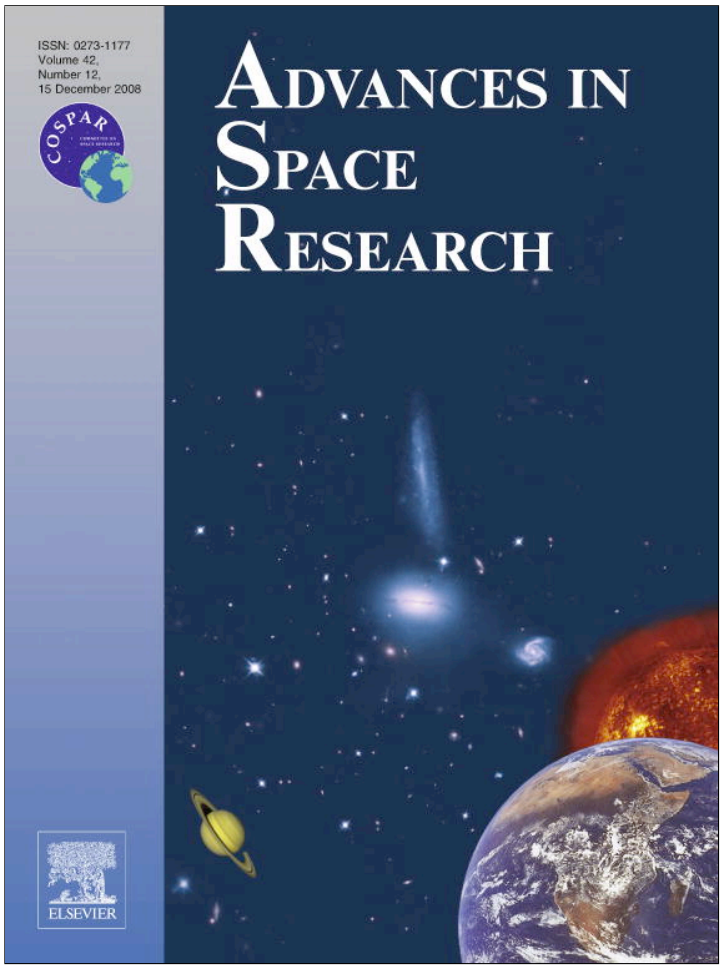

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Physiologic and metabolic responses of wheat seedlings to elevated and super-elevated carbon dioxide

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Abstract

The metabolic consequence of suboptimal (400 μmol mol^{-1} or ppm), near-optimal (1500 ppm) and supra-optimal (10,000 ppm) atmospheric carbon dioxide concentrations [CO_2] was investigated in an attempt to reveal plausible underlying mechanisms for the differential physiological and developmental responses to increasing [CO_2]. Both non-targeted and targeted metabolite profiling by GC–MS and LC–MS were employed to examine primary and secondary metabolites in wheat (*Triticum aestivum*, cv Yocoro rojo) continuously exposed to these [CO_2] levels for 14, 21 and 28 days. Metabolite profile was altered by both [CO_2] and physiological age. In general, plants grown under high [CO_2] exhibited a metabolite profile characteristic of older plants under ambient CO_2. Elevated [CO_2] resulted in higher levels of phosphorylated sugar intermediates, though no clear trend in the content of reducing sugars was observed. Transient starch content was enhanced by increasing [CO_2] to a much greater extent at 10,000 ppm CO_2 than at 1500 ppm CO_2. The percentage increase of starch content resulting from CO_2 enrichment declined as plants develope. In contrast, elevated [CO_2] promoted the accumulation of secondary metabolites (flavonoids) progressively to a greater extent as plants became mature. Elevated [CO_2] to 1500 ppm induced a higher initial growth rate, while super-elevated [CO_2] appeared to negate such initial growth promotion. However, after 4 weeks, there was no difference in vegetative growth between 1500 and 10,000 ppm CO_2-grown plants, both elevated CO_2 levels resulted in an overall 25% increase in biomass over the control plants. More interestingly, elevated atmospheric [CO_2] reduced evapotranspiration rate (ET), but further increase to the supra-optimal level resulted in increased ET (a reversed trend), i.e. ET at 1500 ppm < ET at 10,000 ppm < ET at 400 ppm. The differential effect of elevated and super-elevated CO_2 on plants was further reflected in the nitrogen dynamics. These results provide the potential metabolic basis for the differential productivity and stomatal function of plants grown under elevated and super-elevated CO_2 levels.

Keywords: Elevated carbon dioxide; Evapotranspiration (ET); Metabolite profile; Primary metabolites; Secondary metabolites; Super-elevated carbon dioxide; Wheat (*Triticum aestivum*)

1. Introduction

The steady increase of CO_2 levels in the atmosphere during past 150 years, and the prediction of its doubling over the next 50 years, have stimulated great interest regarding the impact of elevated CO_2 on photosynthesis and plant growth. Over the last two decades, elevated CO_2 plant physiology became a very extensively studied field, and a wealth of information on this subject has been accumulated (Urban, 2003). However, nearly all of these studies have

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examined the effects of CO_2 enrichment up to the vicinity of 1200 μ mol mol^{-1} (i.e. ppm as will be used hereafter); with relatively fewer studies dealing with CO_2 concentration above this level (Reuveni and Bugbee, 1997; Wheeler et al., 1993, 1999; Bugbee et al., 1994). Findings on the impact of super-elevated CO_2 on plants were intriguing in many ways. CO_2 enrichment up to 1200 ppm often brought about an initial increase in photosynthetic rate and enhanced vegetative growth and crop yield (Drake et al., 1997; Reuveni and Bugbee, 1997; Kauder et al., 2000). On the other hand, CO_2 enrichment above 1200 ppm appeared to have no further positive effect, but rather leads to decreased seed/fruit yield and harvest index in wheat (Grotenhuis and Bugbee, 1997; Reuveni and Bugbee, 1997), bean plants (Julliffe and Ehret, 1985), soybean (Wheeler et al., 1993), tomato (Madsen, 1974), and potato (Mackowiak and Wheeler, 1996). Reuveni and Bugbee (1997) attributed reduced seed yield to reduced dark respiration. In addition, super-elevated [CO_2] tended to induce foliar symptoms of chlorosis or even necrosis at the late developmental stages (van Berkel, 1984; Ehret and Jolliffe, 1985; Wheeler et al., 1993; Mackowiak and Wheeler, 1996), foliar deformation (Tripp et al., 1991), and toxicity (Bugbee et al., 1994). Moreover, plants grown under super-elevated CO_2 had greater transpiration than those grown under elevated CO_2; clearly the opposite of what would be expected (Pallas, 1965; Jarvis and Davies, 1998). This increased transpiration was found to be associated with increased stomatal conductance during both the light and dark cycles (Mackowiak and Wheeler, 1996; Wheeler et al., 1999).

While there are mechanisms proposed linking to plant acclimation after prolonged exposure to elevated CO_2, little is known on the mechanism why there is no further growth enhancement from CO_2 enrichment above the 1200 ppm level or why stomata re-open during the day but fail to close-down at night. Although atmospheric CO_2 on Earth is unlikely to exceed this level for many decades, CO_2 could easily reach much higher levels in closed ecological systems (e.g. 6000 ppm during the US Skylab mission, and between 3000 and 7000 ppm in the Mir Space Station), in natural environments near geothermal vents (Badiani et al., 1993; Miglietta and Raschi, 1993) and Martian environment where 95% of the atmosphere is composed of CO_2. There is therefore great interest in understanding the mechanisms underlying the differential effect of elevated and super-elevated CO_2 on plant function and yield. The knowledge of differential response mechanisms will not only provide insights to the limit of plant productivity, but also to the possibility for *in situ* utilization of CO_2 as atmospheric make-up gas in Mars green houses. Furthermore, most studies involving elevated CO_2 have focused on whole plant physiological responses, with fewer studies on molecular responses (Nie et al., 1995; Ludewig and Sonnewald, 2000, and references cited in a review by Urban, 2003). Studies on the metabolic consequence of CO_2 enrichment are even more limited and fragmented, with the emphasis frequently being placed only on carbohydrate metabolism (Grimmer et al., 1999), or a small group of compounds from a specific biosynthetic pathway. While such studies are essential and pivotal, they may be unable to reveal the coordinating networks of metabolic responses to elevated CO_2. Therefore, simultaneous quantitative and qualitative analyses of as many metabolites as possible are required to better understand the cascading effects of elevated CO_2 on the plant and the coordinated regulation necessary to maintain its homeostasis. This study coupled whole plant observations with both non-targeted and targeted analyses of primary and secondary metabolites using GC–MS and LC–MS to address the following specific questions: (1) What is the effect of elevated CO_2 on plant metabolism under a non-water limiting condition? (2) Is there any difference in metabolic response to elevated and super elevated CO_2? (3) What are the adaptive responses of metabolites to continuous exposure to elevated CO_2?

2. Materials and methods

2.1. Experiment design and plant cultivation

Wheat (*Triticum aestivum*, cv Yocoro rojo) was grown in a custom-designed root module consisting of a porous tube embedded in Turface (1–2 mm particle size) substrate containing 5 g Osmocote time release ferilizer L^{-1} (Levine et al., 2002). Twenty four seeds were glued to a capillary mat that was wrapped around a bottom-situated porous tube in the root module. Germination was initiated by saturating the substrate with organic-free de-ionized (DI) water in total darkness. Relative humidity (RH) was maintained at 90% for two days (germination phase). Substrate water level (SWL) was set at 1 g water per g substrate (defined here as 100%), which was higher than the field capacity of Turface (about 70%, w/w) to ensure a well-watered condition. The SWL was maintained by supplying DI water manually once a day to the substrate through the porous tube to re-establish the desired SWL set point. The amount of water required to establish the water level set point was determined by weighing the root module containing wheat seedlings every day, the changes in weight within a 24 h period was considered as the water loss via evapotranspiration and used for calculation of evapotranspiration rate.

Four root modules were housed in a Plexiglas chamber (33 × 37 × 33 cm L × D × H) with atmospheric CO_2 controlled to either 400 (i.e. 0.4 K), or 1500 (i.e.1.5 K) or 10,000 (i.e. 10 K) ppm. The CO_2 treatments were selected to simulate the conditions of enclosed habitats, and 1.5 K also represented the level at which most plants reach a CO_2 saturation point. All three Plexiglas chambers with both CO_2 control and humidity control were placed in a controlled environment chamber (CEC), thus plants were exposed to the same environmental conditions other than CO_2 level. CO_2 treatments were initiated on the third day. A light level of 185 μmol m^{-2} s^{-1} was delivered by

cool-white fluorescent light bulbs with a photoperiod of 18/6 light/dark. Air RH inside the Plexiglas chamber and CEC, and temperature were maintained at 75% and 23 °C from day 3 to the end of the experiment.

Six to eight plants were harvested from each treatment 5 h into the light cycle on 14, 21 and 28 days after planting (DAP). All the leaf blades, excluding tillers, from 6 to 8 plants were pooled, and immersed in liquid nitrogen immediately. This strategy of sample pooling was adopted to increase the representation of the entire plant leaf blade population, minimize biological variation and reduce the number of samples to be processed at the same time. Samples were lyophilized and ground to pass a 40-mesh screen. Ground tissue was stored over desiccant in a −20 °C freezer until analysis.

2.2. Total nitrogen content

Lyophilized and ground plant tissue (2–3 mg) was weighed and analyzed in triplicate for total nitrogen using a 2400 Series II CHNS/O elemental analyzer (Perkin Elmer Instruments, Shelton, CT).

2.3. Nitrate and phosphate concentration in tissue

The same plant material (ca. 20 mg) was quantitatively extracted with de-ionized water (two replicates per sample). The extract was filtered through a 0.45 μm pore size membrane filter, diluted to an appropriate concentration range, and analyzed for nitrate and phosphate using a DX-500 ion chromatograph system (Dionex Corp. Sunnyvale, CA).

2.4. Determination of starch and soluble carbohydrate

A well-established methodology based on enzymatic hydrolysis followed by enzyme-UV determination of liberated glucose (Boehringer Mannheim, 1979) was adopted for the determination of starch content in small sample sizes (Levine et al., 2005). About 15 mg of dried tissue was extracted with 3 mL 80% ethanol three times to remove soluble carbohydrates that were determined as described below. The sugar-free pellet was gelatinized in 0.2 N KOH in a boiling water bath. Upon neutralizing and cooling, 3 mL of amyloglucosidase solution (10 U mL^{-1} in 50 mM citrate buffer, pH 4.5) were added, and the mixture was incubated at 55 °C for 1 h after thorough mixing. Glucose resulting from the starch hydrolysis was determined by following the procedure provided with a glucose determination kit (R-Biopharm GmbH, Darmstadt, Germany).

For quantification of soluble carbohydrates, the ethanol extracts (see above) were combined and diluted to a total volume of 10 mL. One milliliter of extract was transferred to a vortex-evaporation tube from which the solvent was evaporated to dryness under vacuum at 40 °C. The dried extract was reconstituted in 5 mL of de-ionized water. The solution was filtered through a 0.45 μm pore size filter into an HPLC auto-sampler vial and analyzed using a DX-500 chromatography system (Dionex Corp. Sunnyvale, CA). Soluble carbohydrate was separated on a CarboPac (PA-10) column using 52 mM carbonate free sodium hydroxide at 1.3 mL min^{-1} and selectively detected by a pulsed amperometric detector (Dionex Technical Note 20, 1989).

2.5. Non-targeted metabolite profiling

Metabolites were extracted using a procedure described by Wagner et al. (2003) and modified for freeze-dried tissue. To approximately 9 mg of dried plant material, 350 μL of 85% methanol (pre-cooled to −20 °C) and 30 μL of internal standard mixture consisting of 0.2 mg mL^{-1} ribitol, 1 mg mL^{-1} d4-alanine, 0.5 mg mL^{-1} D-(-)-isoascorbic acid in 33% aqueous methanol was added. The mixture was mixed for 15 min at 70 °C at 750 rpm in an Eppendorf thermomixer (Brinkmann Instruments, Inc. Westburry, NY). Upon cooling to room temperature, 200 μL $CHCl_3$ was added and mixed for 5 min at 37 °C, which was followed by the addition of 400 μL H_2O for the partition of lipophilic and hydrophilic components. After vigorously shaking, the mixture was centrifuged at 14,000 rpm for 5 min in an Eppendorf centrifuge (model 5415C) to facilitate partitioning. A 160 μL aliquot from the polar phase (upper layer) was transferred to a clean micro vial (1.5 mL), and reduced to complete dryness in a Savant Speed Vac at room temperature for 4 h. Vials containing dried extract were flushed with argon and closed immediately for storage at −80 °C.

Polar metabolites in extracts were derivatized by methoxyamination and subsequent trimethysilylation as described by Fiehn et al. (2000) and Roessner et al. (2000). One microliter of metabolite derivative solution was subsequently injected into the GC–MS system (MD 800 ThermoQuest, Manchester, UK). Four separate extracts were prepared from each sample and served as analytical replicates.

Data acquisition and processing were accomplished using the MassLab version 1.4 software (ThermoQuest). AMDIS and NIST02 mass spectral search and comparison software programs were employed for mass spectral analysis (Ausloos et al., 1999; Stein, 1999). Metabolite identity was established by comparison of retention time indices (RI) and mass spectra from complex wheat extracts with a customized GC–MS database of authentic metabolite standards (Schauer et al., 2005) with accepted RI deviation of ±3.0 and a minimum similarity score of 750 on a scale of 0–1000. Currently non-identified compounds were designated as Mass Spectral Tags (MSTs). Selected ion peak areas of either identified metabolites or non-identified MSTs were normalized per plant dry weight and to the peak area of the internal standard ribitol. The resulting normalized response values were used as an estimate of metabolite abundance.

2.6. Targeted secondary metabolite (flavonoids) profiling

Freeze-dried plant tissues (10 mg) were pre-extracted with hexane and hexane/methanol (1:1) mixtures consecutively. Subsequently, flavonoids were quantitatively extracted with 80% methanol in an ultrasonic bath. Quercetin was added as an internal standard. The ratio of extraction solvent to plant materials was maintained at 5 mg mL^{-1}. Extracts were filtered through 0.45 μm pore size disc filters and directly subjected to a reverse phase HPLC/MS/MS analysis. A Thermo Separation Product HPLC system coupled with a ThermoFinnigan (San Jose, CA, USA) LCQ^{Deca} mass spectrometer was used. Separation of the metabolites of particular interest in the extract was carried out on an Alltech (Deerfield, IL, USA) Altima C18 column (5 μm particle, 2.1 × 150 mm) using gradient elution at 200 μL min^{-1}. A segment gradient that changed the solvent composition from 90% solvent A (94% H_2O + 5% MeOH + 1% AcOH) at time 0 to 90% solvent B (5% H_2O + 94% MeOH + 1% AcOH) at 65 min was employed to maximize the separation of metabolites. Both positive and negative electrospray ionization (ESI) full scan mass spectra, and tandem mass spectra were collected to unambiguously determine molecular weight and provide structural information, while the total ion counts of the product ions that resulted from negative ESI MS/MS of respective molecular ions were used for quantification.

2.7. Statistical analysis of data from non-targeted metabolite profiling

The normalized responses (see above) of metabolites from plants grown under 0.4 K ppm CO_2 served as a control group, and plants grown under elevated CO_2 (four analytical replicates) were subjected to statistical analysis of variance based on the SAS GLM Procedure. The parameters such as CO_2-effect, time-effect, and CO_2 by time interaction were assessed for each metabolite.

Principal component analysis (PCA) was performed with the S-Plus 2000 software package standard edition release 3 (Insightful, Berlin Germany) as described in more detail by Desbrosses et al. (2005). In short, for the purpose of PCA normalization, responses were converted to normalized response ratios using the average of the control group as quotient denominator. Normalized response ratios were $\log_{10}$ transformed. Missing data were replaced by 0 after $\log_{10}$ transformation.

3. Results

3.1. Whole plant responses

Fig. 1 shows 14-day-old plants grown under three CO_2 levels. Plants grown under 1.5 K CO_2 were substantially larger than 10 K CO_2 and control plants, and 10 K CO_2 plants exhibited slight leaf tip chlorosis (yellowing) indicative of stress. Shoot fresh weight (FW) at 14, 21 and 28 days after planting were also determined to assess plant growth rates. Plants grown at 0.4 K CO_2 doubled in biomass during the interval from DAP 14 to DAP 21, but exhibited insignificant increases from DAP 21 to 28 (Table 1). As expected, increasing [CO_2] from 0.4 to 1.5 K stimulated plant growth, especially during early plant development as evident by twice of the biomass compared to 0.4 K plants at DAP 14. However, such stimulation was not observed from further CO_2 enrichment to 10 K. Plants grown under 10 K CO_2 had similar biomass to that of the control plants, and less than that grown under 1.5 K CO_2 at DAP 14. By DAP 28, plants exposed to high CO_2 (both 1.5 and 10 K) had an overall 25% increase in biomass compared to 0.4 K treated plants. The highest growth rate for 1.5 K CO_2 occurred at a younger age (prior to DAP 14), while that for the 0.4 and 10 K treatments occurred between DAP 14 and 21. That is, elevated CO_2 accelerated plant development, but not super-elevated CO_2.

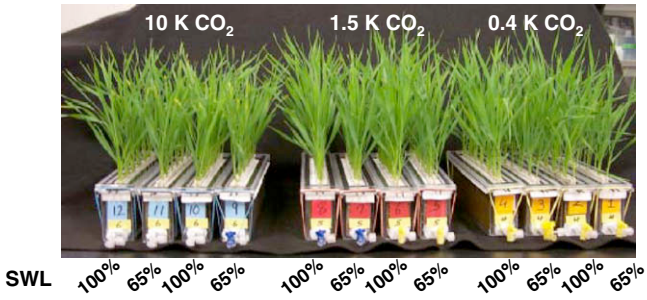

Fig. 1. Fourteen-day-old wheat seedlings grown in substrate compartments under substrate water level (SWL) 65% or 100% and three atmospheric CO_2 concentrations. Elevated atmospheric CO_2 enhanced vegetative growth, while super-elevated CO_2 negated such initial promotion and induced stress symptoms (e.g. tip-yellowing).

Water loss through evapotranspiration (ET) was measured daily beginning at DAP 4, not only to provide the basis for re-hydration of the growth medium, but also as an indirect measure of the effect of atmospheric CO_2 on stomatal conductance. The CO_2 enrichment to 1.5 K resulted in a decrease in ET rate as expected, but further enrichment to 10 K led to higher ET relative to the 1.5 K grown plants (Fig. 2).

3.2. Primary metabolites

A non-targeted metabolite profile of the polar fraction of wheat extracts revealed more than 200 metabolites,

Table 1
Biomass of wheat seedlings grown under three CO_2 levels

CO_2 (μmol mol^{-1}) (K)	Average shoot fresh weight (gram per plant)		
	DAP 14	DAP 21	DAP 28
0.4	0.5	1.0	1.1
1.5	0.9	1.1	1.4
10.0	0.5	1.0	1.4

Data represent the mean of 6–8 plants.

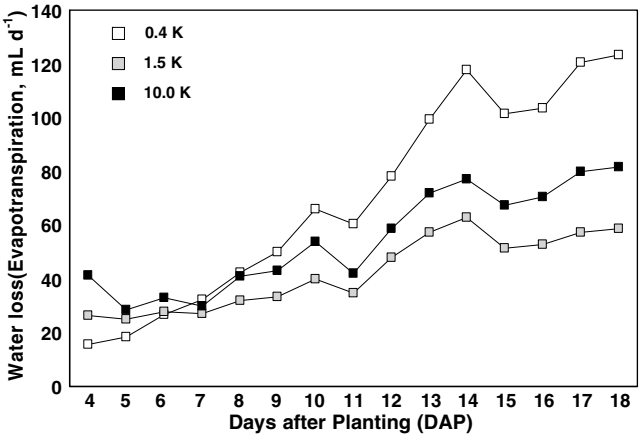

Fig. 2. Evapotransporation (ET) of wheat seedings over the course of 18 days, showing that plants grown in 1.5 K ppm CO_2 instead of 10 K ppm CO_2 had the lowest ET, indicating that stomata developed under the super-elevated CO_2 were more open than normal elevated CO_2 and potentially inhibited stomatal movement.

among which 68 were identified. Principal component analysis (PCA) revealed major metabolites that contributed the most to the effect exerted by CO_2 concentration and developmental stage. They were monosaccharides, oligosaccharides and amino acids. Comparison of CO_2 treatments at a specific sampling day demonstrated the profound CO_2 influence on metabolites (Fig. 3). Replicates (each dot represents a replicate) were clustered according to CO_2 treatments except for a few outliers indicated by arrows. At DAP 14, principal component 1 (PC1) resolved 0.4 and 1.5 K plants from 10 K plants, while PC2 resolved 0.4 K from 1.5 and 10 K plants. Similar cluster groupings were seen at DAP 21 and 28.

Further statistical analysis (ANOVA) identified those exhibiting statistically significant signal intensity changes in response to time of growth and/or atmospheric CO_2 concentration. Of special interest were soluble sugars, hexose phosphates and free amino acids. At 0.4 K ppm CO_2 (control), maltose, an immediate product of hydrolytic starch degradation, increased nearly 5-fold at DAP 21 and held steady from DAP 21 to 28 (Fig. 4A). With the exception of glucose-6-phosphate (G-6-P), downstream metabolites, fructose-6-phosphate (F-6-P) and mannose-6-phosphate (M-6-P) (Fig. 4A and C) also exhibited a developmental dependent change similar to maltose. In response to increasing CO_2, maltose content changed very little from 0.4 to 1.5 K CO_2, increasing approximately 3-fold from 0.4 to 10 K (Fig. 4B) on DAP 14. Furthermore, glucose, fructose and sucrose concentrations followed a similar trend (Table 2) across CO_2 treatments at DAP 14 and across different developmental stages under 0.4 K CO_2 (control). Prolonged exposure (DAP 21 and 28) to either 1.5 K or 10 K ppm CO_2 resulted in reduced glucose, fructose and sucrose. Data in Table 3 also showed that plants grown under elevated CO_2 reached equivalent levels of these carbon metabolites

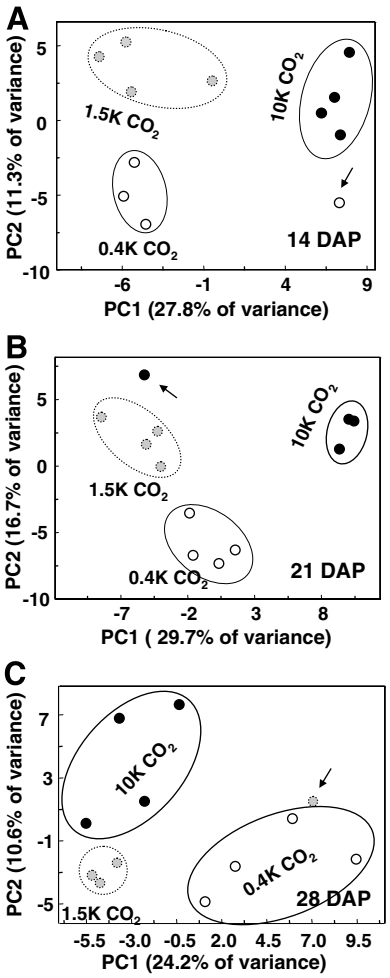

Fig. 3. Principal component plots of Log 10 transformed normalized response ratios of metabolites from GC–MS metabolite profiles in DAP 14 (A), DAP 21 (B) and DAP 28 (C) plants. Arrows indicate outlier samples which were ommitted from statistical analysis of variance.

at an earlier time point of development compared to plants grown under ambient CO_2 as indicated by color shade.

Starch content increased as [CO_2] increases from 0.4 to 1.5 K and to 10 K regardless of the developmental stages examined (Fig. 5). The CO_2 influence on starch accumulation was much more pronounced in younger plants. Under both elevated CO_2 conditions, starch content increased from DAP 14 to 21, followed by a decrease in DAP 28 plants, while under ambient CO_2 (0.4 K), there continued a small steady increase from 14 to 28 DAP.

The concentrations of most amino acids did not show any consistent trend in response to elevated CO_2 (data not shown). Only tyrosine content showed a marginally significant effect of CO_2. It increased as the CO_2 rose from 0.4 to 1.5 K at any given sampling point (e.g. from not detectable to relative signal intensity of 4 at DAP 14, 7 to 12 at DAP 21 and 10 to 19 at DAP 28). A further increase of CO_2 to 10 K did not continue the upward trend except for DAP 14 when it increased to 12.

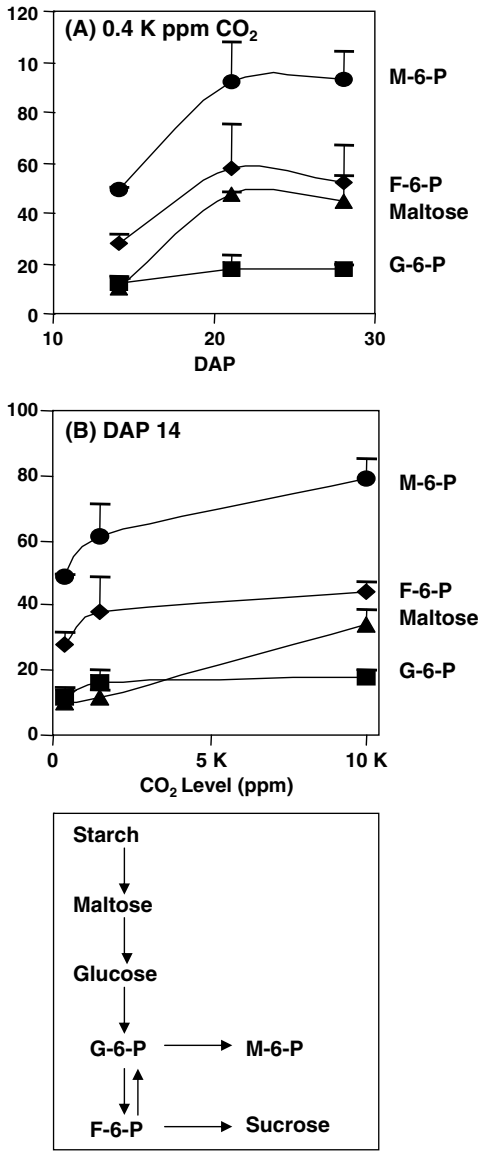

Fig. 4. Key metabolites responding to plant age at ambient CO_2 (400 ppm) (A) and elevated CO_2 (1500 and 10,000 ppm) at DAP 14 (B). ♦, Fructose-6-phosphate; ■, glucose-6-phosphate; ▲, maltose; •, mannose-6-Phosphate.

3.3. Secondary metabolites

Flavonoids were determined because they represent a large group of abundant secondary metabolites. Eleven abundant flavonoids were detected using liquid chromatography coupled with tandem mass spectrometry (Supplemental Fig. A1). The major flavonoid in wheat seedlings was found to be 6-C-glucosyl luteolin (i.e. homoorientin) with a nominal mass of 448. The other flavonoids were found to have corresponding molecular weights listed in the insert of Fig. A1, and to be structurally correlated with the most abundant flavonoid homoorientin in a way shown in Supplemental Fig. A2. No free aglycones (i.e. non-glycosylated phenolics) were found. All flavonoids identified in wheat in this study had a carbon to carbon linked apigenin glucoside core structure, and were likely derived from apigenin through hydroxylation, glycosylation with pentose, rhamnose, hexose, or methylation of one of the hydroxy groups in the aglycone (Fig. A2).

Table 2
Soluble sugars determined by IC/PAD (mg g^{-1} dry material)

CO_2 levels (μmol mol^{-1}) (K)	DAP 14	DAP 21	DAP 28
Glucose			
0.4	9.4 ± 0.07	39.2 ± 0.29	28.8 ±0.95
1.5	5.3 ± 0.24	20.0 ± 0.04	19.1 ±0.35
10.0	23.0 ± 0.53	19.1 ± 0.18	12.1 ±0.10
Fructose			
0.4	5.7 ± 0.30	36.3 ± 0.59	32.2 ± 1.90
1.5	0.7 ± 0.15	10.8 ± 0.10	18.5 ± 0.08
10.0	10.3 ± 0.4	24.1 ± 0.62	21.6 ± 0.32
Sucrose			
0.4	39.7 ± 0.54	68.0 ± 1.31	67.8 ± 1.49
1.5	32.3 ± 1.08	81.9 ± 1.04	56.0 ± 0.50
10.0	59.4 ± 1.92	75.7 ± 0.66	52.0 ± 0.51

Shading was used to show that plants grown under elevated CO_2 reached a similar level of metabolites at a younger age than plants grown under ambient CO_2. Values in parenthesis are standard deviations of three analytical replicates.

Table 3
Tissue phosphate content (mg g^{-1} dry tissue)

CO_2 (μmol mol^{-1}) (K)	DAP 14	DAP 21	DAP 28
0.4	10.9 ± 0.3	12.3 ± 2.6	7.4 ± 0.8
1.5	11.8 ± 0.1	11.5 ± 0.8	11.1 ± 1.4
10.0	11.5 ± 1.1	11.6 ± 0.7	8.0 ± 0.5

Values represent the average of two analytical replicates of samples pooled from 6 to 8 plants and standard deviations.

The concentration of these secondary metabolites was affected by both elevated CO_2 and developmental stage. The content of total leaf flavonoids kept climbing as the CO_2 increased to 1.5 and 10 K ppm (Fig. 6). Older plants had higher levels of flavonoids and greater increases in flavonoids in response to CO_2 enrichment (Fig. 6A). Indi-

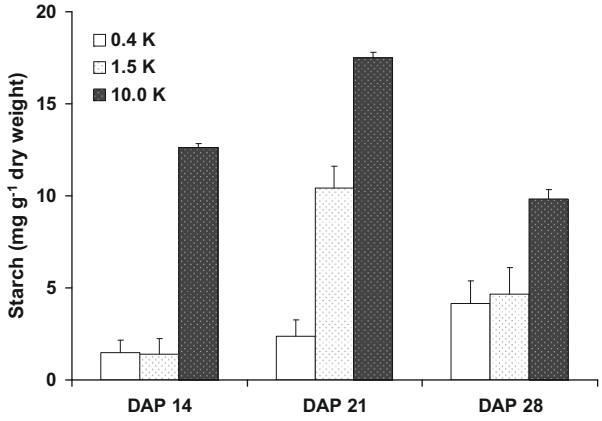

Fig. 5. Transient starch concentration in wheat leaves increased in response to increased atmospheric CO_2 level (also affected by leaf age). Unit for CO_2 concentration was μmol mol^{-1} (i.e. ppm) for this and all other graphs.

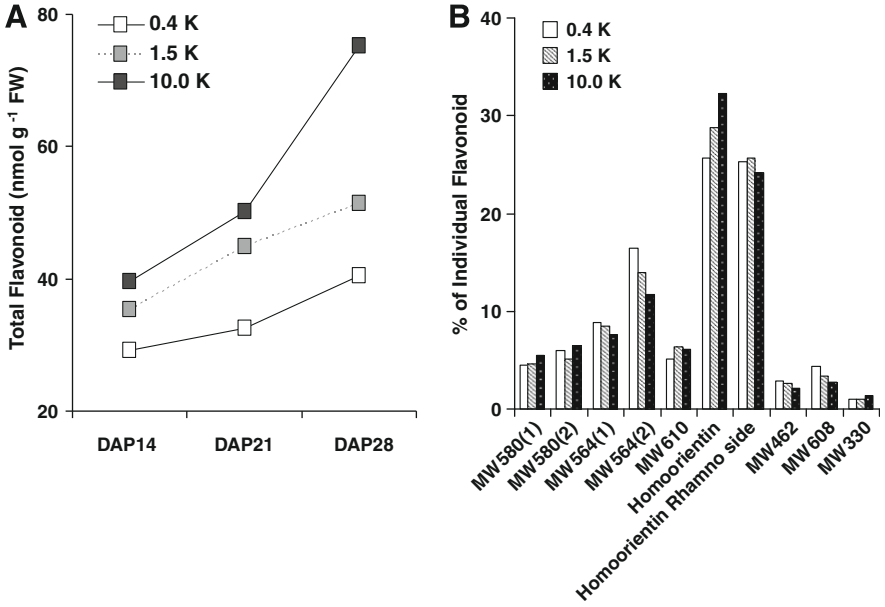

Fig. 6. Total flavonoid content in wheat leaves at three sampling times and three CO_2 levels (A) and relative abundance of different flavonoids on DAP 28 (B). Although elevated CO_2 enhanced the overall accumulation of flavonoids, it had a differential effect on individual flavanoids.

vidual flavonoids were affected by the enrichment of CO_2 differently. Mono glycosylated flavonoids (especially hormooorientin) increased in elevated CO_2-grown plants, while diglycosylated flavonoids (MW 564, MW 608 and 462) decreased. Homoorientin and its rhamnoside were most abundant and constituted more than 28–64% of the total flavonoids depending on the CO_2 levels.

The pool size of two precursors of the phenylpropanoid pathway, tyrosine (Tyr) and *trans*-caffeic acid, were also affected by increased CO_2 levels. Tyr increased as CO_2 concentration increased in DAP 14 plants, while *trans*-caffeic acid content increased as CO_2 concentration was increased in DAP 28 plants.

3.4. Phosphorous and nitrogen status

Phosphate concentration was relatively constant for all CO_2 treatments at DAP 14 and 21 (Table 3). At DAP 28, the phosphate levels of 0.4 and 10 K plants appeared to be slightly reduced, while 1.5 K plants were unchanged.

The level of nitrate and organic nitrogen in leaf tissues was affected by both the developmental stage and atmospheric CO_2 level (Fig. 7). The organic nitrogen level mirrored the change of the tissue nitrate. Both nitrate-N and organic-N were strikingly high in 14-day plants, and drastically reduced from DAP 14 to 21, with much smaller changes thereafter. There was a general downward trend as CO_2 level increased with the exception of DAP 14 plants. In the younger plants, both nitrate and organic nitrogen increased with CO_2 enrichment to 1.5 K, but dropped substantially in the 10 K CO_2 treatment. The ratio of organic nitrogen to nitrate nitrogen (Fig. 7 insert) increased with the age at any given CO_2 level, peaking at DAP 28 for the control CO_2 and DAP 21 for elevated CO_2-grown plants. The ratio was generally increased as CO_2 level increased especially for the earlier vegetative growth (DAP 14 and 21).

4. Discussion

4.1. Whole plant response and primary metabolism

Elevated CO_2 induced higher growth rate initially, but the percentage increase in growth was reduced by the continuous exposure to the elevated level of CO_2 (Table 1). In contrast, further increase to 10 K ppm CO_2 (super-elevated CO_2) did not promote such an initial increase, although it resulted in an overall 25% increase in biomass similar to 1.5 K CO_2 after 4 weeks (DAP 28). This suggests that super-elevated CO_2 may result in temporary stresses in non-acclimated and young plants. This observation is consistent with previous findings (Grotenhuis and Bugbee, 1997; Reuveni and Bugbee, 1997) that there was no overall benefit in terms of biomass accumulation by increasing CO_2 above 1.5 K. The differential response to elevated and super-elevated CO_2 levels at early developmental stages was repeatedly observed, and exacerbated at lower substrate water level. Under less than optimal water condition, e.g. 65% SWL, 1.5 K CO_2 led to 44% increase, while 10 K CO_2 led to 7% decrease in shoot biomass by 18 days of growth. Under well-watered conditions (e.g. 100% SWL), 1.5 K CO_2 resulted in similar increases, but 10 K CO_2 resulted in a 19% increase instead of a decrease. Furthermore, in spite of well-watered, well-fertilized and ample light conditions, it was consistently found that super-elevated CO_2 had a reduced effect on promotion of plant vegetative growth compared with the CO_2 levels ranging from double to triple of the ambient CO_2. Although photosyn-

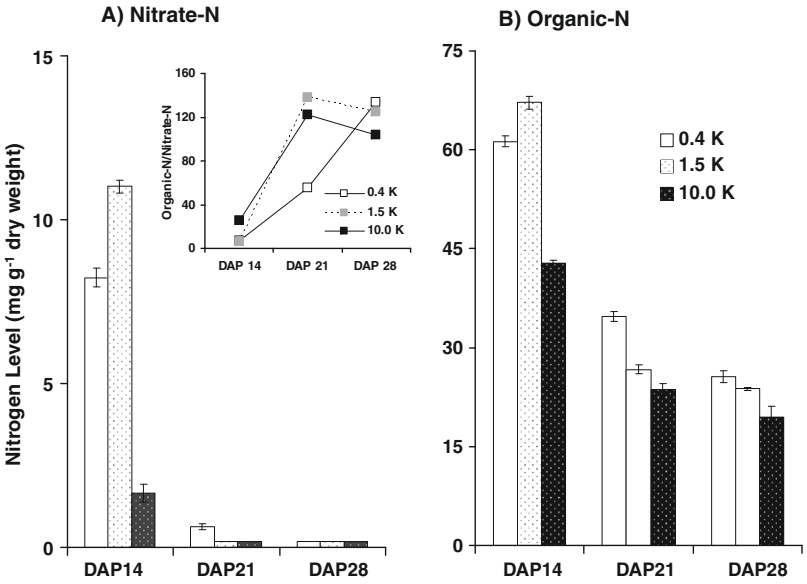

Fig. 7. Nitrate nitrogen and total organic nitrogen content affected by developmental stage and elevated CO_2. Error bars represent standard deviations of three analytical replicates of the pooled samples.

thetic rate was not measured directly in this study, the reduced growth rate (or net carbon gain) from DAP 14 to 28 implied reduced photosynthesis under 1.5 K CO_2 compared with the control.

A decreased photosynthetic capacity or down-regulation of photosynthesis has been observed in many CO_2 enrichment studies and is termed acclimation. The mechanism underlying this acclimation is still very elusive. Based upon findings mostly from doubling of the CO_2 concentration, several factors have been proposed to explain this phenomenon: (1) the sink-limiting or sugar-mediated down-regulation of photosynthesis (Stitt, 1991; Stitt and Krapp, 1999; Paul and Pellny, 2003), (2) limitation by N-availability (Pettersson and McDonals, 1994; Stitt and Krapp, 1999), and (3) accelerated senescence (Miller et al., 1997; Ludewig and Sonnewald, 2000). The first hypothesis is substantiated by the correlation between increased accumulation of soluble sugars under elevated CO_2 and down-regulated photosynthetic gene transcripts (Nie et al., 1995; van Oosten and Besford, 1996; Paul and Driscoll, 1997; Pego et al., 2000), and by the evidence obtained from experiments involving sugar addition to the growth medium, cold-girding of leaf petioles to reduce the export of sugars and other metabolites, as well as the utilization of transgenic plants that express invertase (Pego et al., 2000 and reference cited therein). However, the effect of elevated CO_2 on sugar accumulation is inconsistent and a negative correlation between soluble sugar content and photosynthetic rate has not always been found (Ludewig and Sonnewald, 2000 and references therein, Wingler et al., 2006). In our study, although the accumulation of starch and hexose phosphates (Figs. 5 and 4) increased in response to elevated CO_2 and to a greater extent in 10 K than 1.5 K CO_2, glucose and fructose levels (Table 2) were lower in elevated CO_2 grown plants compared with control plants at DAP 21 and 28. Thus the reduced growth rate at elevated CO_2 is not associated with soluble sugar accumulation. The pattern of secondary metabolite accumulation (Fig. 6A) as well as our recent finding that Arabidopsis reached the 1.14 leaf stage four days earlier under high CO_2 than their control counterparts suggest that plants grown under elevated CO_2 are somewhat accelerated developmentally. Therefore, should samples be taken on the same day after planting for high CO_2 and ambient CO_2-grown plants, the result would reflect the comparison between plants of different developmental stages, and thereby different photosynthetic capacity. Furthermore, the fact that tissue nitrogen declined from above 50 mg g^{-1} dry matter (recorded for healthy wheat plants) to below 30 mg g^{-1} in elevated CO_2 grown plants at 21 and 28 DAP (Fig. 7) supports the view that nitrogen supply plays a key role in the acclimation to elevated CO_2.

Water use efficiency is known to be affected by atmospheric CO_2 concentration. Under elevated CO_2 stomatal conductance (in most species) would decrease, resulting in less transpiration per unit leaf area. The typical 40% reduction in stomatal conductance induced by a doubling of CO_2 has generally resulted in only a 10% (or less) reduction in crop canopy water use in chamber or field experimental conditions. An interesting finding from this study is that increasing CO_2 from ambient to 1.5 K reduced the evapotranspiration (ET) rate as it would be expected, while a further increase in CO_2 levels from 1.5 to 10 K increased the ET rate (Fig. 2). This result is consistent with the direct measurement of stomatal conductance of wheat grown under similar CO_2 levels (Wheeler et al., 1999), that is, the conductance in the mid-day period is the highest in the 0.4 K ppm treatment followed by 10 K and the 1 K ppm treatments. In addition, Wheeler et al. (1999) also found that super-elevated CO_2 not only

resulted in higher stomatal conductance than elevated CO_2 during the light period, but that the rate of stomatal closure declined during the dark period and stomata failed to close-down as much as that of the control or elevated CO_2 grown plants. It was reported that the mid-dark period conductance for wheat averaged 3%, 13% and 22% of that of mid-light period for 0.4 K, 1 K and 10 K CO_2 treatments, respectively. It is the combination of higher stomatal conductance in both light and dark periods for the super-elevated CO_2 treatment compared to moderately elevated CO_2 that resulted in the higher transpiration observed in this study. Although the mechanism underlying the re-opening of stomata and failure to achieve complete closure at night in plants exposed to super-elevated CO_2 is unknown, this result once again demonstrates that the effect of CO_2 concentration on plants is fundamentally different depending on its level.

4.2. Nitrogen dynamics

Although it has been reported that tissue nitrate level decreases with plant age for other plant species such as lettuce (Blom-Zandstra and Eenink, 1986), the 10-fold decrease from DAP 14 to 21 found in this study is quite unique. Rapid biomass accumulation and increased nitrate assimilation as plants develop will no doubt contribute to the reduction of tissue nitrate level, but this cannot account for the observed difference. The temporal change in tissue nitrate level may be in part due to the fact that Osmocote (a time release fertilizer) releases nitrate at a constant rate, while nitrate uptake and assimilation rate varies as plants develop. Therefore, there was an accumulation of nitrate in younger seedlings when the nitrogen assimilatory capacity and demand were low (nitrate assimilation rate $\ll$ nitrate uptake rate), and a draw-down when the nitrogen demand was high at rapid growth phases (nitrate assimilation rate $\gg$ nitrate uptake rate) as shown in Fig. 7A.

There was a general trend that elevated CO_2 decreased nitrate nitrogen and total nitrogen in leaves with an exception at DAP 14 (Fig. 7A and B). This is largely due to a higher rate of carbon accumulation than of nitrate uptake as well as a higher rate of nitrate assimilation under elevated CO_2. This phenomenon has been reported before by Theobald et al. (1998). It was found that elevated CO_2 (double the ambient) not only reduces leaf nitrogen content in spring wheat, but also reduces the amount of Rubisco required to maintain enhanced rates of photosynthesis. It is worth noting the pattern of nitrate content at DAP 14. CO_2 enrichment from 0.4 K to 1.5 K enhanced accumulation of nitrate in spite of a doubled biomass (Table 1) and reduced transpiration rate (Fig. 2), while 10 K CO_2 exposure led to a significant reduction in nitrate concentration in spite of the abundant supply of nitrate in the growth medium, higher transpiration than 1.5 K CO_2 plants, and no obvious increase in biomass compared to ambient control plants. We have previously observed this differential effect of elevated and super-elevated CO_2 on tissue nitrate level in green onion (*Allium fistulosum* L. cultivar) grown hydroponically where there was continuously high nitrogen supply due to the fact that nitric acid was used to maintain the pH of the growth medium (unpublished data). In the instance of green onion, elevating CO_2 to 1200 ppm resulted in a 20% increase in nitrate content, while elevating it to 4000 ppm led to a 6% decrease under 450 $\mu mol\ m^{-2}\ s^{-1}$ of cool-white fluorescent lamps. This result is consistent with nitrate uptake not being governed by transpiration rate alone, but by active nitrate transporters (Harrison et al., 2004). Moreover, this result implies that nitrate transporters are influenced by CO_2 concentrations, and may be enhanced by elevated CO_2, but suppressed by super-elevated CO_2. Our results also seem to suggest that the influence of CO_2 concentration on tissue nitrogen level is developmental stage dependent. However, Farage et al. (1998) elegantly demonstrated that it was not the total level of nitrogen availability, but the means by which nitrogen was supplied resulting in different influences on elevated CO_2 relative to tissue nitrogen level and plant growth.

To a less extent compared with nitrate nitrogen, organic nitrogen decreased as the plants developed and declined at a higher rate at an earlier stage (e.g. from DAP 14 to 21) (Fig. 7B). This is partially due to the dilution effect accompanying the greater increase in biomass accumulation from DAP 14 to 21 than that from DAP 21 to 28. Furthermore, the result that increasing CO_2 resulted in lower levels of tissue nitrogen (Fig. 7) is in agreement with the previous findings by Farage et al. (1998) in wheat grown in sand culture with N supplied at a fixed concentration. The drop in organic nitrogen from 50 to 60 $mg\ g^{-1}$ dry mass to below 30 $mg\ g^{-1}$ may have contributed to the reduced growth rate (or net carbon gain) from DAP 14 to 28 under elevated CO_2. On the other hand, the ratio of organic nitrogen to nitrate nitrogen (Fig. 7 insert) increased with increasing age of the vegetative tissue at any given CO_2 level, which is likely the result of increased nitrate assimilation and the decreased nitrate uptake due to declining availability of free nitrate supply in the growth medium. Elevated CO_2 had a greater impact on nitrate nitrogen concentration than organic nitrogen concentration, as indicated by the general downward trend in both forms of nitrogen (Fig. 7) and an increase in the ratio of organic nitrogen to nitrate nitrogen in DAP 14 and 21 plants. Since the ratio indicates the units of reduced-N present for every unit of nitrate-N in tissue at a given time, it can be used as an indirect measure of assimilatory capacity. Consequently, the results suggest the plants grown under high CO_2 reached equivalent assimilatory capacity at a younger age than the control plants.

It was somewhat surprising that there was a lack of responsiveness of most amino acids to increasing CO_2 and developmental stage. We attribute this result to the fast turnover rate and small pool size of these primary metabolites. Therefore, other nitrogen containing compounds (e.g.

polyamines) and proteins might be the main contributor to the reduced total organic nitrogen with increasing maturity and CO_2 concentration.

4.3. Secondary metabolism

Previous studies using free air CO_2 enrichment (FACE) to 550 ppm have demonstrated that elevated CO_2 induces the accumulation of carbon-based secondary compounds (Peñuelas et al., 1996, 1997), for example, flavonoids (Estiarte et al., 1999). The result from this study not only confirms these observations that elevating CO_2 to a moderate level increases flavonoid accumulation, but also extends our current knowledge to the super-high CO_2 (10 K or 1%) level as well (Fig. 6). Moreover, plants grown under high CO_2 consistently have the flavonoid phenotype of older plants grown under ambient levels of CO_2. The increased accumulation of secondary metabolites in plants grown under elevated CO_2 may have implications regarding plant–herbivore interactions, decomposition rates for inedible biomass, and potential beneficial effects on plant tolerance to water stress (Idso, 1988) and cold stress (Solecka and Kacperska, 2003) due to their potentials for the scavenging of reactive oxygen species (ROS).

Observations regarding the effect of elevated CO_2 on primary and secondary metabolites support the carbon-nutrient balance hypothesis (Bryant et al., 1983), which predicts that changes in source-sink relationships are accompanied by variations in relative partitioning of carbon to growth, total non-structural carbohydrate and carbon-based secondary (structural or non-structural) compounds. According to this hypothesis, either high CO_2 that increases carbon supply, or nutrient stress that decreases carbon demand, would promote a relative increase in carbon availability and accumulation of total non-structural carbohydrate in source leaves. This accumulation exceeding growth requirements would provide carbon for boosting secondary compound synthesis. The present findings that not only starch (Fig. 5), but also flavonoids (Fig. 6) and C/N ratio (Table 4) increased with elevated CO_2 levels support this hypothesis.

Table 4
Ratio of carbon to nitrogen in plant tissue

CO_2 levels (μmol mol^{-1}) (K)	DAP 14	DAP 21	DAP 28
0.4	7.0 ± 0.1	12.1 ± 0.3	15.9 ± 0.5
1.5	6.3 ± 0.1	15.6 ± 0.4	17.0 ± 0.1
10.0	10.2 ± 0.2	17.9 ± 0.6	21.9 ± 1.8

5. Conclusions

Plants respond to elevated and super-elevated CO_2 in different ways. The differential response is not only limited to structural carbon accumulation and stomatal function, but also extends to the metabolism of small molecules (i.e. metabolites). Some of them may function as signaling molecules in regulating plant growth and stomatal movement. High CO_2-grown plants have the metabolic phenotype characteristic of older plants grown at ambient CO_2. Further study is required to address: (a) what ultimately limits photosynthesis; (b) can plants be selected for and/or adapt to the efficient use of super-high CO_2 concentration? (c) if so, how long (number of life cycles, and slope and duration of CO_2 increase) would the acclimation process be? Studies are underway in our laboratory to address these questions by integrating of global gene and metabolite profiles.

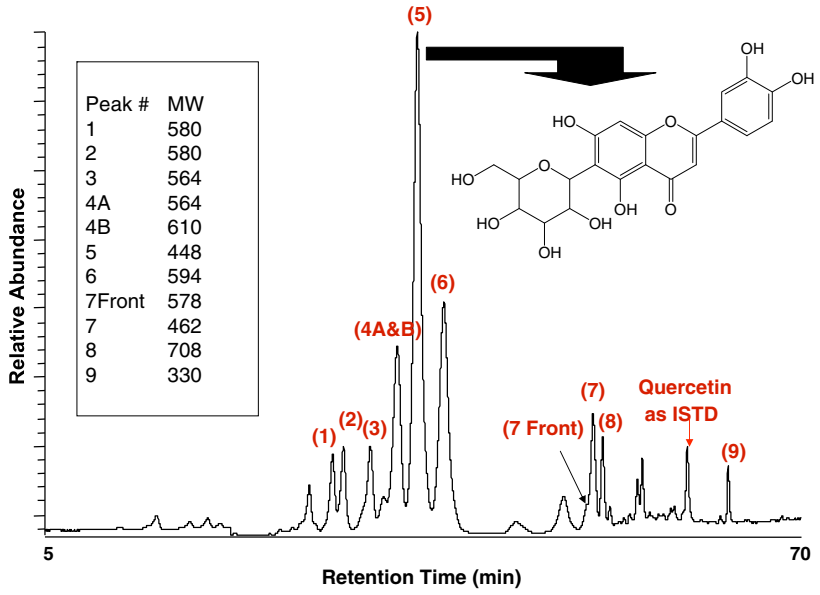

Fig. A1. Liquid chromatography/total ion chromatogram of wheat extract revealing qualitative and quantitative diversity of flavonoids in wheat, as well as that the most abundant flavonoid was homoorientin with a nominal mass of 448.

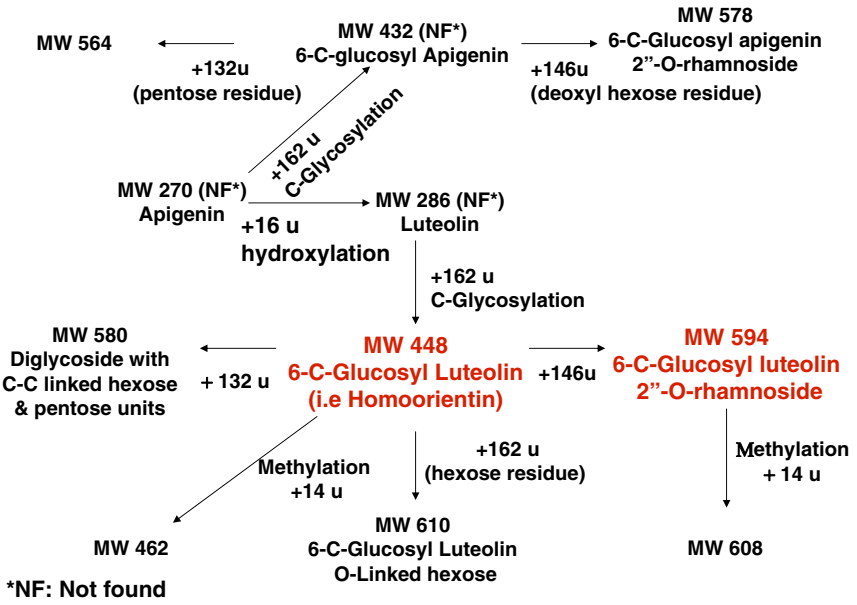

Fig. A2. Flavonoids found in wheat seedlings and their structural relationship to the predominant flavonoid, homoorientin. The +132 u, + 162 u and so forth indicate the mass difference between flavonoids, and were determined by tandem mass spectrometric procedures. This suggests all the flavonoids identified here might be derived from apigenin through hydroxylation, glycosylation or methylation. *NF designates "not found".

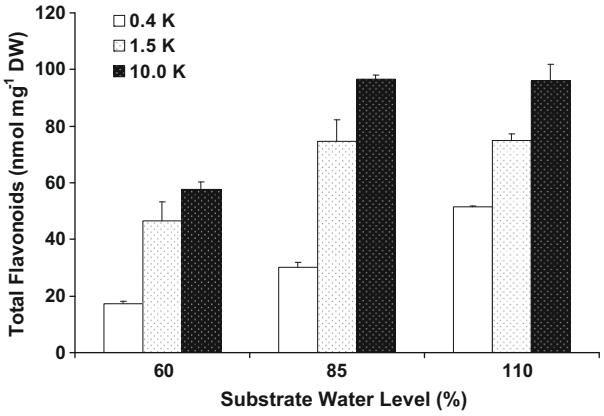

Fig. A3. Total flavonoid content in the leaf samples of 18-day-old wheat seedlings affected by the substrate water and atmospheric CO_2 levels. Error bars are standard deviations of three analytical replicates of pooled samples.

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Appendix A. See Figs. A1–A3.

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