO2 is a direct substrate for photosynthesis, the increase in [CO2] would greatly affect photosynthesis, growth and yields of plants, which has been discussed in several reviews (Long et al. 2004, Ainsworth and Rogers 2007, Leakey et al. 2009a). The accumulation of carbohydrates at elevated [CO2] has been well documented for many species (Long et al. 2004), but changes in other primary metabolites have not been examined comprehensively. Responses of primary metabolites to elevated [CO2], which strongly relate to photosynthesis and growth responses, have not been extensively discussed, except for by Stitt and Krapp (1999), and Misra and Chen (2015). In the former, the data were mainly for tobacco leaves, while the latter reviewed recent studies but did not focus on each primary metabolic pathway. Recently, several metabolome analyses have been reported for leaves of Arabidopsis thaliana that were cultivated at elevated [CO2] (Li et al. 2006, Li et al. 2008, Kaplan et al. 2012, Hachiya et al. 2014, Sato and Yanagisawa 2014, Takatani et al. 2014, Watanabe et al. 2014). Metabolome analyses using A. thaliana leaves that were grown under low nitrogen (N) conditions have also been conducted (Watanabe et al. 2010, Krapp et al. 2011, Hachiya et al. 2012). Low N conditions induce carbohydrate accumulation similar to that of elevated [CO2], but the primary metabolite responses are different between the two conditions (Stitt and Krapp 1999). In some studies, elevated [CO2] enhanced plant growth, and, thus, the plant N concentration became lower at elevated [CO2] than at ambient [CO2] (Li et al. 2008, Aranjuelo et al. 2013). The primary metabolite responses to elevated [CO2] in such studies cannot be separated from the responses to low N levels.

Here, we examined responses of primary metabolites to elevated [CO2] in leaves of A. thaliana grown with sufficient N availability. Responses of primary metabolites to elevated [CO2] appeared different depending on whether the rooting medium contained NH4+ or only NO3− (Sato and Yanagisawa 2014, Takatani et al. 2014). Therefore, we separately summarized metabolome data depending on whether the nutrient medium contained NO3− as the sole N source (NO3−-medium) or NH4+ in addition to NO3− (NH4+ + NO3−-medium). These results were compared with plant primary metabolite responses to low NO3− at an ambient [CO2] (e.g. Krapp et al. 2011) and to an NH4+−containing medium at an ambient [CO2] (e.g. Hachiya et al. 2012). Through these comparisons, we investigated similarities and differences in the responses between elevated [CO2] and low N conditions.
between the responses of primary metabolites and those of photosynthesis/respiration are discussed. Data from A. thaliana leaves that were cultivated with unknown N forms (Li et al. 2006, 2008, Kaplan et al. 2012) were not incorporated in this review. However, these data were compared with the present results. The data from leaves of species other than A. thaliana, such as tobacco, were compared with the present results from A. thaliana leaves.

Calculation of Metabolome Data of A. thaliana Leaves

Available metabolome data from the leaves of A. thaliana accession Col-0 were collected from the literature and examined for four conditions: elevated [CO2] in NO3− media, elevated [CO2] in NH4+ + NO3− media, low NO3− and ambient [CO2] conditions, and NH4+−containing media and ambient [CO2] conditions. The ratio of the level of each primary metabolite at elevated [CO2] to that at ambient [CO2] (E/A ratio) was calculated in NO3− media and NH4+ + NO3− media, respectively. Additionally, the ratio of each primary metabolite in plants grown in low NO3− media to that in high NO3− media, and the ratio in plants grown in NH4+−containing media to that in only NO3− media, at ambient [CO2] were calculated. These ratios were transformed to log2-fold changes. Detailed calculations of metabolome data are shown in the Appendix.

Description

The mean values of log2-fold changes are shown as heat maps in the primary metabolic map (Fig. 1). In Fig. 2, the actual numbers are listed. The log2-fold changes of the metabolites for each condition in each study can be seen in Supplementary Figs. S1 and S2. We also calculated the E/A ratios using the data in Li et al. (2006, 2008) and Kaplan et al. (2012), and the ratios of low to high NH4NO3 for the data in Tschoep et al. (2009). All of these studies used A. thaliana leaves. These data were transformed to log2-fold changes and are shown in Supplementary Figs. S1 and S2.

Statistics

The mean values of log2-fold changes for each condition were statistically analyzed by Student’s t-test. In the analysis, the null hypothesis is that the mean value of the log2-fold change is zero. The statistical results are shown in Supplementary Table S1.

Responses of Primary Metabolite Levels to Elevated [CO2] and Comparison with the Responses to a Low N Condition

Responses of primary metabolite levels to elevated [CO2] in each pathway were summarized and compared with the responses to low N conditions in the media (Figs. 1, 2). In some studies, primary metabolite levels were measured under several conditions. The relevant data were pooled, and the mean value was used for each study (see Appendix; Supplementary Figs. S1, S2). Growth conditions, such as light intensity and light period, were different among the studies. Thus, the mean values of log2-fold changes were statistically analyzed. Many primary metabolite levels were not statistically different between the ambient and elevated [CO2], probably because of the limited number of studies (Supplementary Table S1). In this review, the statistical results are carefully discussed.

In Figs. 1 and 2, we compared the primary metabolite levels in each metabolic pathway. Some metabolites, such as fructose bisphosphate, are involved in multiple pathways. In the studies analyzed in this review, metabolite levels were measured using the whole leaf tissues. Therefore, the same value was shown for one metabolite in different metabolic pathways.

Carbohydrates and sugar alcohols

At elevated [CO2], starch accumulates in the leaves of many species (Long et al. 2004), whereas the degree of sucrose accumulation varied depending on the species (Stitt and Krapp 1999). In A. thaliana leaves, sucrose accumulated to a moderate extent under elevated [CO2] (Figs. 1, 2). Monosaccharides, such as glucose and fructose, accumulated in A. thaliana leaves at elevated [CO2] in NH4+ + NO3− media, while their accumulation was not marked in NO3− media (Sato and Yanagisawa 2014). Similar monosaccharide accumulations under elevated [CO2] were observed in the leaves of tall fescue (Festuca arundinacea; Yu et al. 2012), radish (Raphanus sativus; Urbonaviciute et al. 2006), Scots pine (Pinus sylvestris; Jach and Ceulemans 1999) and silver birch (Betula pendula; Lavola and Julkunen-Tiitto 1994).

The degree of carbohydrate accumulation changed depending on various factors. In tobacco leaves at elevated [CO2], sugars accumulated more markedly at high N than at low N levels (Geiger et al. 1999). Aranjuelo et al. (2013) examined primary metabolite levels in the flag leaves of two wheat cultivars with contrasting sink sizes and harvest indices (HIs). Patterns of carbohydrate accumulation at elevated [CO2] were different between the cultivars. Starch and glucose accumulated more at elevated [CO2] than at ambient [CO2] in the low HI cultivar leaves, whereas the levels of the two carbohydrates were similar in the high HI cultivar leaves under both CO2 conditions. Carbohydrates tended to accumulate under the conditions where photosynthetic rates were high, such as at moderate pH (Hachiya et al. 2014) and at high light (HL; Sato and Yanagisawa 2014). In some cases, maltose and galactose accumulated in A. thaliana leaves at elevated [CO2] (Supplementary Fig. S1; Li et al. 2008, Kaplan et al. 2012, Sato and Yanagisawa 2014).

At low N and ambient CO2 conditions, carbohydrates accumulated but the accumulation patterns differed depending on the N forms in the media (Urbanczyk-Wochniak and Fernie 2005, Tschoep et al. 2009, Watanabe et al. 2010, Krapp et al. 2011). At low NO3− levels, not only starch but also other carbohydrates accumulated in tomato (Urbanczyk-Wochniak and Fernie 2005) and A. thaliana leaves (Watanabe et al. 2010), but under low NH4NO3 conditions, only starch accumulated.
Fig. 1 Heat map of differences in the levels of intermediates in the primary metabolic pathway. Red and blue indicate log₂-fold increases and decreases, respectively, in primary metabolite levels. A box with a slash denotes unavailable data in the literature. ‘E/A in NO₃⁻’ and ‘E/A in +NH₄⁺’ denote log₂-fold changes in the ratio of elevated to ambient [CO₂] in NO₃⁻ media and in NH₄⁺ + NO₃⁻ media, respectively. ‘LN/HN’ and ‘+A/N’ denote log₂-fold changes in the ratio of low to high NO₃⁻ conditions at ambient [CO₂], and in the ratio in NH₄⁺ + NO₃⁻ media to only NO₃⁻ media at ambient [CO₂].
**Fig. 2** Differences in the levels of intermediates in the primary metabolic pathway. Log₂-fold changes in the ratios are shown. A box with a slash denotes unavailable data in the literature. ‘NO₃⁻’ and ‘NH₄⁺’ in E/A denote log₂-fold changes in the ratio of elevated to ambient [CO₂] in N O₃⁻ media and in NH₄⁺ + NO₃⁻ media, respectively. ‘LN/HN’ and ‘+ A/N’ denote log₂-fold changes in the ratio of low to high NO₃⁻ conditions at ambient [CO₂], and in the ratio of NH₄⁺ + NO₃⁻ media to only NO₃⁻ media at ambient [CO₂]. For amino acids, we showed the ratios of N to C numbers. For calculation of ‘Major AA’ and ‘Minor AA’, we summed the contents of major and minor amino acids, respectively, and calculated the log₂-fold changes for each ratio. Total AA/Total OA denotes the ratio of total amino acids to total organic acids in the TCA cycle. * and ** denote statistically significant differences between each value and zero at \( P < 0.1 \) and \( P < 0.05 \), respectively.
in *A. thaliana* leaves (Tschoep et al. 2009). At low NO$_3^-$, sucrose accumulated in the leaves of many *A. thaliana* accessions (Sulpice et al. 2013). At low NO$_3^-$ levels, starch accumulated more than under elevated [CO$_2$] and sufficient N conditions (Figs. 1, 2). Therefore, the N level should be carefully considered in elevated [CO$_2$] experiments. In some studies (Levine et al. 2008, Aranjuelo et al. 2011, Aranjuelo et al. 2013), carbohydrates markedly accumulated concomitantly with the decrease in the plant N level. Under such conditions, the low N may induce a marked accumulation of carbohydrates. Under low NO$_3^-$ conditions, glucose 1-phosphate (G1P), the precursor of starch synthesis, also accumulated. At elevated [CO$_2$], G1P did not accumulate markedly, except under HL, when G1P accumulated to some extent (Sato and Yanagisawa 2014). ADP-glucose, another precursor of starch synthesis, accumulated at elevated [CO$_2$] in NH$_4^+$ + NO$_3^-$ media. There were few data on sugar alcohols. Raffinose accumulation was observed in *A. thaliana* leaves at elevated [CO$_2$] (Supplementary Fig. S1; Li et al. 2008, Kaplan et al. 2012) and under low NO$_3^-$ conditions (Supplementary Fig. S2; Krapp et al. 2011).

In summary, at elevated [CO$_2$], starch and monosaccharides accumulated in leaves. In particular, in NH$_4^+$ + NO$_3^-$ media, not only monosaccharides but also some sugar phosphates tended to accumulate. Under low NO$_3^-$ and ambient CO$_2$ conditions, most of the carbohydrates accumulated more than under elevated [CO$_2$], and G1P, a precursor of starch synthesis, also accumulated.

**Glycolysis, the Calvin cycle and photorespiration**

Analyses of the levels of glycolytic intermediates were limited even under low N conditions. At elevated [CO$_2$], metabolites upstream of glycolysis tended to accumulate but metabolites downstream of glycolysis did not (Figs. 1, 2). In contrast, under low NO$_3^-$ and ambient CO$_2$ conditions, glycolytic metabolites, except for pyruvate, accumulated. The pyruvate level decreased both under elevated [CO$_2$] and at low N conditions. Pyruvate is a respiratory substrate for mitochondria. The low level of pyruvate may affect the respiratory CO$_2$ efflux, but there was no clear relationship between the pyruvate level and CO$_2$ efflux rate in *A. thaliana* leaves both at elevated [CO$_2$] (Watanabe et al. 2014) and at low NO$_3^-$ levels (Watanabe et al. 2010). The response of pyruvate was different from those of lactate, alanine, valine and leucine, although the precursors of these compounds is pyruvate. The leucine level at low NO$_3^-$ levels increased but did not change under elevated [CO$_2$].

At elevated [CO$_2$], the dihydroxyacetone phosphate (DHAP) level did not change in *A. thaliana* leaves (Figs. 1, 2), but the glyceraldehyde 3-phosphate (GAP) level decreased (Supplementary Fig. S1; Watanabe et al. 2014). Triose phosphate isomerase catalyzes the equilibration reaction between DHAP and GAP, producing methyl glyoxal (MG) and glyoxal (GLO) as by-products. MG and GLO are also produced by the non-enzymatic reaction between DHAP and GAP (Phillips and Thornalley 1993). They are reactive carbonyls (RCs) that can impair the functions of multiple proteins. Takagi et al. (2014) showed that the MG and GLO levels increased in wheat leaves at elevated [CO$_2$]. Saito et al. (2013) reported that the gene expression of AKR4C, which functions in RC detoxification, was induced in leaves of *A. thaliana* at elevated [CO$_2$]. At low NO$_3^-$ conditions, DHAP increased markedly (Watanabe et al. 2010; Supplementary Fig. S2), but it is unknown whether MG and GLO accumulate at low N levels.

Analyses of the levels of intermediates in the Calvin cycle and oxidative pentose phosphate pathway were also limited. Under HL and elevated [CO$_2$] conditions, ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P) accumulated, in particular in NH$_4^+$ + NO$_3^-$ media (Supplementary Fig. S1; Sato and Yanagisawa 2014). Under these conditions, the level of sedoheptulose 7-phosphate decreased. At elevated [CO$_2$], photosynthetic CO$_2$ assimilation rates increased in many species, including *A. thaliana* (Markelz et al. 2014), but the metabolite levels of the Calvin cycle did not show marked changes. At low NO$_3^-$ conditions, the Ru5P level increased but the ribulose 1,5-bisphosphate (RuBP) level decreased in *A. thaliana* leaves. The Ru5P level did not show a clear change at low NO$_3^-$ conditions. The reaction from Ru5P to RuBP is catalyzed by phosphoribulokinase (PRK) in the Calvin cycle. Geiger et al. (1999) measured PRK and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activities in leaves of tobacco that had been cultivated at elevated [CO$_2$] under various N conditions. In their study, PRK activity did not decrease compared with Rubisco activity, and, thus, the changes in Ru5P and RuBP levels could not be explained by the changes in the activity levels of these enzymes. RSP is considered to be a precursor of histidine, but the histidine level did not correlate with the Ru5P level.

Because the oxygenation reaction of Rubisco is suppressed by elevated [CO$_2$], photorespiration is suppressed at elevated [CO$_2$] (Long et al. 2004). The levels of glycine and serine, intermediates in the photorespiratory pathway, decreased at elevated [CO$_2$]. Because the glycine level often decreased more than the serine level, the ratio of glycine to serine (Gly/Ser) decreased at elevated [CO$_2$] (Fig. 2). In tobacco leaves, the levels of glycine and serine also decreased (Geiger et al. 1998, Geiger et al. 1999). In the study of Geiger et al. (1999), the levels of amino acids other than glycine and serine did not decrease at elevated [CO$_2$]. The level of another intermediate of the photorespiration pathway, glycerate, also decreased at elevated [CO$_2$]. Yu et al. (2012) and Aranjuelo et al. (2013) reported decreases in the levels of glycine, serine and glycerate in leaves of tall fescue and wheat at elevated [CO$_2$]. The response of glycine to elevated [CO$_2$] was the same, irrespective of the N forms. At low N and ambient CO$_2$ conditions, the glycine level decreased in the leaves of all *A. thaliana* accessions (Sulpice et al. 2013) and in the leaves of tobacco (Tschoep et al. 2009), but the level of serine did not always decrease (Tschoep et al. 2009).

In summary, at elevated [CO$_2$], the intermediates upstream of glycolysis accumulated. These intermediates also accumulated at low NO$_3^-$ conditions. However, the responses of 3-phosphoglyceric acid and phosphoenolpyruvate, intermediates downstream of glycolysis, were different between elevated [CO$_2$] and low NO$_3^-$ levels. At low NO$_3^-$ levels, Ru5P increased...
The TCA cycle

Based on their diurnal change patterns, organic acids in the tricarboxylic acid (TCA) cycle were divided into two groups (Watanabe et al. 2014). Citrate, cis-aconitate and succinate decreased but fumarate and malate increased during the daytime. Citrate and cis-aconitate response levels to elevated [CO2] varied depending on the study (Supplementary Fig. S1). In Sato and Yanagisawa (2014), citrate accumulated at elevated [CO2], but in Kaplan et al. (2012), Yu et al. (2012), Aranjuelo et al. (2013) and Hachiya et al. (2014), the citrate levels decreased at elevated [CO2]. The level of 2-oxoglutarate (2OG) decreased to a small extent in many studies using A. thaliana leaves (Figs. 1, 2). In tobacco leaves (Matt et al. 2001), the 2OG level hardly changed, irrespective of the conditions. The supply and consumption of 2OG may be well balanced compared with those of other organic acids. Fumarate accumulated to a level similar to that of malate in A. thaliana leaves (Watanabe et al. 2014). The succinate, fumarate and malate levels did not show clear trends at elevated [CO2], but, with HL, succinate and fumarate tended to accumulate at elevated [CO2] (Supplementary Fig. S1; Sato and Yanagisawa 2014). However, Aranjuelo et al. (2013), using wheat leaves, reported that the levels of all TCA cycle-associated organic acids decreased at elevated [CO2]. In their study, the N level was low. Under such conditions, the levels of TCA cycle-associated organic acids decreased, similar to under low NO3− conditions (Fig. 2). Miyagi et al. (2011) examined primary metabolite levels in leaves of Rumex obtusifolius that was cultivated at elevated [CO2]. Rumex obtusifolius accumulates and exudes oxalate that is mainly synthesized from isocitrate, one of the TCA cycle intermediates (Miyagi et al. 2013). At elevated [CO2], the levels of oxalate increased concomitantly with increases in the TCA cycle intermediates, particularly under low nutrient conditions (Miyagi et al. 2011). The authors hypothesized that R. obtusifolius will proliferate in the future under elevated [CO2]. In A. thaliana leaves, oxalate levels were much lower than in R. obtusifolius. In the study of Li et al. (2008), oxalate levels increased at elevated [CO2].

At low NO3− and ambient CO2 conditions, the levels of citrate, cis-aconitate and malate markedly decreased in A. thaliana leaves compared with at high NO3− and ambient CO2 conditions. With lower N levels in the media, the levels of TCA cycle-associated organic acids decreased (Watanabe et al. 2010). At ambient [CO2], the levels of TCA cycle-associated organic acids and two major amino acids, glutamate and aspartate, decreased markedly in NH4+ media (Hachiya et al. 2012). However, at elevated [CO2], the decreases were alleviated to some extent, thereby increasing the levels of glutamate and aspartate (Figs. 1, 2).

In summary, at low NO3− levels, TCA cycle-associated organic acid levels decreased, but at elevated [CO2] such large decreases were not observed. Even at elevated [CO2], the low N level induced decreases in levels of TCA cycle-associated organic acids.

Amino acids

We summarized the responses of five amino acid groups to elevated [CO2] (Figs. 1, 2).

Aspartate group. Aspartate and asparagine are abundant compared with the other amino acids in this group (major amino acids). In A. thaliana leaves, threonine also accumulates (Watanabe et al. 2014). At elevated [CO2], aspartate and asparagine accumulate in A. thaliana leaves. Both amino acids accumulated more in the NH4++NO3− media than in the NO3− media (Figs. 1, 2). In tobacco leaves at elevated [CO2], both amino acids accumulated (Geiger et al. 1998). The levels of methionine and homoserine, a precursor of methionine synthesis, tended to increase, but the levels of lysine and threonine tended to decrease at elevated [CO2].

At low N levels, the amounts of all of the amino acids in this group (aspartate, asparagine, threonine and methionine) decreased, and only the lysine level increased in A. thaliana leaves. Such changes were observed in tomato leaves (Urbaczewski-Wochniak and Fernie 2005) and the leaves of many A. thaliana accessions (Sulpice et al. 2013). In wheat leaves, under low N and elevated CO2 conditions, the levels of all of these amino acids decreased (Aranjuelo et al. 2013). In the presence of NH4+, the level of asparagine, which had a high N/C ratio, increased in A. thaliana leaves at ambient [CO2].

Glutamate group. The glutamate group includes glutamate, glutamine, proline, ornithine, citrulline and arginine. Glutamate and glutamine are abundant compared with the other amino acids in this group (major amino acids). In A. thaliana leaves, asparagine accumulates more in the NH4++NO3− media than in the NO3− media (Figs. 1, 2). In tobacco leaves at elevated [CO2], both amino acids accumulated (Geiger et al. 1998). The levels of asparagine are decreased, but under NH4++NO3− conditions, this ratio decreased, but under NH4+ and ambient CO2 conditions this ratio increased. Under NH4+ -sufficient conditions, NH4+ is incorporated into glutamine, which prevents a free NH4+ increase. Otherwise, NH4+ toxicity is induced (Hachiya et al. 2012).
The ornithine and arginine levels decreased at elevated \([\text{CO}_2]\) irrespective of the N forms (Figs. 1, 2). At low \(\text{NO}_3^-\) conditions, the ornithine and arginine levels did not decrease greatly, although both amino acids have a high N/C ratio (Fig. 2).

**Branched-chain amino acid groups.** Levels of branched-chain amino acids, valine, leucine and isoleucine, are low and the N/C ratios of these amino acids are also low. In *A. thaliana* leaves at elevated \([\text{CO}_2]\), changes in the levels of these branched-chain amino acids were small (Figs. 1, 2). However, under low \(\text{NO}_3^-\) and ambient \(\text{CO}_2\) conditions, leucine and isoleucine accumulated in *A. thaliana* leaves. In plants, a leucine response gene regulatory network is suggested (Hannah et al. 2010). Accumulated leucine may have a regulatory function in gene expression in *A. thaliana* leaves at low \(\text{NO}_3^-\) levels.

The alanine and serine group. Alanine, glycine and serine are the major amino acids in this group. Alanine accumulated in *A. thaliana* leaves at elevated \([\text{CO}_2]\) in certain cases, such as during late developmental stages (Kaplan et al. 2012), in the evenings (Watanabe et al. 2014) and under HL conditions (Sato and Yanagisawa 2014). In leaves of tall fescue, alanine accumulated under elevated \([\text{CO}_2]\) (Yu et al. 2012). At low N levels, the alanine level decreased in *A. thaliana* leaves (Figs. 1, 2). The responses of glycine and serine to elevated \([\text{CO}_2]\) are already summarized above (see ‘Glycolysis, the Calvin cycle and photorespiration’).

Glutathione (GSH) and glutathione disulfide are synthesized from cysteine. At elevated \([\text{CO}_2]\), the GSH level was much decreased. The mechanism responsible for this is unknown, but because GSH is used to detoxify MG, an RC (Takagi et al. 2014), the GSH level may be consumed during the dissipation of MG, which is known to accumulate in wheat leaves at elevated \([\text{CO}_2]\) (Takagi et al. 2014).

Aromatic group. The aromatic amino acids, tryptophan, phenylalanine and tyrosine, have low N/C ratios. At elevated \([\text{CO}_2]\) in \(\text{NH}_4^+ + \text{NO}_3^-\) media, tryptophan and tyrosine accumulated in *A. thaliana* leaves (Figs. 1, 2). A precursor, shikimic acid, also accumulated under elevated \([\text{CO}_2]\) (Fig. 2). Tryptophan and tyrosine also accumulated in *A. thaliana* leaves under low \(\text{NO}_3^-\) conditions. However, the phenylalanine level did not show such a change in *A. thaliana* leaves at elevated \([\text{CO}_2]\) or low \(\text{NO}_3^-\) levels. Phenylalanine is a precursor for flavonoids such as anthocyanin. Although the phenylalanine level did not change with elevated \([\text{CO}_2]\) or low \(\text{NO}_3^-\) levels, the anthocyanin level increased in *A. thaliana* leaves (Takatani et al. 2014), in particular under low N and elevated \(\text{CO}_2\) conditions (Aoyama et al. 2014). The increase in flavonoid levels at elevated \([\text{CO}_2]\) was also observed in wheat leaves (Levene et al. 2008). Responses of plant secondary metabolites to elevated \([\text{CO}_2]\) were summarized in a recent review (Misra and Chen 2015).

**Histidine and other primary metabolites.** At elevated \([\text{CO}_2]\), the histidine level showed a small decrease in *A. thaliana* leaves.

A precursor of histidine synthesis, histidinol, also decreased (Figs. 1, 2). Ascorbate and 4-amino butanoic acid (GABA) accumulated under elevated \([\text{CO}_2]\) irrespective of the N nutritional forms. Yu et al. (2012) also showed GABA accumulation in leaves of tall fescue at elevated \([\text{CO}_2]\). Urea, having a high N/C ratio, accumulated at elevated \([\text{CO}_2]\), especially in \(\text{NH}_4^+ + \text{NO}_3^-\) media.

Takatani et al. (2014) measured three polypamines, putrescine, spermidine and spermine, in *A. thaliana* leaves and showed the accumulation of putrescine at elevated \([\text{CO}_2]\). The physiological roles of putrescine at elevated \([\text{CO}_2]\) are unclear, but this polypamine often accumulates under various stress conditions (Alcazar et al. 2010). Putrescine is degraded to GABA. Thus, the accumulation of GABA at elevated \([\text{CO}_2]\) may be partly owing to the accumulation of putrescine at elevated \([\text{CO}_2]\).

In summary, major amino acids other than glycine and serine, such as aspartate, asparagine, glutamate, glutamine and alanine, accumulated under elevated \([\text{CO}_2]\) and sufficient N conditions, and their levels decreased under low \(\text{NO}_3^-\) and ambient \(\text{CO}_2\) conditions. Changes in the glutamate level were smaller than those of the other major amino acids. The total minor amino acid amount showed a small decrease at elevated \([\text{CO}_2]\). Among the minor amino acids, the methionine level increased at elevated \([\text{CO}_2]\), and decreased at low \(\text{NO}_3^-\) levels. Lysine, leucine and isoleucine responded differently from methionine. The levels of these amino acids decreased at elevated \([\text{CO}_2]\) and increased at low \(\text{NO}_3^-\) levels. What physiological mechanism determines these changes in amino acid levels at elevated \([\text{CO}_2]\)? At elevated \([\text{CO}_2]\), a decrease in photorespiration and an increase in photosynthetic \(\text{CO}_2\) assimilation directly change the levels of photorespiratory intermediates and carbohydrates. In the situation where a C skeleton for N assimilation is readily available, inorganic N levels may determine the major amino acid levels.

Responses of Photosynthesis and Respiration to Elevated \([\text{CO}_2]\) and their Relationships to Primary Metabolite Responses

Here, we briefly summarize relationships between primary metabolite responses and photosynthetic/respiratory responses to elevated \([\text{CO}_2]\). At elevated \([\text{CO}_2]\) where carbohydrates accumulate in leaves, gene expression levels in the photosynthetic pathway are often suppressed, which is often related to sugar repression (Moore et al. 1999). The transcript levels of many photosynthesis-related genes decreased in the leaves of three *A. thaliana* accessions at elevated \([\text{CO}_2]\) (Li et al. 2006). However, the repression of photosynthetic gene expression levels is not always linked to decreases in the photosynthetic rates in mature source leaves because most of the photosynthetic genes are transcribed in young sink leaves at the early developmental stage (Seneweera et al. 2011).

The Rubisco content based on leaf area is often decreased at elevated \([\text{CO}_2]\) (Nakano et al. 1997, Sun et al. 2002). However, because the Rubisco content may not limit the photosynthetic
rate at elevated [CO2], this decrease may not be related to changes in the photosynthetic rate (Makino and Mae 1999). The decrease in the Rubisco content at elevated [CO2] may be because of changes in the N allocation pattern in plants and/or due to early leaf senescence at elevated [CO2] (Nakano et al. 1997, Seneweera et al. 2011, Sudo et al. 2014). When the starch accumulation is intense in leaves owing to a small sink size, then the starch accumulation may decrease the photosynthetic rate (Makino and Mae 1999). The N level also affects the photosynthetic rate. A low N level and small sink size induced a large decrease in the photosynthetic rate, even at elevated [CO2] (Aranjuelo et al. 2013).

Elevated [CO2] affects stomatal and mesophyll conductance (Chen et al. 2014). Both conductance levels decreased in rice (Oryza sativa) leaves at elevated [CO2], but these decreases do not limit photosynthetic rates (Chen et al. 2014). Elevated [CO2] decreases the Rubisco oxygenase reaction, leading to a suppressed photosynthetic pathway. In the photosynthetic pathway, NH3 is produced during glycerol decarboxylation. In rice leaves at elevated [CO2], the NH4+ content decreased in a similar manner to the other intermediates of the photosynthetic pathway (Miyazawa et al. 2014).

At elevated [CO2], photoassimilated translocation from source leaves to sink organs is enhanced. In A. thaliana leaves, elevated [CO2] induced the up-regulation of the gene expression levels of some components, such as SUT4 and SWEET12, relating to phloem loading (Duan et al. 2014). At elevated [CO2], gene expression levels of respiratory components were up-regulated and dark respiratory rates were increased in soybean (Leakey et al. 2009b), rice (Fukayama et al. 2011) and A. thaliana leaves (Markelz et al. 2014). Changes in mRNA levels and/or maximal enzymatic activities of respiratory components were not always directly related to dark respiratory rates in leaves (Gonzalez-Meler et al. 2004). In the leaves of some species, respiratory substrates directly affect dark respiratory rates (Noguchi 2005), and, thus, increases in carbohydrate or organic acid levels may influence leaf respiratory rates at elevated [CO2]. However, in A. thaliana leaves at elevated [CO2], leaf respiratory rates were not determined by maximal enzymatic activities or primary metabolite levels, but by respiratory ATP-consuming processes (Watanabe et al. 2014).

**Conclusion**

In this review, we analyzed the responses of primary metabolite levels to elevated [CO2] in the leaves of A. thaliana Col-0. The response of each metabolite strongly depends on the N level and N forms in the rooting media. Therefore, we should be careful when interpreting metabolite changes in the leaves of field-grown plants if the N level or N forms in the soil are not examined. A low N level and/or small sink size affects the metabolite response pattern to elevated [CO2]. In this case, not only starch accumulation but also decreased levels of major amino acids and TCA cycle-associated organic acids were observed, and were similar to their responses under low N conditions. At elevated [CO2], can the sufficient N increase plant biomass and yield? Results from free air CO2 enrichment experiments suggest that photosynthetic N use efficiency, the net amount of CO2 assimilation per unit of leaf N, is improved in C3 plants at elevated [CO2], but the increase is mainly determined by enhanced photosynthetic CO2 assimilation rather than the saving of nutritional N (Leakey et al. 2009a). Therefore, under future elevated CO2 conditions, more N fertilization may increase the biomass and yields of crop species.

Among the primary metabolites, the levels of 2OG, glutamate, valine, phenylalanine and histidine were affected by elevated [CO2] or low N levels to smaller extents than those of the other primary metabolites. The detailed mechanisms of the insensitivity remain unknown, but a balance of these metabolites should be important in A. thaliana leaves. Arabidopsis thaliana mainly accumulates starch as a carbohydrate in leaves (starch-accumulating species). However, rice and wheat accumulate sugars more than starch in their leaves (sugar-accumulating species) (Makino and Mae 1999). At elevated [CO2], sugar-accumulating species may show responses different from A. thaliana. Further studies are needed to elucidate species-dependent responses of primary metabolites to elevated [CO2] and their relationships with photosynthetic/respiratory rates.

**Supplementary data**

**Supplementary data** are available at PCP online.

**Funding**

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan [grant No. 25440127].

**Acknowledgements**

We thank Dr. Nobuyuki Takatani and Professor Tatsuo Omata for providing their metabolome data.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


Aranjuelo, R., Cabrera-Bosquet, L., Morcuende, R., Avice, J.C., Nogués, S., Araus, J.L., et al. (2011) Does ear C sink strength contribute to


**Appendix**

Detailed calculation of metabolome data for each condition.

**Elevated [CO2]**

We calculated the ratio of the level of each primary metabolite at elevated [CO2] to that at ambient [CO2] (E/A ratio), using the data of *A. thaliana* accession Col-0 leaves (Hachiya et al. 2014, Sato and Yanagisawa 2014, Takatani et al. 2014, Watanabe et al. 2014), and the E/A ratio was transformed to log2-fold change. For the data of Hachiya et al. (2014), we calculated the mean log2 values of the data obtained at the two pH levels. Sato and Yanagisawa (2014) examined metabolite levels at three CO2 levels. We used the data of 1,200 p.p.m. for the elevated [CO2], and calculated the E/A ratios. Using the data at 3,600 p.p.m. [CO2], we calculated the ratio (SE/A) and show the values in Supplementary Fig. S1. For their data, we separately treated the data of plants grown in NO3− media, in which NO3− was the sole N source, from those of plants grown in NH4+ + NO3− media, which contained NH4+ in addition to NO3−. We also calculated their data for high (HL) and low light (LL) conditions, and then calculated the mean log2 values. For the data of Watanabe et al. (2014), we calculated the mean log2 values of the data in three different periods of the day. We pooled the mean values of these four studies and calculated the mean value of log2-fold changes of the E/A ratio.

**Low NO3−-containing media and ambient [CO2]**

We calculated the ratio of each primary metabolite in plants grown in low NO3−-containing media to that grown in high NO3−-containing media using the data of Watanabe et al. (2010), Krapp et al. (2011) and Hachiya et al. (2012), and the ratio was transformed to log2-fold change. For the data of Watanabe et al. (2010), we calculated the mean log2 values of the ratios of the data at 0.5 mM to those at 10 mM NO3− and of the data at 0.1 mM to those at 10 mM NO3−. For the data of Krapp et al. (2011), we calculated the mean log2 values of the ratios of the data at low NO3− and those at high NO3− conditions, at 2 and 10 d after an N starvation treatment. For the data of Hachiya et al. (2012), we calculated log2 values of the ratios of the data at 0–10 mM NO3−. We pooled mean values of these three studies, and calculated the mean log2-fold change in the ratios of low to high NO3− conditions.

**NH4+-containing media and ambient [CO2]**

Hachiya et al. (2012), Sato and Yanagisawa (2014) and Takatani et al. (2014) grew plants under various N conditions. For each primary metabolite, we calculated the ratio of its level in the plants grown in NH4+ ± NO3− media to that in the plants grown in only NO3− media. Using the data of Hachiya et al. (2012), we calculated the mean log2 values of the ratios of the data obtained at 10 mM NH4NO3 to those at 10 mM NO3−, and of the data at 10 mM NH4+ to those at 10 mM NO3−. Using the data of Sato and Yanagisawa (2014), we calculated the mean log2 values of the ratios of the data at 10 mM NO3− + 1 mM NH4+ to those at 10 mM NO3−, and of the data at 10 mM NH4NO3 to those at 20 mM NO3−. Using the data of Takatani et al. (2014), we calculated the log2 values in the ratios of data at 5 mM NH4NO3 to those at 15 mM NO3−. We pooled the mean values of these three studies and calculated the values of log2-fold changes.