Molecular mechanisms of growth responses to elevated atmospheric carbon dioxide in wheat (*Triticum aestivum* L.)

A Thesis submitted by

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BSc (Agriculture) (Hons)

For the award of

Doctor of Philosophy

2018

Abstract

The carbon dioxide concentration [CO_2] in the current atmosphere is increasing at a significant rate and is predicted to reach up to 700 µmol mol^{-1} by the end of this century. Elevated ([CO_2]) has the potential to increase the growth and yield of crops. Being the primary substrate for photosynthesis, increased CO_2 levels significantly promote the growth of most C_3 crops through increased photosynthesis capacity and reduced stomatal conductance. This stimulation of photosynthesis is central to other post-photosynthetic key metabolic processes such as carbon and nitrogen metabolism, cell cycle functions and hormonal regulation, which may lead to changes in whole plant growth. The magnitude of these responses to elevated [CO_2] varies even within same species, indicating a significant genetic variation for CO_2 responsiveness within plant communities. In addition, the CO_2 responsiveness of plants depends on their ontogeny and is found to be more pronounced in the early development stages of the crops. However, there is a limited understanding of underlying molecular mechanisms of plant growth responses to elevated [CO_2] which is crucial for developing crop breeding strategies to improve crop productivity in a changing climate. Therefore, this project broadly aimed to dissect the molecular mechanisms of plant growth responses to elevated [CO_2] in wheat (*Triticum aestivum* L.), focusing on three unexplored aspects of this underlying mechanism. This project focused primarily on the early vegetative stage of wheat to study the increased CO_2 responsiveness of crops in their early ontogeny.

First, putative quantitative trait loci (QTL) for major early growth traits at elevated [CO_2] were identified using a doubled haploid population of a cross between RAC875 and Kukri, to identify the genetic regions potentially associated with CO_2 responsiveness. In total 24 putative QTL for CO_2 responsiveness were identified for different growth traits. Three QTL, worthy for future research, were identified on chromosome 2A, 1B and 4B that showed an increased response for biomass accumulation at elevated [CO_2]. Secondly, the role of photosynthesis and post-photosynthetic metabolic processes in moderating growth responses to elevated [CO_2] was investigated through developing an understanding of the source and sink interaction of wheat. Transcript abundance of key genes involved in carbon and nitrogen metabolism, and cell cycle functions varied greatly among CO_2 levels (400 and 700 µmol mol^{-1}), organ types (last fully expanded leaves, expanding leaves, leaf cell elongation zone and shoot apex region) and genotypes. Finally, the interplay of different regulatory mechanisms involved in plant growth at elevated [CO_2] was investigated through a comparative proteomics analysis. Most of the differentially expressed proteins at elevated [CO_2] were involved in carbon metabolism, energy pathways, protein synthesis and cell cycle functions. However, the leaf proteome responses to elevated [CO_2] were highly genotype dependent. Overall, the results indicated that post-photosynthetic metabolic processes play a significant role in moderating plant growth responses at elevated [CO_2]. Molecular level responses of these processes are subject to developmental regulation and thus, are involved in determining the source and sink integration of plants. This study has demonstrated the intraspecific variability of growth responses to elevated [CO_2] at the genetic, transcriptomic and proteomic level. These variable responses provide valuable targets for the selection of genotypes that can thrive well in the future CO_2 enriched atmosphere.

Certification of Thesis

This Thesis is the work of Dananjali Gamage except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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Statement of Authorship

Chapter 2: New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide. **Gamage D**, Thompson M, Sutherland M, Hirotsu N, Makino A, Seneweera S 2018, Plant, Cell & Environment, 41(6), 1233-1246.

Dananjali Gamage (DG) contributed to 60% of the paper, consisting of the paper's design, literature collection and interpretation, and writing of the manuscript. Saman Seneweera (SS) contributed to 20% of the paper, consisting of the paper's design, revision of the paper and editorial input. Michael Thompson (MT), Amane Makino (AM), Mark W. Sutherland (MWS) and Naoki Hirotsu (NH) contributed to 20% of the paper, consisting of revision and editorial input.

Chapter 3: Early growth mechanism of wheat (*Triticum aestivum* L.) at elevated atmospheric carbon dioxide: New physiological evidence with quantitative trait loci data. **Gamage D**, Thompson M, Okamoto M, Moriyama N, Sutherland M.W., Hirotsu N, Seneweera S. (Prepared for publication)

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Chapter 4: Elevated carbon dioxide mediated early growth responses of wheat (*Triticum aestivum* L.): an analysis of source and sink interactions. **Gamage D**, Thompson M, Dehigaspitiya P, Fukushima A, Sutherland M.W., Hirotsu N, Seneweera S. (Prepared for publication)

DG contributed to 60% of the paper, consisting of experimental design; acquisition, analysis and interpretation of data; drafting and revising the manuscript. SS and NH contributed 20% and 10%, respectively consisting of the study conception and design, analysis and interpretation of data, critical revision of the manuscript. Prabuddha Dehigaspitiya (PD), Ayaka

Fukushima (AF), MT and MWS contributed 10%, consisting of data collection, revision of the manuscript and editorial input.

Chapter 5: Leaf proteome responses of wheat (*Triticum aestivum* L.) to elevated atmospheric carbon dioxide during early vegetative growth. **Gamage D**, Thompson M, Dehigaspitiya P, Faou P, Rajapaksha KH, Downs R, Sutherland M.W., Hirotsu N, Seneweera S. (Prepared for publication)

DG contributed to 60% of the paper, consisting of experimental design; acquisition, analysis and interpretation of data; drafting and revising the manuscript. SS contributed 20% a conception and design, analysis and interpretation of data, critical revision of the manuscript. NH and Harinda Rajapaksha (HR) contributed 10% consisting of data analysis, interpretation and revision of the manuscript. Pierre Faou (PF), Rachael Downs (RD), MWS, MT and PD contributed 10%, consisting of data collection, analysis, revision of the manuscript and editorial input.

Acknowledgment

This PhD has been one of the most amazing experiences of my life and I am truly grateful for all those who have supported me throughout this journey. First and foremost, I would like to acknowledge the University of Southern Queensland for providing me this great opportunity to pursue my doctoral degree along with a USQ Postgraduate Research Scholarship. I would like to express my special appreciation and sincere gratitude to my principal supervisor, Professor Saman Seneweera, for his tremendous academic support, valuable guidance and constant encouragement throughout this journey. I am also indebted to Associate Professor Naoki Hirotsu, associate supervisor of my PhD project for his great scholarly input into my research experiments and constructive discussions on data analysis and interpretation. Whenever I had doubts and questions, Prof. Hirotsu was always made himself available to clarify them despite his busy schedule. Similar profound gratitude goes to Professor Mark Sutherland, my associate supervisor, for his academic support and critical revision of all the scholarly outputs generated from this PhD study. I consider it a great opportunity to pursue my doctoral degree under the great supervision of these three exemplary academics.

I am also hugely appreciative to Dr. Anke Martin from the University of Southern Queensland who has been a dedicated mentor of molecular biology laboratory work and for her kind support during tough times in the PhD pursuit. Further, I would like to express my gratitude for Prof. Peter Terry, Director/Training and Research, USQ for his kind support during my studies at USQ. I also extend my sincere thanks to Professor Achini De Silva, Professor Seiji Nagasaka and Dr. Hao for their support in different stages of my PhD. In addition, I would like to thank Dr. Mamoru Okamoto for supplying the wheat population used in my experiments and Dr. Kolin Harinda Rajapaksha for his great support with the proteomics analysis. I'm truly grateful for Professor Amane Makino and Professor Paul Milham for their support extended during scholarly article preparation. Further, I would like to appreciate the support of Nao Moriyama and Ayaka Fukushima from Toyo University, Japan for their kind support with some of my data analyses. I'm also grateful for Dr. Barbara Harmes, Dr. Marcus Harmes and Dr. Ananda Abeysekara for their great support in improving my academic writing. Additionally, I would like to thank all the staff members in the CCH who have given support over the years.

My deepest thanks go to my research buddy, Michael Thompson who has started his PhD along with me and worked together throughout this entire PhD journey. His great support with my

experiments and as a co-author of my research outputs was remarkable. I would like to extend my love and thanks to all my friends at CCH: Sriram, Barsha, Kiruba, Mela, Joe, Ahmed, Rio and Motiur who were there to share my happy and bitter moments throughout this long journey. Also, I want to give a big shout-out to my junior colleague, Prabuddha who helped and encouraged me in every possible way during this challenging period and pushed me to finish my research and thesis writing work on time. Additionally, I thank Indika, Madhubhashitha and Prasanna for their support.

I am indebted to all my friends in Brisbane and Toowoomba, Australia who were always so helpful in numerous ways. A huge appreciation goes to Dr. Jay Bandara, Gayani Bandara, Dumila Perera, Chaturika Gunatunga, Piyum Herath, Mythree Seneweera and Pramesha Madurangi for their kind support.

I would also like to extend my sincere thanks to my home university; the University of Ruhuna, Sri Lanka for granting me permission to study abroad and strengthen my knowledge and skills in plant science. I must acknowledge all the staff members of the Department of Agricultural Biology, Faculty of Agriculture for taking care of my academic duties while I was on study leave to complete this PhD. My sincere thanks go to all my cousins back home: Thiwanka, Niluka, Tharaka, Nandika and Sampath for taking good care of my family while I was away from Sri Lanka.

Last but not least, my heartfelt love and appreciation go to my dad and sister, Danu who have been by my side throughout this long academic journey from primary school to grad school. Also, today I deeply miss my darling mum who gave me all the courage and inspiration to start this challenging journey. Without their love and encouragement, I would not have finished my thesis successfully.

For me, this PhD journey was a life-changing experience. I deeply treasure every single minute of it and I am truly grateful for all the people who supported me to turn this big dream into a reality.

List of publications and submitted articles

Gamage, D., M. Thompson, M. Sutherland, N. Hirotsu, A. Makino and S. Seneweera (2018). "New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide." *Plant, cell & environment* 41(6): 1233-1246.

Gamage D, Thompson M, Okamoto M, Moriyama N, Sutherland M.W., Hirotsu N, Seneweera S. Early growth mechanism of wheat (*Triticum aestivum* L.) at elevated atmospheric carbon dioxide: New physiological evidence with quantitative trait loci data (Currently under review in *Plant, Cell and Environment*)

List of conferences attended and other co-authored scholarly articles during PhD candidature

Gamage D, Thompson M, Okamoto M, Moriyama N, Sutherland M, Hirotsu N, Seneweera S 'Early growth response of wheat (*Triticum aestivum* L.) to elevated atmospheric carbon dioxide: Quantitative trait loci for major growth traits'. Participated in the poster presentations at the *American Society of Plant Biologists' Plant Biology 2017* conference in Honolulu, Hawaii, USA.

Nagai Y, Matsumoto K, Kakinuma Y, Ujiie K, Ishimaru K, **Gamage D**, Thompson D, Milham PJ, Seneweera S and Hirotsu N (2016). "The chromosome regions for increasing early growth in rice: role of sucrose biosynthesis and NH4+ uptake." *Euphytica* 211(3): 343-352.

Thompson M, **Gamage D**, Hirotsu N, Martin A & Seneweera S 2017, 'Effects of Elevated Carbon Dioxide on Photosynthesis and Carbon Partitioning: A Perspective on Root Sugar Sensing and Hormonal Crosstalk', *Frontiers in Physiology*, 8:578, 10.3389/fphys.2017.00578.

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Chapter 1

Introduction and Literature Review

1.1 Climate change and future projections

Climate change is currently exerting additional pressure on global agricultural productivity and thereby threatens future global food security (Ainsworth *et al.*, 2008). The causes behind climate change are diverse. Increased emission of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and halocarbons into the atmosphere is considered to be one of the main causes behind global climate change (IPCC, 2007). Among all greenhouse gases, CO_2 contributes more than 70% to global climate change (IPCC, 2007). The carbon dioxide concentration ([CO_2]) of the earth's atmosphere has increased since the industrial revolution, with human activities consequently contributing strongly to climate change (IPCC, 2007, Qaderi & Reid, 2009).

Since the industrial revolution, the atmospheric [CO_2] has increased by more than 40%, and it is predicted to further increase by the middle of the century (IPCC, 2013). The current ambient [CO_2] has already exceeded 400 µmol CO_2 mol^{-1} (Tans & Keeling, 2018) and is expected to reach between 730 to 1020 µmol mol^{-1} by 2100 (Solomon *et al.*, 2007). At present, the CO_2 concentration in the atmosphere is linearly increasing. For example, in the past ten years, increase in atmospheric [CO_2] was recorded at a rate of 2.1 µmol mol^{-1} per year, double what was observed in the 1960's (IPCC, 2007). The increasing atmospheric [CO_2] has a significant impact on global temperature and thus precipitation patterns; projections predict global temperature will increase by 1.5-4.5^{o}C while the distribution of rainfall decreases (IPCC, 2007). Changes in [CO_2], temperature and water balance will influence climate and consequently the balance of ecosystem processes.

1.2 Wheat production under future climate

Wheat is the most widely cultivated cereal crop, an important carbohydrate source, and the most important grain protein source for a majority of the human population. Wheat contains

essential amino acids, minerals, vitamins, beneficial phytochemicals and dietary fiber components required for human nutrition (Shewry, 2009). It is predicted that the human population will increase to 9.1 billion by 2050, which will require an increase in cereal production of 3 billion tonnes to feed the growing world population (FAO, 2009). In order to satisfy the global demand for wheat, the production of more than 850 million tonnes by 2030 (FAO, 2003) is required. However, achieving these targets while conserving the quality will become a challenge under a changing climate. Australia is one of the largest wheat exporters in the world and a majority of arable lands are employed for wheat production. Climate change, including an increase in [CO_2] in the atmosphere together with high temperatures and reduced average annual rainfall, will affect the Australian wheat industry. It has been demonstrated that increasing temperature together with low precipitation is predicted to reduce the wheat yield by 7 to 16% (Kokic *et al.*, 2005). However, some of the negative effects of climate stress variables such as water and temperature on plants can be offset by elevated [CO_2]. This is favorable for the global food production. Gaining a better understanding of the genetic and molecular nature of crop responses to e[CO_2] will improve breeding programs that can ultimately increase future crop productivity (Ainsworth *et al.*, 2008, Semenov & Halford, 2009, Tausz *et al.*, 2013).

The majority of plant species, including wheat, fix CO_2 via the C_3 photosynthetic pathway. When [CO_2] increases in the atmosphere, photosynthesis increases until [CO_2] reaches the saturation limit (Sharkey, 1985, Seneweera *et al.*, 2011a). The relationship between photosynthesis to the changing [CO_2] concentration is well described by the biochemical model of photosynthetic CO_2 assimilation of Farquhar *et al.* (1980). The model indicates that conserved properties of ribulose 1-5 bisphosphate carboxylase/oxygenase (Rubisco), the primary carboxylating enzyme of C_3 plants, is the key to such unique photosynthetic responses to rising [CO_2]. Current ambient [CO_2] is very low at the site of fixation and any increase in [CO_2] will increase the net photosynthesis of C_3 plants (Drake *et al.*, 1997, Bloom, 2015). Laboratory and field studies have shown that the rate of photosynthesis of C_3 plants is approximately doubled when plants were grown at about 700 µmol mol^{-1} in comparison to 400 µmol mol^{-1} (Drake *et al.*, 1997, Ainsworth & Long, 2005, Kant *et al.*, 2012). However, the long-term photosynthesis response to elevated [CO_2] is highly unpredictable (Drake *et al.*, 1997, Seneweera *et al.*, 2005, Seneweera *et al.*, 2011a) and depends on nutrient availability, ontogenic stage and genetic background.

1.3 Plant responses to rising atmospheric [CO_2]

The increase of atmospheric [CO_2] directly and indirectly affects both the net rate of photosynthesis and stomatal conductance and thereby regulates the growth and development of plants (Seneweera & Conroy, 2005). As a result of these fundamental processes, changes in other post-photosynthetic metabolic processes that may lead to changes in plant growth responses are observed and are significant; however, the underlying mechanism is not well understood (Taylor *et al.*, 1994, Seneweera *et al.*, 2003, Gamage *et al.*, 2018)

1.3.1 Plant photosynthetic responses to elevated [CO_2]

Photosynthetic rate of most C_3 plants increases when plants are grown under elevated [CO_2] (Makino & Mae, 1999). In a high [CO_2] environment, the rate of photosynthesis increases as a result of an improvement in the carboxylation efficiency of Rubisco. The carboxylation efficiency of Rubisco rises due to an increase in the CO_2/O_2 ratio at the site of CO_2 fixation (Bowes, 1993). Further, changes in the CO_2/O_2 ratio leads to a reduction in photorespiration of C_3 plants, i.e., at the present atmospheric oxygen concentration of 21 kPa and [CO_2] of 400 µmol mol^{-1}, a considerable amount of light energy is used up for photorespiration, which reduces photosynthetic rates by 20 to 50% depending on temperature (Sharkey, 1985). These physiological responses vary between species and within species. Therefore, growth response to elevated [CO_2] is determined by the plant's capacity to adjust to the [CO_2] assimilation (Thilakarathne *et al.*, 2013, Thilakarathne *et al.*, 2015).

1.3.2 Photosynthetic acclimation of C_3 plants to long-term elevated [CO_2] exposure

When plants are exposed to elevated [CO_2] for an extended period, initial photosynthetic rates are increased. However, the initial stimulation of photosynthetic rates is not always maintained for a longer period (Sharkey, 1985, Sage *et al.*, 1989, Seneweera *et al.*, 2011c). This phenomenon is known as "photosynthetic acclimation" and has been widely reported in the literature (Bowes, 1991, Makino & Mae, 1999, Seneweera *et al.*, 2002, Nowak *et al.*, 2004). A number of mechanisms have been proposed to explain photosynthetic acclimation. Among them, change in the nitrogen supply to growing leaf blades (Drake *et al.*, 1997, Nakano *et al.*, 1997, Seneweera *et al.*, 2011b); accumulation of non-structural carbohydrates, leading to the suppression of the photosynthesis-related gene expression through the hexokinase signalling pathway (Nakano *et al.*, 1997, Stitt & Krapp, 1999, Thompson *et al.*, 2017); lower nitrogen

demand in leaves due to changes in nitrogen influx/efflux balance in growing tissues (Seneweera *et al.*, 2011b) and lower nitrogen in shoots due to suppression of NO_3^- photo-assimilation under e[CO_2] (Bloom *et al.*, 2012) are considered the main possible caused for accentuate photosynthesis acclimation. However, there is a very limited understanding of how these processes relate to plant nitrogen metabolism, and whether these processes are directly, or indirectly, associated with the impact of [CO_2] on photosynthesis. This understanding is essential to establish proper breeding approaches to breed crops to adapt to high atmospheric [CO_2].

1.3.3 Post-photosynthetic responses of plants to elevated [CO_2]

Increased photosynthetic capacity directly influences several post-photosynthetic key metabolic processes which play key roles in promoting plant growth and development under elevated [CO_2]. It is suggested that additional carbon supply at elevated [CO_2] primarily contributes to increased plant growth; however, how this additional carbon supply influences growth and what mechanisms are affected in the leaf or root tissues are still not clearly understood.

Plant growth and development is largely influenced by changes in plant carbon and nitrogen metabolism (Seneweera & Conroy, 2005). This argument is fully supported by Leakey *et al.* (2009). Changes in carbon metabolism are inevitable at elevated [CO_2], as the additional carbon supply influences carbohydrate synthesis, respiration, storage and remobilization. A proper balance among these mechanisms determines the carbon partitioning to different plant organs and maintains plant growth at the whole plant level. On the other hand, many aspects of plant growth and metabolism are regulated by nitrate, and signals derived from nitrogen metabolism (Moore *et al.*, 1999, Ainsworth *et al.*, 2012). Nitrate acts as a resource that accelerates nitrate assimilation, which results in higher levels of amino acids, proteins and decreased levels of starch (Scheible *et al.*, 1997a, Scheible *et al.*, 1997b) thus promoting the growth rate of plants. With additional nitrogen supply, plants tend to increase their growth response to elevated [CO_2], which is mainly regulated through increased plant sink capacity (Rogers *et al.*, 1996, Ainsworth *et al.*, 2004, Leakey *et al.*, 2009). These findings suggest that the capacity for photosynthesis needs to be matched with other indirect processes that control plant growth, such as plant carbon, nitrogen and hormonal metabolism at elevated [CO_2].

Research also suggests that elevated [CO_2] stimulates primary growth of shoots by influencing cell cycle properties, such as increasing the proportion of rapidly dividing cells, shortening the duration of the cell cycle (Kinsman *et al.*, 1997) and promoting cell production and expansion through cell wall loosening as a result of increased xyloglucan endotransglycosylase (XET) activity under elevated [CO_2] (Ranasinghe & Taylor, 1996). Masle (2000) indicated that cell division, cell expansion and cell patterning of plants under elevated [CO_2] may be influenced by increased substrate availability, which could potentially influence the expression of genes involved in the cell cycle and cell expansion.

Plant growth regulators, including auxins, gibberellins, cytokinins, ethylene and abscisic acid mainly regulate these cellular processes within a cell cycle. Hence, changes in hormonal levels may play a significant role in determining plant growth responses at elevated [CO_2] (Yong *et al.*, 2000). Results of several studies suggest that elevated [CO_2] promotes the synthesis of auxins, gibberellin, cytokines and ethylene by stimulating cell cycle processes and protein synthesis, and thereby plant growth (Yong *et al.*, 2000, Seneweera *et al.*, 2003, Teng *et al.*, 2006). However, the metabolism of plant growth regulators also depends on the availability of carbohydrate substrate (Farrar, 1996, Jitla *et al.*, 1997, Taiz & Zeiger, 1998, Kalve *et al.*, 2014). Under elevated [CO_2], the carbon substrate availability is higher as a result of increased photosynthesis, which is likely to increase hormonal metabolism leading to increased plant growth and development.

Genetic factors can also play a major role in regulating plant growth response to elevated [CO_2] by influencing both photosynthetic and post-photosynthetic processes. The photosynthetic response is driven through adjustments in the light reaction of photosynthesis and the carbon reduction process. The photosynthetic rate, potential acclimation propensity, carbohydrate synthesis and nitrogen use efficiency of plants varies remarkably between species and within species (Tausz *et al.*, 2013, Thilakarathne *et al.*, 2013, Thilakarathne *et al.*, 2015). These processes are interlinked and their genetic and physiological plasticity determines the final growth responses observed under elevated [CO_2] (Rae *et al.*, 2006, Leakey *et al.*, 2009). Therefore, understanding the underlying genetic mechanisms that determine the growth response to elevated [CO_2] is essential in order to increase crop growth under rising atmospheric [CO_2]. However, development of improved crop varieties suitable for future climates will require intensive models that combine phenotypic characteristics with their

underlying genetic architecture; which is lacking in the current scientific literature (Sadras & Calderini, 2015).

1.4 CO_2 responsiveness and plant growth stages

CO_2 responsiveness of plants is largely determined by the ontogenetic stage of exposure (Centritto *et al.*, 1999, Stitt & Krapp, 1999). The majority of C_3 species show very high growth response to elevated [CO_2], especially during the early stages of vegetative development (Poorter, 1993). Early growth responses to elevated [CO_2] are usually characterized by accelerated leaf growth and expansion resulting in increased leaf area ratios and relative growth rates (Poorter, 1993, Makino *et al.*, 1997) which positively correlate with biomass accumulation and the final yield (Thilakarathne *et al.*, 2015). It is suggested that increased growth at elevated [CO_2] results from the initial stimulation of photosynthesis and may decline or disappear over time (Masle *et al.*, 1993, Centritto *et al.*, 1999, Trevisan *et al.*, 2014). For example, relative growth rates tend to decline, as plants grow older at elevated [CO_2]. Hence, the effect of elevated [CO_2] on relative growth rate (RGR) is often time-dependent and occurs only during the early stages of plant growth (Poorter & Navas, 2003). Studies conducted in wheat (Neales & Nicholls, 1978, Hikosaka *et al.*, 2005), *Arabidopsis* (Van Der Kooij & De Kok, 1996) and tobacco (Geiger *et al.*, 1998) showed that exposure to elevated [CO_2] leads to a stimulation of RGR in young plants whereas RGR in older plants remains unaffected. Also, it is suggested that even a 10% increase in RGR at the exponential growth phase of the plant can be converted up to a 50% growth enhancement at elevated [CO_2] (Kirschbaum, 2010). Therefore, it is necessary to understand the genetic variation in early growth responses to elevated [CO_2] in order to improve crop productivity.

1.5 Knowledge gaps in the understanding of plant growth responses to elevated [CO_2]

Despite the greater understanding of plant growth and morphological response to elevated [CO_2], there is a significant knowledge gap in understanding the underlying molecular and physiological mechanisms that drive plant growth. Furthermore, the degree of contribution from photosynthetic and post-photosynthetic processes towards plant growth at elevated [CO_2] is not clearly understood (Gordillo *et al.*, 2001). Most of the studies on dissecting plant growth mechanisms at elevated [CO_2] have been focused on the photosynthetic responses, but there has been limited focus on the post-photosynthetic events that lead to subsequent growth and

development of plant tissues, organs and the whole plant (Taylor *et al.*, 1994, Nunes-Nesi *et al.*, 2010, Gamage *et al.*, 2018).

Several studies have been conducted to investigate plant metabolism (Leakey *et al.*, 2009), biomass accumulation ((Wang *et al.*, 2012), morphological and structural changes (Pritchard *et al.*, 1999, Teng *et al.*, 2006, Benlloch-Gonzalez *et al.*, 2014), stress tolerance (Vanaja *et al.*, 2011, van der Kooi *et al.*, 2016) and changes to different agronomic traits (Bourgault *et al.*, 2013, O'Leary *et al.*, 2015) under high [CO_2]; however, little is known about the molecular and physiological mechanisms of how plants respond to elevated [CO_2] (Taylor *et al.*, 2001, Huang & Xu, 2015, Thilakarathne *et al.*, 2015).

Understanding the genetic variation of plant responses to elevated [CO_2] will assist in the selection of plants for fitness and long-term adaptation to climate stress. A clear understanding of the genetic and physiological traits that contribute to an increase in crop productivity under high [CO_2] environments is also required (Ward & Kelly, 2004, Tausz *et al.*, 2013). Physiological traits that are important for plant responses to environmental stimuli are mostly quantitative in nature (Ferris *et al.*, 2002) and determined by a range of gene products. Therefore, identification of quantitative trait loci (QTL) that respond to elevated [CO_2] can be used as a genetic tool to utilize carbon richness to increase crop productivity (Rae *et al.*, 2006). This will provide information on whether the trait of interest has an associated genetic component, which can be utilized to capture high [CO_2] in the atmosphere to increase the crop productivity through plant breeding. Identifying QTL for CO_2 responsiveness will also be a major starting point for future studies on individual genes, genomic regions and inheritance of traits of interest (Rae *et al.*, 2006). However, only a few studies have been published in order to identify [CO_2] responsive QTL. Rae *et al.* (2006) mapped QTL for leaf growth, development, and senescence of poplar trees. Results showed that there was a differential genetic control for traits under elevated [CO_2]. Furthermore, they suggested that candidate genes co-located in the regions where response QTL were mapped. Ferris *et al.* (2002) mapped QTL for stomatal initiation, stomatal density, epidermal cell size, number and area in hybrid poplar under elevated [CO_2]. QTL analysis for above and below ground tree growth and biomass was conducted by Rae *et al.* (2007) using F_2 hybrids of poplar and they identified important areas of the genome which determine above ground growth response and root growth response to elevated [CO_2]. To our knowledge, no research studies have been published to date that elucidates the CO_2 responsive genomic regions for plant growth related traits in wheat.

When analyzing the plant growth responses to elevated [CO_2], changes in the nutritional status, cell cycle regulations and hormonal metabolism cannot be excluded because a dynamic change in C/N relations occurs during plant development. Consequently, changes in carbon and nitrogen metabolism are likely to have a major impact on plant growth processes. For example, a sharp decline in nitrogen uptake occurs at elevated [CO_2], particularly when plants reach the reproductive stage, as most of the photosynthate is used primarily for syntheses of storage and structural carbohydrates. However, there is a limited understanding on how changes in C/N metabolism interact with source-sink integration and thereby influence plant growth processes at elevated [CO_2] (Stitt & Krapp, 1999, Luo *et al.*, 2004, Lekshmy *et al.*, 2013). With the provision of additional carbon at elevated [CO_2], significant changes associated with other post-photosynthetic processes such as cell cycle functions and hormonal metabolism, especially at the molecular level is not clearly understood so far (Gamage *et al.*, 2018). Also, much less attention has been paid to investigate the interactions of all these post-photosynthetic processes and their contribution towards determining the source and sink organ integration at the whole plant level (Gamage *et al.*, 2018).

1.6 Contribution of the present study to scientific theory and knowledge

Since there is a significant knowledge gap in understanding the molecular mechanisms of how plants respond to elevated [CO_2], the experiments in this PhD thesis were designed and conducted to elucidate the underlying molecular mechanisms. This study focussed on dissecting the growth mechanisms of wheat (*Triticum aestivum* L.) in the early vegetative growth stages, as the CO_2 responsiveness of C_3 crops is comparatively high at this growth stage. Knowledge generated from this project will provide new insights into improving early vigor and growth characteristics of wheat, in a changing climate. With this purpose, three different strategies were employed to characterize the molecular growth mechanisms: (i) putative chromosomal locations regulating CO_2 responsiveness were determined through QTL mapping for different plant growth traits under elevated [CO_2]; (ii) the impact of post-photosynthetic processes to enhanced growth responses at elevated [CO_2] were determined using physiological and molecular approaches; (iii) interactions among photosynthetic and post-photosynthetic processes together with their contribution to improving plant growth at elevated [CO_2] was investigated using physiological and proteomics approaches. Overall, this

project will advance our knowledge on mechanisms controlling plant growth response to elevated [CO_2] and provide insights into the genetic factors that can be selected to increase crop productivity in a future CO_2 enriched atmosphere.

1.7 Objectives of the study

Wheat is one of the most cultivated cereal crops showing a variable response to increasing levels of [CO_2] in the atmosphere. Growth and yield stimulation of wheat in response to elevated [CO_2] have shown a high intraspecific variation, suggesting that enhanced photosynthetic capacity may not be the only factor determining final growth and yield of a crop at elevated [CO_2]. Therefore, it is necessary to establish the overall physiological and molecular mechanisms of how plant growth changes in response to rising [CO_2]. Although there have been significant efforts to characterize the underlying physiological mechanisms of growth responses to elevated [CO_2], not much is known about these mechanisms. Thus, this PhD project broadly aims to identify the underlying molecular mechanisms that regulate the plant growth responses to elevated [CO_2]. To achieve this overall aim, this study was broken down into three specific objectives as outlined below.

1. Elucidate QTL associated with plant growth responses to elevated [CO_2] in wheat using a doubled haploid (DH) population

It has been suggested that the identification of new functional traits associated with higher responses to elevated [CO_2] is essential to improve crop productivity under a changing climate. Therefore, identification of QTL that respond to elevated [CO_2] will be useful in identifying the genetic components associated with different growth traits and their regulation under rising atmospheric CO_2. Incorporation of CO_2-response QTL into marker-assisted selection will be beneficial in crop breeding strategies for future climate. To date, there has been no research conducted to elucidate genomic regions/QTL determining enhanced growth traits observed at elevated [CO_2] in wheat. As CO_2 responsiveness is higher during the early vegetative stage, identification of QTL governing early growth will aid in improving growth habit and early vigour of wheat under CO_2 enriched conditions.

2. Determine the contribution of post-photosynthetic metabolic processes to enhanced plant growth responses at elevated [CO_2] using selected DH lines that differ in CO_2 responsiveness

It is well established that growth stimulation at elevated [CO_2] arises from the increased photosynthetic capacity of plants. This photosynthetic stimulation regulates other post-photosynthetic processes such as carbon and nitrogen metabolism and cell cycle functions that collectively contribute to whole plant growth. Elevated [CO_2] impacts on plant growth even at the transcript level and thereby determines the protein synthesis that governs these important metabolic activities. However, there is limited understanding of how these key metabolic processes change in response to increased photosynthetic capacity, especially at the transcript level and their association with enhanced plant growth responses observed under high [CO_2]. Therefore, to serve this objective, a series of experiments were conducted to develop an understanding of transcript level changes to post-photosynthetic processes, plant response to increased sugar supply and growth responses at elevated [CO_2].

3. Investigate the interplay among photosynthesis and post-photosynthesis metabolic processes and their impact on improving plant growth at elevated [CO_2] using physiological and proteomic approaches

A number of research studies have been conducted to understand the underlying physiological mechanisms of plant growth and development at elevated [CO_2]. Plant response to environmental stimuli such as elevated [CO_2] is a result of a complex interplay of different metabolic processes through various regulatory enzymes, metabolic proteins and other biomolecules. Analysis of the whole proteome of particular plant organs will provide an opportunity to identify the protein profile expressed at a given time. This will provide important information regarding the functionality of the proteome and thus, determine the protein network associated with the growth of a particular plant organ at elevated [CO_2]. To our knowledge, no previous research studies have investigated leaf proteome changes in response to elevated [CO_2]. The results of this study will serve as a preliminary report in this regard and will help to better understand the underlying molecular mechanisms of plant growth at elevated [CO_2].

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Chapter 2

Literature Review

In addition to the literature review in Chapter 1, this thesis also has a published literature review entitled "New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide". This review discusses the effect of elevated [CO_2] on photosynthetic and post-photosynthetic metabolic processes and their relationship with the growth enhancement observed in a high CO_2 environment. This chapter focusses on the cellular and molecular level responses associated with plant growth at elevated [CO_2]. The review first discusses the changes of two fundamental metabolic processes, photosynthesis and stomatal conductance, and how they are affected by elevated [CO_2] at the cellular level. Then, this review focussed on the effect of elevated [CO_2] on post-photosynthetic key metabolic processes that govern plant growth, such as carbon metabolism, nitrogen metabolism, cell cycle functions and hormonal regulation. Additionally, we discuss how plants integrate these mechanisms with source and sink activity and thereby alter plant anatomy and morphology. Finally, the knowledge gaps and potential future research are highlighted to provide the readers an understanding of what needs to be further investigated to further elucidate the molecular mechanisms underlying plant growth responses to elevated [CO_2].

Gamage D, Thompson M, Sutherland M, Hirotsu N, Makino A, Seneweera S (2018) New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide. Plant, cell & environment 41(6):1233-1246.

Received: 10 October 2017 | Revised: 21 March 2018 | Accepted: 22 March 2018
DOI: 10.1111/pce.13206

REVIEW

WILEY Plant, Cell & Environment

New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide concentrations

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Abstract

Rising atmospheric carbon dioxide concentration ([CO_2]) significantly influences plant growth, development, and biomass. Increased photosynthesis rate, together with lower stomatal conductance, has been identified as the key factors that stimulate plant growth at elevated [CO_2] (e[CO_2]). However, variations in photosynthesis and stomatal conductance alone cannot fully explain the dynamic changes in plant growth. Stimulation of photosynthesis at e[CO_2] is always associated with post-photosynthetic secondary metabolic processes that include carbon and nitrogen metabolism, cell cycle functions, and hormonal regulation. Most studies have focused on photosynthesis and stomatal conductance in response to e[CO_2], despite the emerging evidence of e[CO_2]'s role in moderating secondary metabolism in plants. In this review, we briefly discuss the effects of e[CO_2] on photosynthesis and stomatal conductance and then focus on the changes in other cellular mechanisms and growth processes at e[CO_2] in relation to plant growth and development. Finally, knowledge gaps in understanding plant growth responses to e[CO_2] have been identified with the aim of improving crop productivity under a CO_2 rich atmosphere.

KEYWORDS

carbon metabolism, cell cycle, climate change, elevated [CO_2] (e[CO_2]), hormonal metabolism, nitrogen metabolism, photosynthesis, plant growth mechanism, source-sink interactions

1 | INTRODUCTION: OPPORTUNITIES AND CHALLENGES IN A CARBON DIOXIDE ENRICHED ATMOSPHERE

The world's population is expected to increase by 2.3 billion between 2009 and 2050, requiring significant increases in global food production to fulfil future food demand (Alexandratos & Bruinsma, 2012). Current and predicted changes in climate render the achievement of food production targets even more challenging. The continuing rapid increase in atmospheric [CO_2], due to a range of anthropogenic factors, including the burning of fossil fuels and deforestation, is a major driver for the current changes in climate (Qaderi & Reid, 2009). Since preindustrial times, atmospheric [CO_2] has increased more than 40% and a similar magnitude of increase is expected by the end of this century (IPCC, 2013). Current ambient [CO_2] now exceeds 400 μmol/mol (Tans & Keeling, 2018) and is expected to increase even more causing significant changes in global temperature and thereby precipitation patterns. According to current projections, global temperature will increase by 1.4–3.1 °C when atmospheric [CO_2] reaches ~670 μmol/mol (IPCC, 2013). The long-term exposure of plants to elevated [CO_2] (e[CO_2]), high temperature, and water deficits will significantly influence the balance of ecosystem processes both at the regional and global level.

Carbon dioxide is the primary substrate for photosynthesis and thus can be considered as the main driver of global food production.

Abbreviations: [CO_2], carbon dioxide concentration; e[CO_2], elevated [CO_2]; O_2, oxygen; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase

†Author contributions: D. M. G. (50%), M. T. (5%), M. W. S. (10%), N. H. (10%), A. M. (5%), and S. S. (20%). D. G. wrote the manuscript. M. T., M. S., N. H., A. M., and S. S. each contributed to the design and review of the final manuscript.

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Nearly 90% of known plant species belong to the C_3 biochemical type and are not photosynthetically saturated at the current CO_2 partial pressure; therefore, photosynthesis and biomass of these species can be expected to increase under e[CO_2] conditions (Kimball, 2016; Makino & Mae, 1999). In C_3 plants, photosynthesis is catalysed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), an enzyme which reacts with both CO_2 and oxygen (O_2), initiating photosynthetic carbon reduction and photorespiratory carbon oxidation cycles, respectively (Drake, Gonzàlez-Meler, & Long, 1997; Makino & Mae, 1999). The efficiency of both processes is dependent on the relative partial pressures of CO_2 and O_2 at the site of fixation (Lorimer, 1981). Rubisco has a higher affinity for CO_2 than O_2, and often, Rubisco carboxylation is the rate-limiting step in C_3 photosynthesis. Increased carboxylation of Rubisco inhibits photorespiration, indirectly aiding photosynthetic rates (Makino & Mae, 1999). Photorespiration is the process that involves the oxygenation of Ribulose-1,5-bisphosphate by Rubisco, which significantly wastes energy produced by photosynthesis (Makino & Mae, 1999; Walker, VanLoocke, Bernacchi, & Ort, 2016). Photosynthesis, respiration, and water relations are the three primary physiological processes of plants influenced by e[CO_2]. Therefore, understanding the fine and coarse control of photosynthesis, respiration, and water use, and their impact on plant growth under a CO_2 rich atmosphere provides a unique opportunity to improve crop productivity under a changing climate.

Laboratory and field experiments have demonstrated that C_3 photosynthesis approximately doubles when plants grown at ~380 μmol/mol, are exposed to 700 μmol/mol of CO_2 (Drake et al., 1997). Changes in plant growth and phenology at e[CO_2] are well documented across C_3 and C_4 species (Ainsworth & Long, 2005; Ainsworth & Rogers, 2007; Kimball, 2016). However, these growth traits are complex; so an understanding of the different organizational level responses to e[CO_2] is necessary to develop new breeding strategies. Although plant growth and morphology tend to change with increased atmospheric [CO_2], these responses cannot be fully explained by the direct effects of e[CO_2] on photosynthesis, respiration, and water use alone (Geiger et al., 1998; Taylor, Ceulemans, Ferris, Gardner, & Shao, 2001; Thilakarathne et al., 2015). In this review, we have focused on the mechanisms by which increased carbon supply at e[CO_2] influences a range of plant growth processes and cellular functions and their relation to changes in plant growth, development, and phenology.

2 | MECHANISMS OF PLANT GROWTH RESPONSES TO ELEVATED [CO_2]

Elevated [CO_2] directly and/or indirectly affects plant growth and development by modifying a range of physiological processes. Plant growth at e[CO_2] changes due to the immediate effects of e[CO_2] on photosynthesis and stomatal conductance (Ainsworth et al., 2008; Drake et al., 1997; Seneweera & Conroy, 2005; Figure 1). On the other hand, plant growth at e[CO_2] also depends on the post-photosynthetic process that may lead to changes in carbon and nitrogen metabolism, changes in cell cycle properties, and hormonal metabolism as a result of increased supply of carbon to the growing shoots and roots under e[CO_2] (Figure 2). For example, increases in photosynthetic carbon assimilation could be offset by changes in investment in photosynthetic proteins as a consequence of foliar adjustments to plant carbon and nitrogen metabolism at e[CO_2]. Most of the research has tended to focus on changes in photosynthesis and stomatal conductance in response to e[CO_2] whereas very less attention has been paid to variations in other cellular mechanisms that may moderate plant growth response to e[CO_2]. Changes in carbon and nitrogen metabolism, cell cycle properties, and hormonal metabolism together with source-sink optimization at e[CO_2] are significant and largely determine the growth responses of plants to e[CO_2] (Seneweera, Aben, Basra, Jones, & Conroy, 2003; Taylor, Ranasinghe, Bosac, Gardner, & Ferris, 1994). Holistic changes of these processes are complex, closely interrelated, and determine the growth responses by differential allocation of

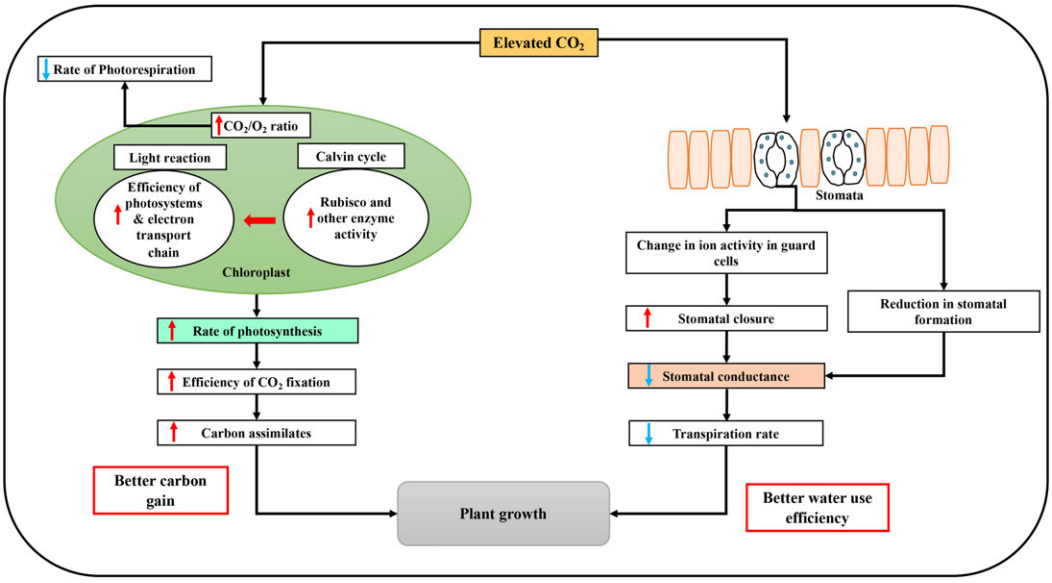

FIGURE 1 Effects of CO_2-induced photosynthesis and stomatal conductance on plant growth responses (Green circle = chloroplast; orange squares = epidermal cells; half circles = stomata; red arrow denotes an increase; and blue arrow denotes a decrease)

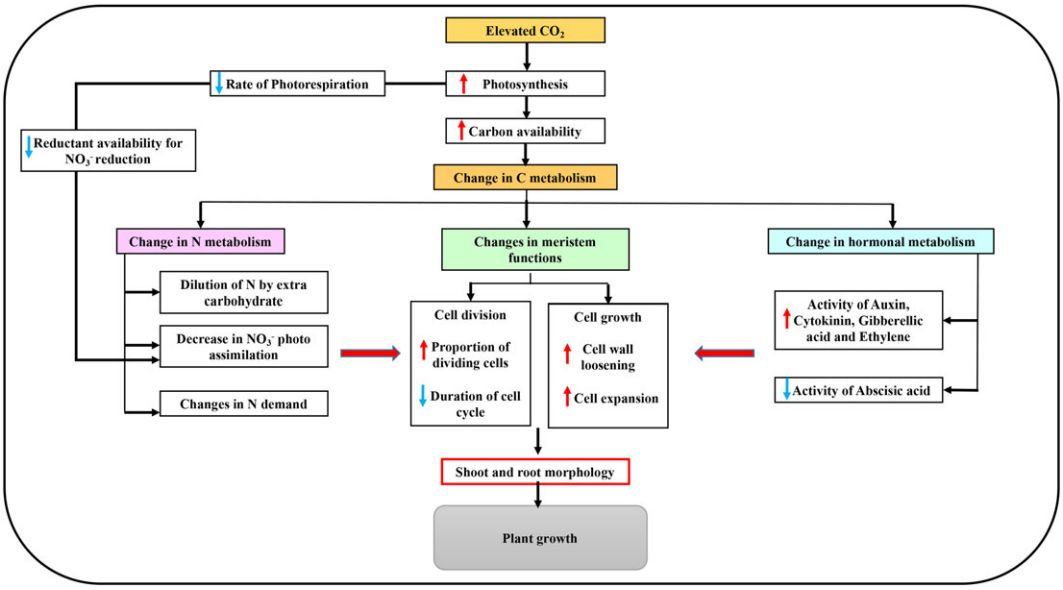

FIGURE 2 Effect of increased carbon supply at elevated [CO_2] on other cellular processes and plant growth responses (C = Carbon; N = Nitrogen; NO_3^- = Nitrate; red arrow denotes an increase; and blue arrow denotes a decrease) [Colour figure can be viewed at wileyonlinelibrary.com]

resources to shoot and root depending on the environmental conditions. A thorough understanding of these processes and their association with high carbon input will advance our knowledge of the mechanistic basis of differential plant phenology observed at e[CO_2].

3 | PRIMARY EFFECTS OF ELEVATED [CO_2] ON PLANTS—PHOTOSYNTHESIS AND STOMATAL CONDUCTANCE

3.1 | Photosynthesis

Elevated [CO_2] increases photosynthetic rates and thereby crop growth and yield. The main reason for this enhanced photosynthesis is the increased carboxylation efficiency of Rubisco, which is relatively low at the current atmospheric [CO_2]. However, at e[CO_2], the increase in CO_2 concentration at the site of CO_2 fixation will increase the CO_2/O_2 ratio; thus, the carboxylation efficiency of Rubisco will be promoted by lowering the rate of photorespiration (Bowes, 1991; Figure 1). The efficiency of photosystems I and II (PSI and PSII) is increased at e[CO_2] and correlates well with the rate of photosynthesis, producing more adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), the two most vital components required to activate photosynthetic enzymes at e[CO_2] (Van Heerden, Swanepoel, & Krüger, 2007; Zhang et al., 2008). It has also been demonstrated that PSII yield (number of photochemical products produced per energy quantum) is higher when plants are exposed to e[CO_2] (Habash, Paul, Parry, Keys, & Lawlor, 1995), suggesting that light utilization efficiency increased at e[CO_2]; consequently, electron flow between PSI and PSII also increased under e[CO_2] (Sekhar, Sreeharsha, & Reddy, 2015). These findings suggest the possibility of improving photosynthesis at e[CO_2] without interfering with the photosynthetic light reaction.

When plants are exposed to e[CO_2], a number of genes and proteins associated with photosynthesis are differentially expressed. Genes involved in the light reaction of photosynthesis, encoding for PSII core proteins such as *PSII protein K*, essential for the function and assembly of the PSII reaction centre, have been up-regulated under e[CO_2] (De Souza et al., 2008; Moore, Cheng, Rice, & Seemann, 1998). In addition, Ferredoxin-1, an electron donor to $NADP^+$ reductase, showed increased expression under e[CO_2] (Oosten & Besford, 1995). In addition, Vicente et al. (2015) reported that genes encoding for putative *PSI P700 Chl a apoprotein A1 (PsaA)*, *apoprotein A2 (PsaB)*, *PSII protein D1* and *D2* are up-regulated in durum wheat under e[CO_2]. Increases in the expression of genes and proteins related to the light reaction of photosynthesis suggest that photochemical efficiency of photosystems is increased under e[CO_2]. However, several studies showed that transcript levels of genes and proteins involved in light dependent (i.e., chlorophyll a/b binding proteins of light harvesting complexes I and II; Nie, Hendrix, Webber, Kimball, & Long, 1995; Oosten, Wilkins, & Besford, 1994) and light independent reactions (several Calvin cycle enzymes) of photosynthesis have been down-regulated in response to prolong exposure to e[CO_2] (S.-H. Cheng, d Moore, & Seemann, 1998; Moore et al., 1998; Nie et al., 1995). However, more detailed studies are required to establish both photosynthesis light reaction and dark reaction responses to e[CO_2]. Further, genes associated with photosynthetic carbon metabolism have been differentially expressed under e[CO_2]. For example, genes encoding fructose-1,6-bisphosphatase, transketolase, and aldolase enzymes involved in ribulose-1,5-bisphosphate regeneration were up-regulated at e[CO_2] (Wei et al., 2013). In C_4 species, several enzymes associated with the light reaction of photosynthesis, for example, NADP-malate dehydrogenase and pyruvate phosphate dikinase, were up-regulated by 117% and 174% respectively at e[CO_2] 14 days after leaf emergence in sugarcane followed by up-regulation of genes associated with sucrose metabolism (Vu, Allen, & Gesch, 2006). This study further

showed that key enzymes involved in photosynthesis and sucrose metabolism are up-regulated at e[CO_2], particularly during the early stages of plant development suggesting that C_4 plants behave like C_3 during early leaf development. All these findings suggest the possibility to improve photosynthesis by taking advantage of the CO_2 rich atmosphere.

The initial stimulation of photosynthetic rates at e[CO_2] is not always maintained over a longer period (Sage, Sharkey, & Seemann, 1989; Seneweera, 2011; Sharkey, 1985). This phenomenon is known as "photosynthetic acclimation" and has been widely reported in the literature (Alonso, Pérez, & Martínez-Carrasco, 2009; Gutiérrez et al., 2009; Martínez-Carrasco, Pérez, & Morcuende, 2005; Nakano, Makino, & Mae, 1997; Seneweera et al., 2002). A number of mechanisms have been proposed to explain plant acclimation to e[CO_2]. These include decline in nitrogen supply to the growing leaf blades (Drake et al., 1997; Nakano et al., 1997; Seneweera, Makino, Hirotsu, Norton, & Suzuki, 2011), accumulation of non-structural carbohydrates that suppress photosynthesis-related gene expression (Nakano et al., 1997; Stitt & Krapp, 1999), lower nitrogen demand in leaves due to changes in nitrogen influx/efflux balance in growing tissues (Seneweera et al., 2011), and lower shoot nitrogen due to suppression of nitrate (NO_3^-) photo-assimilation (Bloom et al., 2012). Overall, it has been suggested that the most reasonable explanation of photosynthetic acclimation to e[CO_2] is the suppression of nitrogen assimilation at e[CO_2] (Bloom et al., 2012). In addition, photosynthetic acclimation will become even more pronounced when there is insufficient sink strength in the plant to accommodate increasing photo-assimilates at e[CO_2] (P. Li, Ainsworth et al., 2008). For example, this phenomenon was evaluated by Ainsworth, Rogers, Nelson, and Long (2004) using soybean (*Glycine max*) lines showing both determinate and indeterminate growth habit and found acclimation was more prominent in lines with determinate growth habit. This suggests that plants that are able to expand their sink capacity to utilize photo-assimilates efficiently may be more likely to sustain increased photosynthetic rates at e[CO_2].

Leaf photosynthesis is closely correlated with both the Rubisco and nitrogen content of the leaf blade (Makino & Mae, 1999; Vicente et al., 2015). The amount of Rubisco in leaves is determined by both synthesis and degradation, and it is regulated by internal and external factors. The decline in Rubisco content at e[CO_2] is known to be often associated with the accumulation of non-structural carbohydrates (Seneweera et al., 2011). However, a negative relationship between photosynthesis and soluble sugar content has not always been reported (Makino, Nakano, Mae, Shimada, & Yamamoto, 2000). Functional Rubisco has a small and large subunit, and down-regulation of genes encoding for both sub-units (*rbc*S and *rbc*L) has been observed for many species under e[CO_2] (Takatani et al., 2014; Vicente et al., 2015). The widely accepted hypothesis for the reduced Rubisco content at e[CO_2] is the repression of the *rbc*S and *rbc*L gene expression (S.-H. Cheng et al., 1998; Moore, Cheng, Sims, & Seemann, 1999; Rolland, Moore, & Sheen, 2002; Sheen, 1994). Rubisco activase, the protein required to activate the Rubisco complex, has been reported to decrease under e[CO_2] (Bokhari et al., 2007), which could compromise the activation state of the enzyme. Further, there have been reports of a decline in transcript abundance of Rubisco activase and two Calvin Cycle enzymes, ribulose-phosphate-3-epimerase and ribose-phosphate isomerase, which may influence the turnover of Rubisco and thus the continuance of the photosynthesis mechanism (Kontunen-Soppela et al., 2010; Leakey et al., 2009). Finally, photosynthesis is largely controlled by its related protein turnover; however, there is a limited understanding of how photosynthesis-related protein turnover is influenced by e[CO_2] (Seneweera, Makino, Mae, & Basra, 2005).

3.2 | Stomatal conductance

Stomatal conductance is mainly controlled by the size of the stomatal aperture (Ainsworth & Rogers, 2007); number of stomata per unit of leaf area (Casson & Gray, 2008; Casson & Hetherington, 2010); and water transport capacity of the guard cell (Leakey et al., 2009). Stomatal conductance is decreased in crops exposed to e[CO_2] (Ainsworth & Long, 2005; Leakey et al., 2009; Figure 1). The size of the stomatal aperture is mainly determined by the turgor pressure of guard cells, which is mediated through ion concentration (Araújo, Fernie, & Nunes-Nesi, 2011). It is reported that e[CO_2] increases the activity of outward rectifying K^+ channels relative to that of inward rectifying K^+ channels, causing stomatal closure (Brearley, Venis, & Blatt, 1997). Elevated [CO_2] also stimulates Cl^- release from guard cells and increases Ca^{2+} concentration within them (Brearley et al., 1997; Webb, McAinsh, Mansfield, & Hetherington, 1996). These changes help to depolarize the membrane potential of guard cells causing stomatal closure (Hanstein & Felle, 2002), which is very common under e[CO_2]. It has also been demonstrated that e[CO_2] increases the concentration of malate, the effector mediating flux flow between CO_2 and these anion channels (Hedrich et al., 1994). Modified malate concentrations under e[CO_2] enhance the activation potential of anion channels, thus indirectly influencing stomatal closure. Because e[CO_2] is known to promote anion channels, attempts have been made to identify genes encoding guard cell anion channels.

Stomatal guard cell responses to e[CO_2] are also driven by multiple signalling components associated with guard cell activity. Because e[CO_2] is known to promote anion channels, attempts have been made to identify genes encoding guard cell anion channels. As a result, *SLAC1* (Slow Anion Channel Associated 1) was identified in *Arabidopsis thaliana* that encodes a protein mediating CO_2 induced stomatal closure through regulating S-type anion channels (Laanemets et al., 2013; Negi et al., 2008; Vahisalu et al., 2008). SLAC1 is activated by the gene OST1 (open stomata 1) that is a SNF-1 related protein kinase 2, a positive regulator of CO_2-induced stomatal closure (Lind et al., 2015; Merilo et al., 2013). Activation of OST1 is triggered by abscisic acid (ABA) signalling at e[CO_2] involving PYR/RCAR family of ABA receptors (Chater et al., 2015; Merilo et al., 2013; Merilo, Jalakas, Kollist, & Brosché, 2015). In another study, Yamamoto et al. (2016) showed that SLAC1 channel perceives CO_2 signals by an ABA-independent pathway in a transmembrane region of the guard cells. In addition, it has been reported that other phytohormones such as jasmonic acids are altered at e[CO_2]. Jasmonic acid is known to play a significant role in mediating stomatal closure at e[CO_2] (Geng et al., 2016). A very recent study by He et al. (2018) revealed that a novel allele of the *Arabidopsis BIG* locus named *cis1* is involved as a signalling component responsible

for controlling stomatal aperture at e[CO_2]. Further, they indicated that loss of *BIG* function compromises activation of guard cell S-type anion channels by bicarbonate at e[CO_2].

Recent studies indicate that redox signalling, which includes reactive oxygen species produced by NADPH oxidases encoded by respiratory burst oxidase homologs, has a key role in determining stomatal conductance and density at e[CO_2] (Chater et al., 2015). Carbonic anhydrases also appear to have a role in the regulation of stomatal movement. These enzymes catalyse the conversion of CO_2 to bicarbonate (HCO_3^-), which functions as a molecular activator of *SLAC1* anion channels in the guard cells (Xue et al., 2011). Consistent with this proposition is the finding that expression of *CA1* and *CA4* (β-carbonic anhydrase 1 and 4) was significantly higher in guard cells at e[CO_2] (Hu et al., 2010; Xue et al., 2011). Mutants that lack these genes showed reduced CO_2-induced stomatal movements and increased stomatal density indicating that these genes play a key role in determining both stomatal movement and development (Hu et al., 2010). Additionally, studies in *Arabidopsis thaliana* revealed that protein kinase HT1 (High Leaf Temperature 1; Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016), RHC1 (resistant to high CO_2, a MATE type transporter that link e[CO_2] with repression of HT1; Tian et al., 2015), and mitogen activated protein kinase (MPK4 and MPK12; Hõrak et al., 2016; Jakobson et al., 2016) are strongly related to CO_2-induced stomatal closure. Another hypothesis for guard cell activity is based on the high levels of sucrose produced at e[CO_2] (Kang, Outlaw, Andersen, & Fiore, 2007; Kelly et al., 2013). When sucrose production rate exceeds the sucrose loading rate to the phloem at e[CO_2], excess sucrose will be transported to the stomata via transpiration flow stimulating stomatal closure through hexokinase mediated expression of ABA-related genes (Kelly et al., 2013). Further, these authors showed that increased expression of hexokinase in guard cells is associated with the CO_2 induced stomatal closure; however, the role of sugars in regulating stomatal regulation deserves further exploration.

Stomatal density is also vital for determining the efficiency of photosynthesis and water use in plants. Decreased stomatal numbers have been observed in a wide range of species at e[CO_2] (Casson & Gray, 2008; Casson & Hetherington, 2010; Woodward & Kelly, 1995). Further, molecular evidence indicates that e[CO_2] impedes stomatal development in plants. Gray et al. (2000) identified the *HIC* (High Carbon Dioxide) gene from *Arabidopsis thaliana* that encodes for an enzyme called putative *3-keto acyl coenzyme A synthase* that negates stomatal development in response to e[CO_2]. In another study, Kim et al. (2006) demonstrated that the *SDD1* gene, which encodes for putative subtilisin serine protease, was down-regulated under e[CO_2]. This protein is involved in signal processing that contributes to guard cell formation and development. These changes at the molecular level are likely to contribute towards the reduction of stomatal formation under e[CO_2]. In addition, the expression of genes encoding water transport proteins, such as aquaporins, is suppressed in the guard cells of some plants under e[CO_2] (Gupta et al., 2005; Wei et al., 2013). Such adjustments of guard cell chemistry may have a major impact on the reduced stomatal conductance and thus an improvement in water use efficiency of the plant under e[CO_2]. At the same time, several other aquaporins, such as tonoplast intrinsic protein 1 (*T1P1*), are up-regulated in stems and leaves (Ainsworth, Rogers, Vodkin, Walter, & Schurr, 2006; Wei et al., 2013). These findings suggest that e[CO_2] can facilitate intercellular and vascular water transport that may eventually contribute to improved water use efficiency of plants.

4 | IMPACT OF ELEVATED [CO_2] ON OTHER CELLULAR MECHANISMS AND ITS INFLUENCE ON PLANT GROWTH

4.1 | Effects of elevated [CO_2] on plant carbon metabolism

Carbon metabolism at e[CO_2] is collectively determined by photosynthesis, respiration, sugar storage, and remobilization capacity (Aranjuelo et al., 2015). In general, C_3 photosynthesis increases at e[CO_2] but the magnitude of the response can change with plant developmental stages and prevailing environmental conditions (Leakey et al., 2009). Also, increased photosynthesis rates at e[CO_2] results in higher sugar production, including glucose, fructose, and raffinose, across a range of plant species (Aranjuelo et al., 2015; Watanabe et al., 2013). These additional sugars are available for development of new sink organs such as leaves, stems, tillers, and seeds. The developmental plasticity of these organs determines the final growth response to e[CO_2]. However, plant responses to e[CO_2] are much greater at early vegetative stages compared with later stages (Makino, Harada, Sato, Nakano, & Mae, 1997; Seneweera et al., 2002). This ontological variation is largely determined by sink activity and varies with both species and environmental conditions (J. Y. Li, Liu et al., 2008).

Enzymes associated with plant carbon metabolism play a key role in determining sink activity. For example, sucrose phosphate synthase (SPS) and sucrose synthase (SUS) are the key enzymes regulating sucrose biosynthesis and transport in most terrestrial plants (Koch, 2004; Rogers, Milham, Gillings, & Conroy, 1996; Seneweera, Basra, Barlow, & Conroy, 1995). It has been reported that starch to sucrose ratio in leaves correlates well with SPS activity (Chávez-Bárcenas et al., 2000), and up-regulation of both SUS and SPS proteins occurs especially in young fully expanded leaves under e[CO_2] (Aoki et al., 2003; W.-H. Cheng, Im, & Chourey, 1996; Gesch, Vu, Boote, Hartwell Allen, & Bowes, 2002; Seneweera et al., 1995). In addition, specific genes related to SUS (*RSus1*) and SPS (*sps1*) were found to be highly expressed under e[CO_2] in rice (J. Y. Li, Liu, et al., 2008). These findings suggest that e[CO_2] increases sucrose biosynthesis in many species. However, the rate of sucrose biosynthesis depends on a range of physiological and environmental factors. Plants have evolved strategies to translocate soluble sugars away from leaves, which reduces starch accumulation in the leaf blade. Starch accumulation in the leaves leads to feedback inhibition of photosynthesis at e[CO_2]. It has also been identified that the ratio of starch to sucrose is a good indicator of plant acclimation to e[CO_2] (Sharkey, Laporte, Lu, Weise, & Weber, 2004) and suggests that lower starch to sucrose ratios could be used as a breeding target to increase crop productivity under e[CO_2].

4.2 | Change of nitrogen metabolism at elevated [CO_2]

Long-term growth responses to e[CO_2] are largely determined by nitrogen supply (Seneweera et al., 2011; Seneweera & Conroy, 2005;Stitt & Krapp, 1999; Takatani et al., 2014). However, the nitrogen concentration of plants decreases at e[CO_2] (Stitt & Krapp, 1999; Takatani et al., 2014), especially in leaf tissues (Seneweera, 2011). The decrease in leaf nitrogen is not due to a simple dilution effect caused by a relative increase in plant carbohydrates but could be due to a decrease in allocation of nitrogen to leaves at the whole plant level. It has been observed that e[CO_2] changes nitrogen allocation to different organs, reduces the partitioning to leaves, while increasing allocation to leaf sheaths and roots (Seneweera, 2011). However, the mechanism(s) by which e[CO_2] decreases tissue concentrations of nitrogen and protein are not fully understood as growth at e[CO_2] can affect multiple processes involved in nitrogen metabolism (Stitt & Krapp, 1999; Takatani et al., 2014). Vicente et al. (2015) showed down-regulation of transcripts for key enzymes of nitrogen uptake, deamination and assimilation [glutamate dehydrogenase (*GDH1*); aspartate synthetase (*ASN1*), ferredoxin-nitrate reductase (*nir*)], and nitrate transporters [low-affinity nitrate transporter (*NRT1.5B*)] may have contributed to a low nitrogen status at e[CO_2]. A widely documented cause for this change in nitrogen is that e[CO_2] increases the rate of carbohydrate production and consequently increases the carbon to nitrogen ratio of plants (Geiger, Haake, Ludewig, Sonnewald, & Stitt, 1999; Paul & Driscoll, 1997; Seneweera et al., 2002; Seneweera & Conroy, 2005). This increase in carbon to nitrogen ratio results in increased initial growth rates of plants, which is later hindered by nitrogen limitation under e[CO_2].

Several mechanisms have been proposed to explain lower nitrogen concentration in plant shoots at e[CO_2] (Figure 2): dilution of nitrogen by extra carbohydrates (Gifford, Barrett, & Lutze, 2000); reduction in nitrogen uptake relative to carbon gain (BassiriRad, Gutschick, & Lussenhop, 2001; Del Pozo et al., 2007; Vuuren et al., 1997); reduction of plant nitrogen demand (Stitt & Krapp, 1999); ontogenetic drift leading to changes in nitrogen balance (Bernacchi, Thompson, Coleman, & Mcconnaughay, 2007; Coleman, McConnaughay, & Bazzaz, 1993); and changes in NO_3^- photo-assimilation (Bloom, 2015; Bloom, Smart, Nguyen, & Searles, 2002). Of these, the most reasonable explanation is the reduction in nitrogen influx to above-ground plant organs by lowered NO_3^- photo-assimilation and down-regulation of gene expression through sugar sensing and signalling pathways. Plants exposed to e[CO_2] accumulate excess soluble sugars, which are known to influence gene expression associated with plant carbon and nitrogen metabolism (Lastdrager, Hanson, & Smeekens, 2014; Smeekens, Ma, Hanson, & Rolland, 2010). Together with these responses, e[CO_2] also reduces the transpiration flow through lowered stomatal conductance which then lowers the passive nutrient transport to the plant shoots (McDonald, Erickson, & Kruger, 2002). Hence, the nitrogen inflow to the plant shoots is reduced significantly at e[CO_2].

Rubisco is the rate-limiting enzyme in photosynthesis, it accounts for nearly 50% of soluble nitrogen in leaves (Makino, Mae, & Ohira, 1984; Spreitzer & Salvucci, 2002). A high turnover of this enzyme is the key to maintaining the photosynthetic rates at e[CO_2], which is influenced by many environmental factors such as nitrogen availability, water availability, and temperature. Synthesis of this enzyme is controlled by transcriptional, posttranscriptional, and translational processes. The degradation is mainly controlled by various chloroplast proteolytic enzymes. It has been reported that Rubisco synthesis is reduced at e[CO_2] whereas its degradation is accelerated. The major cause of decreased synthesis of Rubisco is suggested to be associated with the sugar sensing mechanism based on the hexokinase signalling pathway that can suppress transcription of *rbcS* and *rbcL* genes encoding the small and large subunit of Rubisco, respectively (S.-H. Cheng et al., 1998; Jang, León, Zhou, & Sheen, 1997; Makino et al., 1984). However, Seneweera and Conroy (2005) have suggested that among the number of other mechanisms contributing to lower shoot nitrogen, lower NO_3^- photo-assimilation at e[CO_2] may reduce the nitrogen flux into the leaf blade and thereby reduce Rubisco synthesis. These authors further proposed another possibility via an increase in the proteolytic enzyme activity in the chloroplasts (Seneweera et al., 2005), but its physiological relevance and the regulation of these proteases are still not well understood. However, a greater understanding of Rubisco turnover during leaf development is essential to improve crop productivity under CO_2 rich atmosphere.

4.3 | Influence of elevated [CO_2] on cell cycle and cell wall properties

The cell cycle is the series of events that take place in a cell leading to cell division. All organisms are composed of one or more cells; cells are the fundamental units of structure and function in all living organisms. The cell cycle is influenced by various environmental factors including [CO_2] (Kinsman et al., 1997; Figure 2). For example, increased cell division, shortening of the duration of the cell cycle, promotion of cell production, and expansion through cell wall loosening is reported at e[CO_2] (Ranasinghe & Taylor, 1996). In another study, Masle (2000) demonstrated that cell division, cell expansion, and cell patterning are altered by e[CO_2]. It has been suggested that high carbon supply at e[CO_2] may contribute to accelerated cell division and expansion in meristematic tissues and enhance early growth and development of the plant (Thilakarathne et al., 2015).

Elevated [CO_2] influences the expression of genes associated with cell division, growth, and cell wall properties. In particular, genes encoding for expansins, pectin esterase, and xyloglucan endotransglycosylase, which play a key role in cell wall loosening, are up-regulated at e[CO_2] and thus facilitate leaf expansion (De Souza et al., 2008; Gupta et al., 2005; Taylor et al., 2005; Wei et al., 2013). On the other hand, gene expression of B-xylosidase, an enzyme responsible for hemicellulose metabolism of secondary cell walls, is down-regulated at e[CO_2] (Kontunen-Soppela et al., 2010). Down-regulation of secondary wall construction enzymes is likely to contribute to cell wall extensibility and cell growth. Further, it has been found that cell cycle genes encoding cyclin, cyclin-dependent protein kinase, cyclin-dependent protein kinase regulator, and tubulin were up-regulated at e[CO_2], together with ribosomal protein genes that regulate cytoplasm growth of plant cells (Ainsworth et al., 2006; Wei et al., 2013). Up-regulation of these enzymes involved in the cell cycle and in cytoplasmic growth may contribute to increased division,

growth, and expansion of plant cells observed under e[CO_2]. However, limited information is available on transcriptome level responses to e[CO_2], and such information may allow us to have a better understanding of how the cell cycle changes in response to e[CO_2]. A clear understanding of whether this increased activity related to the cell cycle is just a general downstream effect of more photo-assimilates being available for growth, or occurs because of divergence in gene expression mediated by increased sugar levels, needs to be established.

4.4 | Source-sink relationship and trehalose-6-phosphate signalling at elevated [CO_2]

Plant growth responses to CO_2 enrichment are also linked to the source-sink status of the whole plant (Makino & Mae, 1999). Elevated [CO_2] increases the carbon source activity that results in a higher rate of photosynthetic CO_2 assimilation providing more carbohydrates for metabolism (Paul & Foyer, 2001). As a result of this increase in substrate availability and up-regulation of genes encoding proteins involved in sucrose catabolism, sink activity of the plants is directly promoted (Pollock & Farrar, 1996). High sink activity of plants can be clearly observed at e[CO_2], especially during early stages of growth (Ainsworth & Rogers, 2007; Leakey et al., 2009; Ranasinghe & Taylor, 1996), influencing cell expansion, patterning, and plant structure (Kinsman et al., 1997; Masle, 2000). The increased levels of carbohydrates at e[CO_2] are efficiently used by plants to develop additional sinks such as new tillers and secondary shoots to accommodate the photo-assimilates generated by photosynthesis (Jitla et al., 1997; Makino & Mae, 1999). When the sink strength is not sufficient to accommodate all the assimilates from photosynthesis, sugar accumulation in source tissues will decrease the photosynthesis rates through feedback inhibition. This will reduce the ratio of source to sink activity and adjust the photo-assimilate production towards an equilibrium. Therefore, both source and sink activities are strongly cross-regulated to sustain desirable plant growth rates at different development stages (Körner, 2015).

There is considerable evidence for this proposition that changes in growth responses to e[CO_2] depend on the relationship between the photosynthetic rate of source tissue and the sugar demand of the sink tissue of the plant. For example, canopy defoliation in e[CO_2] treatments that reduced the source to sink ratio, resulted in high photosynthetic rates being maintained in the remaining source leaves alleviating photosynthesis acclimation (Ainsworth et al., 2003; Rogers et al., 1998). Because the production from source leaves was less compared with the demand of the sink tissues, a higher rate of photosynthesis was maintained in the remaining tissues to sustain the equilibrium. On the other hand, a reverse response can be observed when the sink size of the plants is reduced through physical restrictions (Arp, 1991) or genetic manipulation (Ainsworth et al., 2004) that leads to an increase in the source to sink ratio. In this case, plants initially increase photosynthetic rates followed by down-regulation of photosynthetic activity due to the inadequate sink capacity (Ainsworth & Bush, 2011; White, Rogers, Rees, & Osborne, 2015). Hence, photosynthetic stimulation and growth at e[CO_2] directly depend on the sink strength and its ability to make use of additional sucrose supply at e[CO_2].

The production and consumption of the photo-assimilates (predominantly sucrose) is a dynamic process and varies according to environmental conditions, and the metabolic demands of the plant. Thus, plants need to maintain a fast and precise balance between source and sink while adjusting them over the long run to sustain their growth (Lawlor & Paul, 2014). The mechanistic understanding of the integration of source/sink interaction with the processes of carbon assimilation is still a long-running debate (Lawlor & Paul, 2014; Paul & Pellny, 2003). One mechanism linking these two processes involves trehalose-6-phosphate (T6P), a signal molecule that influences the basic metabolism of plant proteins (Paul, 2007; Paul, Primavesi, Jhurreea, & Zhang, 2008). Trehalose-6-phosphate is an intermediate in glucose synthesis, generated from glucose-6-phosphate (G6P) and UDP-glucose in the presence of trehalose-6-phosphate synthase1 (TPS1; Lastdrager et al., 2014). It is essential for the vegetative growth of plants and the concentration of T6P is dependent on the sucrose concentration (Lawlor & Paul, 2014; van Dijken, Schluepmann, & Smeekens, 2004). When sucrose concentration is high, such as in plants grown at e[CO_2], high T6P availability will influence SnRK1, a SNF1-related protein kinase which is a key sensor regulating the balance of anabolic and catabolic processes in cells (Schluepmann, Berke, & Sanchez-Perez, 2011; Y. Zhang et al., 2009). The rising T6P content will inhibit the activity of SnRK1 resulting in an increase in anabolic processes stimulating growth and development of the cells and thereby the plant production (Lawlor & Paul, 2014). Therefore, growth stimulation observed at e[CO_2] could be due to this strong correlation between sucrose, T6P content, and the T6P/SnRK1 signalling process. In response to high sucrose content generated at e[CO_2], plants tend to alter their source-sink balance, and species with increased growth plasticity will thrive well in CO_2 rich conditions (White et al., 2015). This could partly explain the large variation in growth responses that exists between and within species at e[CO_2]. Recently, Paul, Oszvald, Jesus, Rajulu, and Griffiths (2017) have highlighted the possibility of modifying T6P content to alter carbon allocation as an approach to improve the yield potential of cereal crops in a changing climate.

4.5 | Plant hormonal metabolism is influenced by elevated [CO_2]

Plant hormones play a major role in modifying plant development at e[CO_2] (Gupta et al., 2005; Teng et al., 2006; De Souza et al., 2008; Wei et al., 2013; Figure 2). Few studies have investigated the effect of long-term e[CO_2] on endogenous hormone production in plants. Woodrow and Grodzinski (1993) and Seneweera et al. (2003) demonstrated that plants exposed to e[CO_2] produce higher levels of ethylene than plants grown under ambient [CO_2]. Further, the same plants had a greater capacity to convert ACC (1-aminocyclopropane-1-carboxylic acid) oxidase to ethylene (Seneweera et al., 2003). This conversion is mediated by ACC oxidase that was highly expressed when plants were exposed to e[CO_2]. Accumulation of ACC oxidase is higher under e[CO_2] as this enzyme has an absolute requirement of [CO_2] for its activation, and thus contributes to high levels of ethylene production (Finlayson & Reid, 1996). It has been demonstrated that an increase in ethylene production is a key feature of

accelerated growth and development in rice under e[CO_2] that accelerates tiller number and auxiliary bud development, potentially leading to higher grain yield (Seneweera et al., 2003). Despite the importance of ethylene in response to e[CO_2], little in-depth investigation has been carried out exploring its role in plant growth responses to e[CO_2].

Auxins, gibberellic acids, and cytokinins are important plant hormones that synergistically regulate cell division, cell expansion, control shoot meristem development, and stem elongation (Cato, Macedo, & Peres, 2013). Teng et al. (2006) investigated the effects of e[CO_2] on plant hormones using *Arabidopsis thaliana* and concluded that there was a significant increment in indole-3-acetic acid (one of the common plant hormones in the auxin class), gibberellic acid, zeatin riboside, dihydrozeatin riboside, and isopentenyl adenosine of the class cytokinin at e[CO_2]. Transcript abundance of genes related to synthesis and transport of auxins (auxin response factor, auxin influx carrier), cytokinins (cytokinin response factor), and gibberellic acids (gibberellin response modulation protein) have been reported to increase under e[CO_2] (De Souza et al., 2008; Gupta et al., 2005; Taylor et al., 2005; Wei et al., 2013). In addition, e[CO_2] influences the selective transport system of hormones that maintain hormone balance in plants. For example, reduction in gene transcripts related to auxin and cytokinin transport, such as auxin: hydrogen symporter 8 and cytokinin transporter 2 and 3, has been observed under e[CO_2] (Wei et al., 2013).

ABA plays a critical role in environmental stress responses, promoting senescence and eventually abscission (Finkelstein, 2013). Teng et al. (2006) reported that the concentration of ABA was significantly reduced under e[CO_2] and suggested that low levels of ABA are likely to be associated with delayed senescence. Molecular evidence has shown that several genes related to ABA synthesis (genes encoding ABA2 xanthoxin dehydrogenase, ABA-responsive protein) were suppressed, whereas expression of ABA degradation enzymes (ABA hydrolase and glycosyl hydrolase) was up-regulated at e[CO_2] (Wei et al., 2013). Together, these findings suggest that altered ABA metabolism at e[CO_2] may have a significant impact on plant growth and development (Figure 2). In addition, it is well-established that ABA plays a major role in determining stomatal conductance in plants (Chater et al., 2015). Such changes in ABA metabolism is likely to lower the stomatal conductance and thus improve the plant's water use efficiency.

4.6 | Plant morphological and anatomical responses to elevated [CO_2]

The morphology of plants is significantly influenced by e[CO_2], mainly as a result of changes in the activity of primary and secondary meristems of roots and shoots (Pritchard, Rogers, Prior, & Peterson, 1999). Growth characteristics of the shoot apex in the early stage of vegetative growth have provided important information on how e[CO_2] influences plant growth and development. Jitla et al. (1997) demonstrated changes in the shoot apex at e[CO_2] and they observed increased height and diameter of the apical dome, increased length of leaf primordia, and a greater number of tiller buds in the shoot apex of rice grown under e[CO_2]. These dynamic changes of shoot and root meristem occur primarily as a result of increased carbon supply and increased growth of sink tissues stimulated by hormonal signalling. As a consequence of these changes, relative growth rate of the plants increases at e[CO_2] (Lambers, Chapin, & Pons, 2008; Makino et al., 1997) that leads to an increase in biomass and yield.

Leaf morphology and anatomy are influenced by environmental conditions including e[CO_2]. Stimulation of early leaf growth has been identified as one of the key physiological traits associated with final biomass and grain yield (Seneweera, Milham, & Conroy, 1994; Thilakarathne et al., 2015). Under e[CO_2], plants tend to show an increased rate of leaf initiation and accelerated elongation coupled with enhanced leaf area (Seneweera et al., 1995; Taylor et al., 1994; Taylor et al., 2001). Canopy size can be increased by greater leaf number and individual leaf area that leads to greater photosynthetic area per given plant at e[CO_2]. However, leaf area is not always enhanced by CO_2 enrichment. For example, in rice, although the leaf area increases during seedling and early vegetative stages at e[CO_2], it frequently decreases during the later vegetative and reproductive stages (Makino et al., 1997; Seneweera et al., 1995). In addition, when plants are exposed to e[CO_2], leaf thickness and leaf internal anatomy are also substantially altered. For example, in soybean (*Glycine max*) leaf, e[CO_2] induced an extra layer of palisade cells (Rogers, Thomas, & Bingham, 1983; Smith, Lewis, Ghannoum, & Tissue, 2012), and in the leaves of *Pinus radiata*, e[CO_2] increased both the mesophyll cross section and the area of vascular tissue (Araújo et al., 2011; Conroy, Barlow, & Bevege, 1986). Increases in mesophyll and vascular tissue area are considered important parameters that contribute to photosynthetic capacity and assimilate transport efficiency of the plant (Jitla et al., 1997; Pritchard et al., 1999). In addition, plants grown at e[CO_2] have shown a significant variation in leaf mass per unit area, which may partly explain the intraspecific variability of growth responses in CO_2 rich conditions (Evans & Poorter, 2001; Thilakarathne et al., 2013). In wheat (*Triticum aestivum* L.), this trait is associated with increases in photosynthetic rates per unit leaf area, leaf nitrogen content on area basis, plant growth rates and yield at e[CO_2] (Thilakarathne et al., 2013). Differences in leaf mass per unit area indicate the ability of plants to deploy on resources in response to environmental signals (Hikosaka & Shigeno, 2009). It is highly likely that changes in leaf anatomy and morphology at e[CO_2] increase the photo-assimilate supply to the apical meristem, which promotes leaf primordial development (Pritchard et al., 1999). Further, it is suggested that increased availability of soluble carbohydrates from expanding leaf blades is a key factor contributing towards high leaf elongation rates, leaf area expansion, and whole plant growth observed under e[CO_2].

Plant growth is influenced by both above-ground and below-ground processes under e[CO_2]. Below-ground processes of plants facilitate photosynthesis through nutrient and water uptake, which then influence above-ground biomass production (Madhu & Hatfield, 2013). Under e[CO_2], plant root growth is also accelerated (Benlloch-Gonzalez, Berger, Bramley, Rebetzke, & Palta, 2014). Several studies have reported that plants tend to increase root biomass and dry weight, especially in wheat (Benlloch-Gonzalez et al., 2014; Chaudhuri, Kirkham, & Kanemasu, 1990), sorghum (Chaudhuri, Burnett, Kirkham,

& Kanemasu, 1986), and soybean (Del Castillo, Acock, Reddy, & Acock, 1989; Libault, 2014) under CO_2 enriched conditions. Salsman, Jordan, Smith, and Neuman (1999) claim that the increase in root biomass at e[CO_2] is associated with increased starch levels in roots and an increase in the levels of ABA that may have caused more carbon to be allocated to root growth.

4.7 | Future research perspectives on plant growth responses to elevated [CO_2]

There is greater body of knowledge on the changes in photosynthesis and stomatal conductance in response to [CO_2] than of the other cellular processes influenced by the increased carbon supply generated at e[CO_2]. A better understanding of these post-photosynthetic effects of e[CO_2] on plant growth is essential for the development of strategies to improve crop productivity under a CO_2 rich atmosphere. Among these, an improved understanding of plant carbon and nitrogen metabolism, source/sink interactions, cell cycle properties and their crosstalk with hormones during atmospheric CO_2 enrichment is required to establish a holistic overview of the physiological mechanisms modulating plant growth responses under these conditions.

Breeding crops for a changing future climate requires the identification of key physiological and growth traits that help to enhance crop productivity. Further, understanding the genetic variation of plant responses to e[CO_2] will also assist in selecting plants for greater fitness and long-term adaptation to climate stress (Ward & Kelly, 2004). However, very limited information is available on the genetic regulation of plant growth at e[CO_2] (Rae, Ferris, Tallis, & Taylor, 2006). It has been reported that CO_2 responsiveness of plants is a quantitative trait that is determined by a range of gene products (Ferris et al., 2002). Therefore, identification of quantitative trait loci via the collection of phenotypic, biochemical, and physiological data on genetically characterized populations will help to develop an understanding of the cellular mechanism of plant responses to e[CO_2] (Rae et al., 2006) and assist the selection of appropriate germplasm for use in breeding programs. In order to achieve this target, state of the art analytical techniques such as high throughput genomics, proteomics, metabolomics, and transcriptomics are required. Identification of the candidate genes involved in the plant's primary metabolism will also be helpful in understanding the regulatory networks of plant growth and shifts in carbon-nitrogen balance at e[CO_2] (Vicente et al., 2015). Understanding plant sugar—hormonal crosstalk mainly associated with carbon and nitrogen metabolism will be important to adapt to a CO_2 rich atmosphere. However, in the real world, increased atmospheric [CO_2] interacts with higher global temperatures and altered rainfall patterns to provide plants with a "triple whammy" of challenges. With experimentation on e[CO_2] generally conducted under controlled temperatures and adequate water supply, it is difficult to predict the results to these actual situations. Therefore, understanding the interactions of these triple challenges (e[CO_2], altered water availability and increased temperature) from cellular to whole plant level is crucial when addressing future food security targets under a changing climate.

5 | CONCLUDING REMARKS

Climate change is challenging the productivity of global agriculture and thus global food and nutrient security. As CO_2 is the primary substrate for photosynthesis and plant growth, a better understanding of the atmospheric [CO_2] utilization strategies of plants will pave the way for increasing crop productivity. Considerable research has established the adjustments in photosynthesis and stomatal conductance in response to e[CO_2], but not much attention has been paid to understanding the role of other key cellular processes in modifying physiological and plant growth process. The findings from long-term [CO_2] enrichment studies suggest that understanding the cellular processes together with the source-sink interaction is essential to capture the full benefits of rising [CO_2] on crops. Theoretically, a number of bottle necks in C_3 photosynthesis can be overcome with elevated levels of [CO_2]; however, the physiological and molecular mechanisms to achieve this target are still not clearly understood. In particular, understanding is needed of the effects of e[CO_2] on carbon and nitrogen metabolism, the cell cycle, and hormonal metabolism, which are more likely to play a major role in modifying plant growth processes. Comprehensive research in this area of study will open up new avenues for minimizing photosynthetic inefficiencies and thereby potentially improve crop productivity in a future CO_2-enriched atmosphere. However, interactions with elevated temperatures and changes in available water will render this productivity goal very challenging.

ACKNOWLEDGMENT

This work was supported by the University of Southern Queensland, Australia.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How to cite this article: Gamage D, Thompson M, Sutherland M, Hirotsu N, Makino A, Seneweera S. New insights into the cellular mechanisms of plant growth at elevated atmospheric carbon dioxide concentrations. *Plant Cell Environ*. 2018;41: 1233–1246. https://doi.org/10.1111/pce.13206

Chapter 3

Early growth mechanism of wheat (*Triticum aestivum* L.) at elevated atmospheric carbon dioxide: New physiological evidence with quantitative trait loci data

In this study, a quantitative trait loci (QTL) analysis was carried out to identify the potential genetic components associated with plant growth responses to elevated [CO_2]. A doubled haploid population of wheat was used for QTL mapping, focussing on the early vegetative growth stage. Together with QTL mapping, a detailed characterization of the parental lines of the mapping population was also conducted under ambient and elevated [CO_2] conditions. QTL mapping was conducted for several important plant growth traits and the CO_2 responsiveness of each trait was used to determine CO_2-response QTL. This chapter has been submitted as a research article to "Plant, Cell and Environment" and is currently under review.

Gamage D, Thompson M, Okamoto M, Moriyama N, Sutherland M.W., Hirotsu N, Seneweera S, 'Early growth mechanism of wheat (*Triticum aestivum* L.) at elevated atmospheric carbon dioxide: New physiological evidence with quantitative trait loci data' (Under review)

Article title:

Early growth mechanism of wheat (*Triticum aestivum* L.) at elevated atmospheric carbon dioxide: New physiological evidence with quantitative trait loci data

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Abbreviations:

[CO_2] – Carbon dioxide concentration

QTL – Quantitative trait loci

WAP – Weeks after planting

DH – Doubled haploid

Rubisco - Ribulose 1,5-bisphosphate carboxylase/oxygenase

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[1] DG – Study design, acquisition, analysis and interpretation of data, drafting of manuscript, MT- Acquisition of data, NM- interpretation of data, MO & MWS – critical revision, NH – study design, analysis and interpretation of data and critical revision, SS- Study conception and design, analysis and interpretation of data, critical revision

RGR – Relative growth rate

LER – Leaf elongation rate

V_{cmax} – Maximum carboxylation rate of Rubisco

J_{max} – Maximum rate of photosynthetic electron transport

TPU – Rate of triose phosphate utilization

DC – decimal code for wheat growth stage

C_i – Intra-cellular CO_2 concentration

G_s – Rate of stomatal conductance

QTL – Quantitative trait loci

WUE – water use efficiency

NUE – nitrogen use efficiency

Summary statement

Elevated [CO_2] increases biomass accumulation and growth of wheat (*Triticum aestivum* L.) during early ontogeny. Growth at elevated [CO_2] was linked to 24 putative quantitative trait loci (QTL). CO_2 response of biomass production was associated with 3 key QTL on chromosome 2A, 1B and 4B.

Abstract

The underlying genetic variation of plant growth responses to elevated [CO_2] is still not clearly understood. Here, a quantitative trait loci (QTL) analysis was carried out in a doubled haploid population of wheat (*Triticum aestivum* L.) grown at 700 µmol mol^{-1} (elevated [CO_2]) and 400 µmol mol^{-1} (ambient [CO_2]). The mapping population including the parental lines, Kukri and RAC875 showed a significant difference in growth habit under ambient and elevated [CO_2] ($P<0.05$), indicating a differential response to elevated [CO_2]. Data for major early growth traits and their responsiveness to elevated [CO_2] were used for QTL mapping. In total, 24 putative QTL were identified for CO_2 responsiveness. Among them, three QTL showed increased dry weight response to elevated [CO_2], which were located on chromosome 2A (LOD = 4.372), 1B (LOD = 2.538) and 4B (LOD = 2.511). Of these, QTL on 2A and 4B played a role in increasing shoot dry weight (2A: LOD = 4.409, 4B: LOD = 2.506) at elevated [CO_2]. These findings suggest the existence of a major mechanism that controls CO_2 response at the genetic level. Further in-depth analysis of these genomic regions is required to identify candidate genes responsible for enhanced plant growth under elevated [CO_2].

Keywords: CO_2 responsiveness, Elevated [CO_2]; Early growth; QTL; *Triticum aestivum* L.

1. Introduction

The atmospheric carbon dioxide concentration ([CO_2]) has risen at a remarkable rate and currently exceeds 400 µmol mol^{-1} (Tans & Keeling, 2018), which is a more than 30% increment in [CO_2] since the industrial revolution (IPCC, 2013). It is predicted that atmospheric [CO_2] may reach 550 µmol mol^{-1} in 2050 and 730 to 1020 µmol mol^{-1} by 2100 (Solomon *et al.*, 2007) as a consequence of anthropogenic activities such as fossil fuel combustion and deforestation. Although CO_2 is considered a greenhouse gas, it is the primary substrate for plant photosynthesis and substantially stimulates photosynthesis especially in C_3 plants, supporting vigorous growth and development. Plants of C_3 origin are not photosynthetically saturated at the current CO_2 partial pressure due to the properties of Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the primary carboxylation enzyme of C_3 plants (Makino & Mae, 1999). Under current ambient conditions, [CO_2] is very low at the site of CO_2 fixation and any increases in [CO_2] will increase the CO_2/O_2 ratio, thereby increasing the carboxylation efficiency of Rubisco by lowering the rate of photorespiration (Bowes, 1991, Drake *et al.*, 1997). This increase in ambient [CO_2] directly and indirectly affects photosynthesis and stomatal conductance and subsequently increases the growth and development of plants (Seneweera & Conroy, 2005, Ainsworth *et al.*, 2008, Gamage *et al.*, 2018). To improve the crop productivity in a CO_2 rich atmosphere, a thorough understanding of long-term adaptations of plants to elevated [CO_2] at the genetic level is essential (Rae *et al.*, 2006).

Growth response to elevated [CO_2] varies depending on the plant species and growth stage (Seneweera, 2011a, Fitzgerald *et al.*, 2016). The majority of the C_3 species show very high growth responses to elevated [CO_2], especially at early stages of vegetative development (Poorter, 1993). Early growth responses to elevated [CO_2] are usually characterized by accelerated leaf growth and expansion, which will result in increased leaf area ratios and relative growth rates (Poorter, 1993). It is suggested that increased growth response to elevated [CO_2] results from the initial stimulation of photosynthesis and this stimulation may disappear over time (Masle *et al.*, 1993, Centritto *et al.*, 1999, Trevisan *et al.*, 2014). Similar to photosynthesis response, relative growth rate (RGR) also tends to decline as plants grow older at elevated [CO_2] (Makino *et al.*, 1997). Hence, the effect of elevated [CO_2] on RGR is often time-dependent and occurs only at the early stage of plant growth (Poorter & Navas, 2003). It has also been reported with species such as wheat (Neales & Nicholls, 1978, Hikosaka *et al.*, 2005), *Arabidopsis* (Van Der Kooij & De Kok, 1996) and tobacco (Geiger *et al.*, 1998) that exposure to elevated [CO_2] increased the RGR in young plants whereas in old plants, RGR

remains unaffected. Even a slight increase in RGR in the exponential growth phase of the plants can be translated up to 50% absolute growth enhancement at elevated [CO_2] (Kirschbaum, 2010). In addition, increased photosynthetic capacity at elevated [CO_2] leads to accelerated leaf elongation rates (LER) and contributes towards higher green leaf area production under high [CO_2]. Faster rates of RGR and LER observed at elevated [CO_2] strongly correlate with the total biomass production and consequently influence on grain yield production (Jitla *et al.*, 1997, Seneweera & Conroy, 2005). This early advantage of enhanced seedling growth and vigor is vital for the plant's subsequent establishment and, consequently, impacts on crop productivity (Thilakarathne *et al.*, 2015, Nagai *et al.*, 2016). Therefore, an understanding of the fundamental mechanisms of how crops respond to elevated [CO_2] at the early growth stages is crucial to the development of new breeding strategies for increasing crop yield potential under rising [CO_2] in the atmosphere. However, plant growth, or structural development, is a complex process (Ter Steege *et al.*, 2005) and detailed experiments are required to identify the different organizational level responses to elevated [CO_2] and uncover the holistic mechanism of plant growth at elevated [CO_2].

A large intra-specific variation in growth response to elevated [CO_2] has been widely reported (Thilakarathne *et al.*, 2015), however, there is limited information available for the genetic control of plant growth responses to elevated [CO_2] (Rae *et al.*, 2006). Physiological traits that are affected by environmental factors such as elevated [CO_2] are quantitatively determined (Ferris *et al.*, 2002).To identify the genetic response of traits changed by external environmental factors, identification of quantitative trait loci (QTL) for a particular trait is important (Rae *et al.*, 2006). Quantitative trait loci are the regions within a genome that correlate with the phenotypic variation of a particular quantitative trait (Collard *et al.*, 2005) and provides information on whether the trait of interest has an associated genetic component that can be utilized in plant breeding programmes. Identification of new CO_2-responsive QTL provides new tools to improve crop productivity under a CO_2 rich atmosphere and thus adaptation to climate stress (Rae *et al.*, 2006).

There had been few studies focused on identifying CO_2-responsive QTLs with tree species. For example, Rae *et al.* (2006) mapped QTL for increased leaf growth and delayed senescence in hybrid poplar (*Populus spp*) and showed that candidate genes controlling the traits collocate to the regions where QTL were mapped. In another study, Ferris *et al.* (2002) determined QTL for leaf stomatal initiation, stomatal density, epidermal cell size, number and area in hybrid

poplar. In addition, QTL for above ground and below ground tree growth were determined in poplar by Rae *et al.* (2007), showing that there are significant genomic regions in the poplar genome that respond to shoot and root biomass at elevated [CO_2]. However, only a limited number of studies have been carried out to identify QTL responsive to elevated [CO_2] in cultivated crops and there have not been any studies on wheat (*Triticum aestivum* L.) (Gamage *et al.*, 2018), which is the most widely cultivated and consumed cereal crop (Shewry, 2009). Moreover, none of the studies have been focused on the early vegetative growth despite its important role in determining final biomass and yield. Therefore, identifying key genomic regions determining plant growth traits under elevated [CO_2] will provide key information to broaden our knowledge and understanding of how plants respond to high CO_2.

In this study, we aimed to elucidate the genomic regions responsible for growth-related traits under elevated [CO_2], focusing on the early vegetative growth stage in wheat. The main objective was to identify QTL for plant growth responses at ambient [CO_2] and elevated [CO_2]. Further, we tested whether the variation of CO_2 responsiveness in wheat is associated with new QTLs and/or physiological response of the crop. The ultimate goal of this study is to identify the genetic basis of growth responses of wheat to elevated [CO_2] and incorporate these traits into future breeding programs to improve the crop productivity and to maintain ecological success in a changing climate.

2. Materials and methods

2.1 Plant materials – mapping population

A doubled haploid (DH) wheat mapping population (152 wheat lines including parental cultivars) derived from a cross between RAC875 and Kukri (Fleury *et al.* (2010) was used in this study. This mapping population was obtained from the Australian Centre for Plant Functional Genomics, University of Adelaide, Australia. The parental lines of this population were physiologically characterized in detail by Izanloo *et al.* (2008). RAC875 (RAC655/3/Sr21/4*LANCE//4*BAYONET) is a breeder's line which shows good and stable yield potential even under water-limited conditions. On the other hand, Kukri (76ECN44/76ECN36//MADDEN/6*RAC177) is a cultivar that shows less yield potential (less than 44%) under the same conditions (Bennett *et al.*, 2012a, Bennett *et al.*, 2012b). The harvest index (HI) and the nitrogen use efficiency (NUE) of Kukri is 41% and 100.4 kg grain yield kg^{-}

^{1}, respectively. For RAC875, HI and NUE is 43% and 108.1 kg grain yield kg^{-1}, respectively (Mahjourimajd *et al.*, 2015). The differences in NUE in these cultivars is suggested to be a key determinant of variation in growth and yield response (Gamage *et al.*, 2018). Elevated [CO_2] significantly reduces the nitrate photo assimilation (Bloom, 2015), changes the nitrogen allocation to different plant organs (Bernacchi *et al.*, 2007), reduces the nitrogen uptake relative to the carbon gain (Del Pozo *et al.*, 2007) and thereby reduces nitrogen demand (Stitt & Krapp, 1999). On the other hand, both Kukri and RAC875 have different water use efficiencies (WUE) (Izanloo *et al.*, 2008) which was significantly changed under elevated [CO_2] (Conley *et al.*, 2001, Keenan *et al.*, 2013). It is likely that different NUE and WUE of these parental lines provided them with the ability to perform differently at elevated [CO_2].

2.2 Growth conditions

2.2.1. Experiment 01 – Parental lines characterization

Two parental lines of the mapping population, Kukri and RAC875, were characterized for all the growth and physiological traits that were investigated in this study. Plants were grown in a temperature-controlled glasshouse under either 400 or 700 µmol mol^{-1} [CO_2] maintaining a day and night temperature of 22°C and 13°C, respectively. Plants were harvested at two different physiological stages: 4 weeks and physiological maturity. Total final biomass, thousand grain weight, tiller number at anthesis and final yield were recorded. Four weeks after planting (WAP) and at anthesis (DC 65) (Zadoks *et al.*, 1974), gas exchange measurements were carried out in the last fully expanded leaf and flag leaf, respectively using an infrared gas analyzer (IRGA) system (Li-6400, Li-Cor, Lincoln, NE, USA) as described in Seneweera *et al.* (2002). The net photosynthesis rate, stomatal conductance, transpiration rate, and intercellular CO_2 concentration were measured in both growth stages. The [CO_2] in the leaf chamber was maintained at 400 µmol mol^{-1} and 700 µmol mol^{-1} for ambient and elevated [CO_2], respectively. The leaf chamber temperature was maintained at 25°C and irradiance was maintained at 1500 µmol quanta m^{-2} s^{-1} which was supplied by a red and blue light source (6400-02B LED source). Photosynthesis gas exchange measurements were conducted during the day, between 0900 hours and 1430 hours to produce *A-Ci* response curves (photosynthesis (*A*) versus intracellular [CO_2] (*Ci*) curves). Prior to taking the readings for *A-Ci* response curves, each leaf was allowed 10-15 mins to reach a steady state of photosynthesis at 400 µmol mol^{-1} or 700 µmol mol^{-1}. The initial slopes of the *A-Ci* response curves were used to calculate maximum carboxylation rate

of Rubisco ($V_{c.max}$) and maximum rate of photosynthetic electron transport (J_{max}) as suggested by Farquhar *et al.* (1980) and Von Caemmerer and Farquhar (1981), respectively.

2.2.2. Experiment 02 – Glasshouse experiment

One hundred and fifty-two lines of the mapping population, including the two parental lines, were germinated on moistened filter papers in Petri plates. When germinated seedlings reached 2 cm in height, they were planted in pots (140 mm in diameter) filled with 1.5 kg of brown topsoil. The experiment was laid as a completely randomized design with each line of the mapping population replicated three times in three individual pots. The pots were placed in glass houses maintained at either ambient CO_2 (400 µmol mol^{-1}) or elevated CO_2 (700 µmol mol^{-1}). The conditions in the glasshouses were identical except for the varied [CO_2] concentration. Day and night temperature was maintained at 22°C and 13°C, respectively. All pots were randomized at weekly intervals to minimize the glasshouse effect. Standard crop management practices were carried out to ensure optimum crop health and growth throughout the study period. The experiment was maintained in both glasshouses for four weeks during winter 2015 in the University of Southern Queensland, Australia (Latitude: 27° 33' 38.02" S and Longitude: 151° 55' 55.20" E).

2.2.3. Experiment 03 – Growth chamber experiment

The same 152 lines of the mapping population were again grown in two identical growth chambers (Reach in growth chambers, PGC-105, Percival, USA) under controlled environmental conditions for four weeks in 400 mm pots using the same soil type. Both chambers were maintained at a 14-hour photoperiod with day and night temperatures of 23°C and 13°C, respectively. Relative humidity in the chambers was maintained at 70% throughout the growth period. The light intensity was diurnally varied and was 1000 µmol m^{-2} s^{-1} during midday throughout the growing period. Plants were supplied with optimum nutrients and water throughout the growth period. Elevated [CO_2] conditions were maintained at ~700 µmol mol^{-1} and ambient [CO_2] condition was maintained at ~400 µmol mol^{-1} throughout the growing season.

2.3. Growth trait measurements

In this study, shoot dry mass, root dry mass, total dry mass, RGR, seedling height, LER and leaf width were measured as growth traits focusing on the early vegetative stage of wheat seedlings of the mapping population. All the physiological trait measurements were carried out as described by (Pérez-Harguindeguy *et al.*, 2013). In experiment 02, measurements were taken at 2 WAP and 4 WAP in the glasshouse experiment. In both data collection rounds, one plant from each replicate was measured for seedling height, leaf width and LER. Another plant was carefully sampled to determine aboveground biomass accumulation. Plants were oven dried at 70°C for 72 hours and dry weights were measured. Plant growth analysis and RGR calculations were carried out as described by Hunt (2003).

In experiment 03, seedlings were carefully harvested, including roots, at the end of the growth period (4WAP) and washed with water to remove all soil residues attached to the roots. Then, the shoot and root were separated, and oven dried at 70°C for 72 hours to determine the dry weight of shoots and roots separately. Then, the root to shoot ratio of each line of the mapping population was calculated.

2.4. Calculation of CO_2 responsiveness of each growth trait

Percentage responsiveness of each measured trait to elevated [CO_2] was calculated as described in Rae *et al.* (2006). These results were then considered as the responsiveness of each trait and used for QTL mapping.

$$\mathrm{CO_2\ responsiveness} = \frac{(\text{Trait value at elevated } [\mathrm{CO_2}] - \text{Trait value at ambient } [\mathrm{CO_2}])}{\text{Trait value at ambient } [\mathrm{CO_2}]} \times 100\ (\%)$$

2.5. QTL mapping

The genetic map of this mapping population was published by Bennett *et al.* (2012b) employing 456 DArT and SSR markers forming linkage groups representing all 21 chromosomes (Bennett *et al.*, 2012b, Bennett *et al.*, 2012c). This genetic map was used in this study to identify the potential QTL associated with early growth traits of wheat. In this study, traits used for QTL mapping included seedling height, LER, leaf width, shoot dry weight, root dry weight, RGR and root to shoot dry weight ratio. Measurements for these traits were separately taken from ambient [CO_2] and elevated [CO_2] grown wheat seedlings. Then these data were used to map putative QTL under both ambient [CO_2] and elevated [CO_2]. The CO_2 responsiveness of each

trait was calculated separately as mentioned in section 2.4 and these data were used to identify response QTL under elevated [CO_2]. The trait data were tested for normality before they were used in the analysis. The physiological data for each 152 wheat lines together with their relevant genotypic data were fed in to the QGene software for QTL mapping. QTL mapping was conducted using QGene software version 4.3.10 employing single trait multiple interval mapping to obtain LOD scores, additive effects and QTL effect (R^2) values. For each trait, a minimum LOD value of 2.0 was used for the identification of putative QTL.

2.6. Data analyses

The trait differences in plants grown at ambient [CO_2] and elevated [CO_2] were analyzed using the paired t-test to confirm the suitability of data for QTL mapping. Treatment effects and interactions were determined through Analysis of Variance. Differences were considered significant at $P<0.05$. All the data were analyzed using SPSS statistical software version 23 (IBM, Armonk, NY, USA). All the graphical representations were carried out using GraphPad Prism scientific software version 5.01 (GraphPad Software, San Diego, CA).

3. Results

3.1. The response of Kukri and RAC875 to elevated [CO_2]

Elevated [CO_2] increased total dry mass and total grain yield per plant, respectively, by 30% and 82.8% for Kukri compared with 23% and 64.2% for RAC875 (Table 1, $P<0.01$ and Supplementary Figure 1). A significant [CO_2] effect was observed for the rate of photosynthesis at elevated [CO_2] and for the number of tillers at anthesis (Table 1, $P<0.01$ and Supplementary Figure 1). Photosynthetic rates were significantly increased in both parental lines when grown at elevated [CO_2] (Figure 1(a) and Supplementary Table S1, $P<0.01$), however, Kukri showed a slightly higher increase in photosynthetic rate (46.2%) than that of RAC875 (38.13%). The cultivar effect was significant on net photosynthesis at elevated [CO_2] ($P<0.01$) and J_{max} ($P<0.01$). At anthesis, both parental lines showed a decrease in V_{cmax}, in which Kukri showed a comparatively higher decline (Figure 1(c)). However, Kukri showed a higher J_{max} under elevated [CO_2] than the RAC875 and the effect of cultivars on J_{max} was statistically significant (Figure 1(d)). In addition, there was a significant interaction between [CO_2] and cultivar on J_{max} ($P<0.01$). Further, the rate of triose phosphate utilization (*TPU*) of

the two varieties was significantly different when grown at elevated [CO_2] with Kukri showing a slightly lower *TPU* than that of RAC875 (Figure 1 (e)).

In the early growth stages, both parental lines showed a significantly higher photosynthetic rate at elevated [CO_2] (Figure 1(b) and Supplementary Table S1, $P<0.01$) and the magnitude of increase in photosynthetic rates were similar for Kukri and RAC875. Despite the similar photosynthetic rates of these two varieties in the early growth stages, Kukri showed a significantly higher biomass accumulation in shoots (44%) and roots (42%) than RAC875 when plants were grown at elevated [CO_2] (Table 1). Shoot and root biomass accumulation, seedling height, LER and RGR of Kukri were significantly higher than those traits of RAC875 at elevated [CO_2] (Table 2, $P<0.05$)

3.2. Mapping population response to elevated [CO_2]

There was a marked difference between the traits measured in ambient [CO_2] and elevated [CO_2] (Table 2, $P<0.05$), indicating that QTL mapped from these data will be relevant and of value for further detailed analysis. The frequency distributions for all the measured growth traits of the mapping populations grown in both glasshouse and growth chambers (controlled environment chambers) are shown in Figure 2 and 3, respectively, and the trait values of parental lines of the mapping population are marked under both ambient [CO_2] and elevated [CO_2]. All the measured parameters obtained from different CO_2 treatments showed continuous variation and near-normal distribution except for root dry weights, which were positively skewed (Figure 2 & Figure 3). In general, plant growth was stimulated under elevated [CO_2] as assessed from biomass accumulation, seedling height, RGR and LER of different lines either grown at elevated or ambient [CO_2] in glasshouse conditions. For example, the average biomass accumulation of the population was prominent at elevated [CO_2] with an increase of 73 mg per plant when compared with ambient [CO_2] grown plants. However, growth responses of the population to elevated [CO_2] greatly differed under growth chamber conditions. The average biomass accumulation in shoots and roots of the DH lines showed an increase of 175 mg and 62 mg per plant, respectively, when compared with the average trait values of plants grown at ambient [CO_2].

3.3. Detection of QTL for growth traits

A total of 13 QTL were identified for the population in ambient [CO_2] (Table 3), 31 QTL in elevated [CO_2] (Table 4) and 24 CO_2-response QTL (Table 5) for the nine traits investigated

in this study. There was evidence of QTL for all the growth traits measured under ambient and elevated [CO_2]. However, significant CO_2-response QTL were identified in all traits except for LER and RGR. The QTL identified at elevated [CO_2] were distributed across 15 chromosomes, with the chromosomes of the A and B genomes having the most number of QTL (12 each).

Totally, 12 QTL were identified in this study which explained a significant phenotypic variation for biomass accumulation at elevated [CO_2] (Table 4). Of them, four QTL were identified on chromosomes 2A, 3B, 7D and 3D for shoot dry weight, which explained 7.6 – 9.9% variation in this trait. In experiment 03, QTL for shoot dry weight were found on 1A, 3A, 6B and 6D, which explained a total of 42.6% of the phenotypic variation in the population. Of these, the QTL on chromosome 6B could explain 14.4% of shoot dry weight variation in the DH population. In addition, two QTL on chromosome 2A, two QTL on chromosomes 3B and 2D, and two QTL on chromosomes 3B and 6B were identified as putative QTL for controlling root dry weight, root to shoot ratio and total dry weight, respectively (Table 4). Interestingly, the QTL for shoot dry weight on chromosome 6B is collocated with the QTL for total dry weight on the same chromosome. Three CO_2-response QTL associated with increased dry weight were identified on chromosomes 2A, 1B and 4B (Table 5). Of these QTL, two (identified on 2A and 4B) played a role in determining shoot dry weight response to elevated [CO_2].

Six QTL located on 2A, 1B, 3B, 6B and 7D were found to control seedling height (2 WAP) at elevated [CO_2] and the most significant QTL was located on chromosome 3B (Table 4). This was further confirmed by the presence of a QTL on chromosome 3B for the trait of seedling height at 4WAP (Table 4). The location of the QTL for these traits at ambient [CO_2] was different (chromosome 3B and 6D, Table 3). Further, different CO_2 response-QTL for seedling height were identified during plant development. For example, CO_2-response QTL for seedling height at 2WAP were mapped on chromosomes 6A, 7A and 2D while a response QTL for seedling height at 4WAP was mapped on chromosome 1B (Table 5).

For LER, three QTL were identified on chromosomes 4A, 6A and 1B at elevated [CO_2] which accounted for 24% phenotypic variation of this trait (Table 4). For the same trait, only one QTL was identified (chromosome 3B) under ambient [CO_2] (Table 3), however, no CO_2-response QTL was identified. Leaf width of plants also showed differential control under the two CO_2 treatments. At elevated [CO_2], four QTL for leaf width were identified on chromosomes 6A,

1B, 6B and 2D, of which the QTL located on 6B and 2D were found to explain 38.6% phenotypic variation of the trait at elevated [CO_2]. For the same trait, only two QTL were identified at ambient [CO_2] and they were located on chromosomes 6B and 2D (Table 3). Further, the CO_2-response QTL for leaf width was identified on chromosome 6B (Table 4).

Discussion

4.1. Variation of photosynthesis capacity and plant growth responses at elevated [CO_2]

Genetic variation in growth response to elevated [CO_2] has been documented for many crop and pasture species (Shimono *et al.*, 2009, Thilakarathne *et al.*, 2013, Thilakarathne *et al.*, 2015), however, the molecular and genetic mechanism of this response is not fully understood. To the best of our knowledge, this study is the first to report new QTL for key plant growth traits of wheat grown under elevated [CO_2]. We have also established that the parental lines of the mapping population showed contrasting growth responses for elevated [CO_2]. In this study, we identified contrasting growth behavior for two parents, Kukri and RAC875, and among their progenies for different growth traits, particularly at early vegetative growth. The parent line Kukri, showed a higher responsiveness to elevated [CO_2], showing higher trait values for most of the traits tested in this study, especially in biomass accumulation. Significant interactions were found for [CO_2] effect and genotypic effect of the mapping population for main growth traits such as dry mass accumulation and LER suggesting that even the mapping population responded to elevated and ambient [CO_2] in two distinct patterns (Supplementary Table S2). It is likely that such distinct variation in response to elevated [CO_2] could be linked to different water use efficiencies (WUE) of the genotypes, that mapping population was originally generated (Bennett *et al.*, 2012a, Bennett *et al.*, 2012c). On the other hand, the parental cultivars of this mapping population also differ in their NUE which correlates with the final grain yield production (Mahjourimajd *et al.*, 2015). Therefore, the differences in plant growth responses at elevated [CO_2] could be due to the differences in plant nitrogen and water status. In addition to the genetic capacity of these genotypes for differential WUE, the increased levels of [CO_2] generally enhances the rates of photosynthesis and reduces transpiration, resulting in higher WUE (Figure 1(f), Hsiao & Jackson, 1999) and thereby facilitate higher plant growth and productivity. Further, it is worthy to highlight that the physiological parameters of progeny lines perform slightly different manner when plants are grown under glasshouse and controlled environmental conditions. Perhaps, this could relate to the light

condition of the growth chambers. In both experiments, the CO_2 levels were strictly maintained at 400 and 700 µmol mol^{-1} at all the time. However, the temperatures were maintained according to a schedule throughout the day, light intensity and relative humidity were maintained at a constant level throughout the growth period in the controlled growth chambers (Supplementary Table S3). In glasshouse, the temperature and [CO_2] was finely controlled but plant was exposed to sunlight. However, consistently under both environmental conditions, there was a clear increase in plant growth and changes in physiological parameters at elevated [CO_2].

Increased photosynthesis rate largely determines the biomass response to elevated [CO_2] across a range of crops, including wheat (Makino & Mae, 1999, Seneweera, 2011b). In this study, photosynthesis rates of the parental lines were significantly increased at elevated [CO_2], but these rates depended on the plant's developmental stage. The photosynthesis rates at early growth stages were higher than the photosynthesis rates at anthesis across both cultivars. However, there was a marked difference between the biomass accumulations in the two parental lines, suggesting the presence of other regulatory mechanisms that control plant growth at elevated [CO_2]. Generally, the best indicators of the whole photosynthesis process are V_{cmax} and J_{max} which represent Rubisco activity and the electron transport contributing to RuBP regeneration that are crucial for proper functioning of the photosynthesis machinery in plants (Sharkey, 2016). The V_{cmax} and J_{max} parameters of these two parents also showed a considerable difference, with Kukri displaying a comparatively higher reduction of these two important photosynthesis parameters. However, the J_{max} of Kukri was higher than RAC875 suggesting that Rubisco-regeneration limitation of Kukri was less than RAC875 at elevated [CO_2] (Sun *et al.*, 2011). The effect of [CO_2] on *TPU* was significantly reduced suggesting that net photosynthesis is no longer sensitive to increased [CO_2] (Yang *et al.*, 2016). At this stage, both Rubisco and RuBP regeneration activity may have been regulated at a rate that matches with *TPU* (Yang *et al.*, 2016), which was apparent with decreased V_{cmax} values at elevated [CO_2].

Acclimation of photosynthesis to elevated [CO_2] is usually associated with significant decreases in Rubisco activity, which will lead to changes in the photosynthetic efficiency of leaves in response to the biochemical adjustments in the carbon and nitrogen metabolism of plants (Ghildiyal & Sharma-Natu, 2000). In the case of elevated [CO_2], although plants become acclimated to high [CO_2] levels, the acclimated photosynthesis rates of plants are higher than the photosynthesis rate of plants grown at ambient [CO_2]. This may have led to improvements

in the overall performance of the plants in the high CO_2 environment. Despite the large reduction of V_{cmax} in Kukri compared to RAC875, Kukri showed the highest shoots and roots biomass response to elevated [CO_2], suggesting that increased photosynthesis capacity may not be the only factor that drives increased plant growth and development in the high CO_2 environment. This hypothesis was further supported by recent findings that genetic variation in wheat responses to elevated [CO_2] is determined by specific leaf nitrogen status rather than photosynthetic gas exchange characteristics (Thilakarathne *et al.*, 2015). It has also been demonstrated that cultivars with a high tillering capacity/secondary shoots development capacity have a greater ability to thrive well in elevated [CO_2] (Shimono *et al.*, 2009, Tausz *et al.*, 2013), as even a slight stimulation in net assimilation rates and RGR will produce more photosynthetic tissues and leaf area during earlier stages, accelerating their growth at elevated [CO_2]. This could offset the negative impacts of the downward acclimation of photosynthesis in the later stage of plant development at elevated [CO_2] (Van Der Kooij & De Kok, 1996). However, if CO_2 responsiveness is to be capitalized on to improve crop productivity, it is important to instigate both photosynthetic and other important post-photosynthetic related CO_2 responsive traits such as plant nitrogen and water status together. Further, it is also required to develop a greater understanding on the coordination of light capture (Zhu *et al.*, 2008) and energy conversion (Murchie *et al.*, 2009) of plants to cope with the atmospheric CO_2 increase.

Increased photosynthesis at elevated [CO_2] leads to increased biomass accumulation, consequently changing plant growth, development and morphology, which was mainly determined by traits such as shoot to root dry weight ratio, LER and RGR (Masle, 2000, Seneweera & Conroy, 2005). Increased biomass accumulation during early growth is the key determinant of the final grain yield in cereals. The positive relationship between these two traits is widely used across the globe to increase crop yield potential (Villegas *et al.*, 2001). Although increases in photosynthesis rates at elevated [CO_2] have a positive influence on biomass accumulation, our study's results showed that there was a high variation in biomass accumulation in the parents and the progenies of the DH population despite their rate of photosynthesis being similar. However, the variation of this response, whether mediated through improved WUE and/or NUE is not known. If so, these physiological mechanisms independently operate to respond to elevated [CO_2]. In a study conducted by Izanloo *et al.* (2008), Kukri and RAC875 showed a great difference in WUE, in which Kukri performed well only in well-watered conditions and RAC875 produced stable yield even under moderate drought stress conditions, suggesting that Kukri has a lower WUE compared to RAC875. Our

experiments were conducted under proper watering schedules and thus, WUE did not measured, except instantaneous WUE from gas exchange data. However, when plants exposed to elevated [CO_2], Kukri showed a large reduction of stomatal conductance and transpiration rate compared to RAC875 which may have led to higher WUE in Kukri than the RAC875 (Figure 1(f)). This increased WUE, together with the high NUE of Kukri (Mahjourimajd *et al.*, 2015, Mahjourimajd *et al.*, 2016), may have partly contributed to the higher accumulation of biomass in shoots and roots and, consequently, all the growth traits showed a significant response to elevated [CO_2]. These findings suggest that growth at elevated [CO_2] is controlled by multiple physiological processes, mainly improved photosynthesis, WUE, and NUE, and these processes likely play a significant role in determining the final growth and yield response under rising [CO_2].

4.2. Genetic basis of plant growth responses to elevated [CO_2] – identification of promising putative QTL

The progenies of the mapping population differed greatly in all growth traits related to final biomass accumulation. Presence of significant positive correlation coefficients between shoot and root biomass at ambient and elevated [CO_2] suggest that these traits are under genetic control and are susceptible to environmental influence (Supplementary Table S3 and S4). These findings were further confirmed by QTL mapping in which we found a large number of QTL for different growth traits of wheat in ambient and elevated [CO_2]. In this study, totally 68 QTL were determined for plant growth traits, of which 31 were detected at elevated [CO_2], 13 QTL at ambient [CO_2] and 24 QTL for CO_2 responsiveness (Figure 4). Although some QTL from the ambient and elevated [CO_2] treatment collocated on the genetic map, there were many QTL that were solely identified in the different growing conditions. A similar pattern of results was observed in a study by (Rae *et al.*, 2006) in which they identified QTL for enhanced leaf growth of hybrid poplar at elevated [CO_2]. Out of all QTL identified at elevated [CO_2], 13 QTL were related to biomass accumulation suggesting that elevated [CO_2] induced biomass gain is highly genetically controlled. This was further supported by summed percentage phenotypic variance. For example, the variance of QTL related to shoot biomass accumulation was 43% while QTL mapped for root biomass explained 22% of its phenotypic variation. The genetic and environmental interaction for these two traits, shoot and root biomass, was statistically significant and suggest that DH progenies act differently under the two CO_2 conditions. These

findings further indicate that some of the regulatory mechanisms only turn on under elevated [CO_2].

A number of QTL regions identified in this study are worthy of future research. Among the QTL mapped under elevated [CO_2], 11 QTL showed a positive additive effect from Kukri for shoot dry weight (1A: wPt-6568 – wmc0215a, 2A: wPt-2052 – wPt-1139, 6B: wPt-2768 – barc0117, 3D: barc0247 – barc0134, 6D: wPt-9887 – wPt-0745), total dry weight (6B: wPt-2768 – barc0117), root to shoot dry weight ratio (7D: barc0184 – wPt-0934), seedling height (2A: wPt-9809 – wPt-0944, 6A: wPt-6003 – barc0095, 3B:stm0092tctg – wPt-7001) and leaf elongation rate (4A: wPt-7306 – wPt-9277) (Figure 4). Of these, a significant QTL was observed for shoot weight on chromosome 6B (between wPt-2768 – barc0117), which explained 14.4% of the phenotypic variation of shoot biomass accumulation. A major QTL for total plant biomass was also identified in the same locus of chromosome 6B suggesting that there might be some candidate genes that located within this region, which may be largely responsible for biomass gain at elevated [CO_2].

Many traits tested in this study showed a differential control over the two CO_2 treatments, which was evident from the presence of CO_2-response QTL (Rae *et al.*, 2006). Carbon dioxide response effect was mapped using the percentage difference between plants grown at elevated and ambient [CO_2], considering the possibility of using this as a potential trait score that could be used in plant breeding to capitalize on the high CO_2 atmosphere. Out of the CO_2-response QTL detected, 10 showed a positive additive effect (Figure 4) from the parent Kukri, especially for shoot dry weight (2A: wPt-5801 – wPt-3753, 4B: barc0340a – gwm0495, 3D: barc0223 – barc0247), root dry weight (2A: wPt- 2052 – wPt-1139, 3D: barc0223 – barc0247), total dry (2A: wPt-5801 – wPt-3753, 1B: wPt-8093 – wPt-5769, 4B: barc0340a – gwm0495 weight) and seedling height (6A: wPt-6003 – wmc0111, 2D: wPt-2636 – wPt-7027). Interestingly, the CO_2-response QTL for shoot dry weight and total dry weight on chromosome 2A and 4B had collocated each other, which further confirms that this region might harbor important genes that control plant adaptation to elevated CO_2 levels. For example, chromosome 2AS contains phenology genes such as *Ppd-A1* (Laurie, 1997) that play a key role in photoperiod sensitivity which may have an influence and thereby regulate the vegetative growth of wheat (Kamran *et al.*, 2014). These phenology genes highly modulate by environmental stimulations and thus involve in improving plants' adaptability to a certain environment (Miralles & Richards, 2000, Royo *et al.*, 2018). In addition, CO_2-response QTL identified for LER on chromosome 6A and

for RGR on chromosome 3D, also showed a positive additive effect, however, these two QTL were not significant at 95% confidence level.

In a recent study by Mahjourimajd *et al.* (2016), the same DH mapping population has been employed to map the QTL related to grain yield in response to different nitrogen applications, some of which are overlapping with regions identified in our study. For example, some QTL identified on 2A, 6B, 7A and 7D for biomass accumulation and CO_2-response QTL in our study were closer to the QTL regions identified in their study. In addition, QTL for other traits such as seedling height, LER and leaf width identified on 1B, 3B and 6A were in line with their study's results (Mahjourimajd *et al.*, 2016). It is always desirable to compare and contrast the genomic regions that have been previously identified in a similar environment to determine the validity of these genomic regions before further detailed analysis. This was a major challenge for our study as only a very limited number of studies have been conducted to identify the genomic regions responsible for plant growth and development at elevated [CO_2], especially in commercially cultivated crops such as wheat.

Greater early vigor is considered as one of the important traits for improving wheat yield potential under different environmental conditions (Ludwig & Asseng, 2010). This trait is also known to influence biomass accumulation and nitrogen uptake throughout crop development (Reinke *et al.*, 2002, Liao *et al.*, 2004). In our study, we identified a large number of QTL for different plant growth traits at ambient and elevated [CO_2] conditions, mostly on dry matter accumulation at the early vegetative stage. Three CO_2-response QTL identified on chromosomes 2A, 1B and 4B for biomass accumulation can be considered as regions worthy for future research. These QTL are assumed to be involved in sensitivity to [CO_2] responses of biomass production and are likely to be associated with different post-photosynthetic processes. This natural variation provides important information about the adaptation of plants to elevated [CO_2] in the long term. Thus, this information provides new insights for future research on understanding the molecular mechanisms of plant growth responses to elevated [CO_2] and facilitates wheat breeding for the future changing climate. However, before utilizing these QTL into the marker-assisted breeding programs it is required to validate them to confirm their reproducibility. Then, further in-depth research is required to carry out the fine-mapping of the potential QTL to identify the candidate genes responsible for increased growth stimulation at elevated [CO_2]. However, before utilizing these QTL into the marker-assisted breeding programmes it is required to validate them to confirm their reproducibility. Further

in-depth research is required to carry out the fine-mapping of the potential QTL to identify the candidate genes responsible for increased growth at elevated [CO_2].

4. Conclusion

Limited information is available on the genetic basis of plant growth responses to elevated atmospheric [CO_2]. However, this information is essential in determining the ability of plants to adapt to the rapidly changing atmospheric [CO_2]. High biomass accumulation of plants at the early vegetative stage is an important trait that influences their subsequent establishment and productivity in the later stages of development. Therefore, improving this trait will be highly beneficial for plants to enhance CO_2 utilization and thereby stimulate the early vigorous growth of the plants. In this study, we developed an understanding of the physiological and genetic variability of plant growth responses to elevated [CO_2]. Further, we made an effort to identify major QTL that is associated with plant growth at elevated [CO_2] using a DH population of wheat. Here, we identified some unique QTL worthy of future research. Several CO_2-responsive QTL identified in this study were associated with growth traits, mainly biomass accumulation. These QTLs were collocated into the same location, suggesting that these regions might play a significant role in determining growth responses at elevated [CO_2]. However, these genomic regions should be further fine mapped to identify the possible genes responsible for growth at elevated [CO_2]. Also, it is noteworthy to mention that increased atmospheric [CO_2] interacts with higher global temperatures and altered rainfall patterns, which will continuously challenge plant communities. Therefore, understanding the interactions of these environmental conditions on plant growth is crucial in improving crop productivity under a future CO_2 rich atmosphere.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The doubled haploid mapping population was obtained from the Australian Centre for Plant Functional Genomics, University of Adelaide, Australia to carry out the research. This study was funded by the Strategic Research Fund of the University of Southern Queensland, Australia.

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Figure 1 (a) Rate of photosynthesis at anthesis; **(b)** Rate of photosynthesis at 4WAP **(c)** V_{cmax}: Maximum rate of Rubisco carboxylation at anthesis; **(d)** J_{max} :Maximum rate of electron transport at anthesis **(e)** *TPU*: Triosephosphate use limitation **(f)** Rate of transpiration at anthesis in Kukri and RAC875 grown at ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}), respectively. All measurements were carried out at the 4 replication level. Vertical error bars represent standard errors of mean values. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

Figure 2 Frequency distribution of early growth traits in the RAC875/Kukri DH population: **(a)** shoot dry weight (2WAP); **(c)** shoot dry weight (4WAP); **(e)** Seedling height (2WAP); **(g)** seedling height (4WAP); **(i)** relative growth rate; **(k)** leaf elongation rate; **(m)** leaf width at ambient [CO_2] (400 µmol mol^{-1}); and (**b)** shoot dry weight (2WAP); **(d)** shoot dry weight (4WAP); **(f)** Seedling height (2WAP); **(h)** seedling height (4WAP); **(j)** relative growth rate; **(l)** leaf elongation rate; **(n)** leaf width at elevated [CO_2] (700 µmol mol^{-1}) under glasshouse conditions. Open circle denotes the trait value of parent Kukri and closed circle denotes the trait value for parent RAC875.

Figure 3 Frequency distribution of **(a)** shoot dry weight (4WAP); **(c)** root dry weight (4WAP); **(e)** total dry weight (4WAP); **(g)** root to shoot ratio (4WAP) of DH population at ambient [CO_2] (400 µmolmol^{-1}) and **(b)** shoot dry weight (4WAP); **(d)** root dry weight (4WAP); **(f)** total dry weight (4WAP); **(h)** root to shoot ratio (4WAP) of DH population at elevated [CO_2] (700 µmolmol^{-1}) under controlled growth conditions. Open circle denotes the trait value of parent Kukri and closed circle denotes the trait value for parent RAC875.

Figure 4 Distribution of quantitative trait loci (QTL) for different early growth traits in response to ambient [CO_2] (400 µmol mol^{-1}), elevated [CO_2] (700 µmol mol^{-1}) and response QTL to elevated [CO_2] in wheat. Numbers on the bars represent traits**: 1**, Shoot Dry Weight (2WAP); **2**, Shoot Dry Weight (4WAP); **3**, Seedling Height (2WAP); **4**, Seedling Height (4WAP); **5**, Leaf Elongation Rate; **6**, Leaf Width; **7**, Relative Growth Rate; **8**, Shoot Dry Weight (4WAP, Controlled Environment); **9**, Root Dry Weight (4WAP, Controlled Environment), **10**, Total Dry Weight (4WAP, Controlled Environment), **11**, Root to Shoot Dry Weight Ratio (4WAP, Controlled Environment). Red and blue bars represent positive additive

effect and negative additive effect, respectively. Bars with continuous and dotted borders represents QTL at ambient [CO_2] and at elevated [CO_2], respectively. Bars with yellow colour borders represents response QTL to elevated [CO_2].

Supplementary Figure 1 Growth parameters of Kukri and RAC875 grown at ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}); (a) Shoot dry weight, (b) Root dry weight, (c) Total dry weight and (d) root to shoot ration at 4 weeks after planting (WAP) and (e) Total dry mass per plant, (f) 1000 grain weight per plant, (g) Number of tillers per plant and (f) Total grain yield per plant at maturity.

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Table 1. Growth parameters of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) and elevated [CO_2] (e[CO_2], 700 µmol mol^{-1}) at 4 weeks after planting (WAP) and at maturity. ns, not significant; *, P<0.05; **, P<0.01.

Table 2. Mean values of different physiological traits of parental lines and progenies of the DH population grown at ambient and elevated [CO_2] conditions. a[CO_2), ambient [CO_2]; e[CO_2], elevated [CO_2]; WAP, weeks after planting; Min, minimum value; Max, maximum value; *, P <0.05.

Table 3. QTL for growth traits of DH population detected in ambient [CO_2] conditions (400 µmol mol^{-1}). WAP, weeks after planting.

Table 4. QTL for growth traits of the DH population detected in elevated [CO_2] conditions (700 µmol mol^{-1}). WAP, weeks after planting.

Table 5. QTL for CO_2 responsiveness of growth traits of the DH population to elevated [CO_2] conditions (700 µmol mol^{-1}). WAP, weeks after planting.

Supplementary Table S1. Analysis of variance (ANOVA) results of gas exchange measurements of Kukri and RAC875 grown at ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}) at 4 weeks after planting (WAP) and at anthesis (DC65). a[CO_2), ambient [CO_2]; e[CO_2], elevated [CO_2]; G_s, rate of stomatal conductance; C_i, intracellular [CO_2]; V_{cmax}, Maximum carboxylation rate of Rubisco; J_{max}, Maximum rate of photosynthetic electron transport; *TPU*, rate of triose phosphate utilization; ns, not significant; *, P<0.05; **, P<0.01.

Supplementary Table S2. Analysis of variance (ANOVA) for different growth traits investigated in experiment 02 (glasshouse trial) and experiment 03 (growth chamber trial). WAP, weeks after planting; ns, not significant; *, P<0.05; **, P<0.01.

Supplementary Table S3. Controlled environmental conditions provided to the Reach-in plant growth chambers throughout the experimental period.

Supplementary Table S4. Correlation matrix of physiological traits of the DH population grown under different CO_2 concentrations in experiment 02 (glasshouse experiment). a[CO_2], ambient [CO_2); e[CO_2], elevated [CO_2]; WAP, weeks after planting; *, P<0.05; ** P<0.01.

Supplementary Table S5. Correlation matrix of physiological traits of the DH population grown under different CO_2 concentrations in experiment 03 (growth chamber experiment). a[CO_2]; ambient [CO_2]; e[CO_2], elevated [CO_2]; WAP, weeks after planting; *, P<0.05; **, P<0.01.

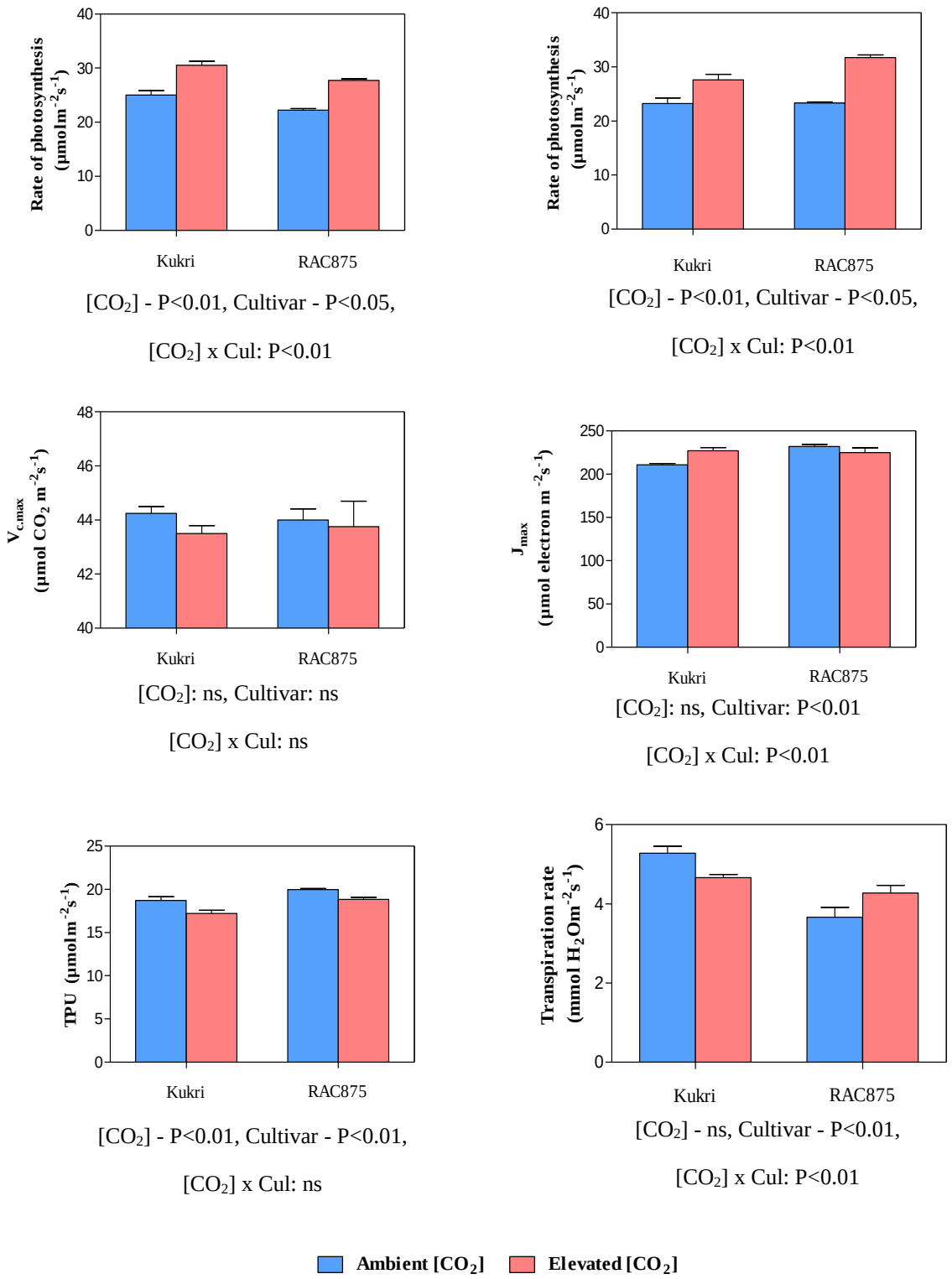
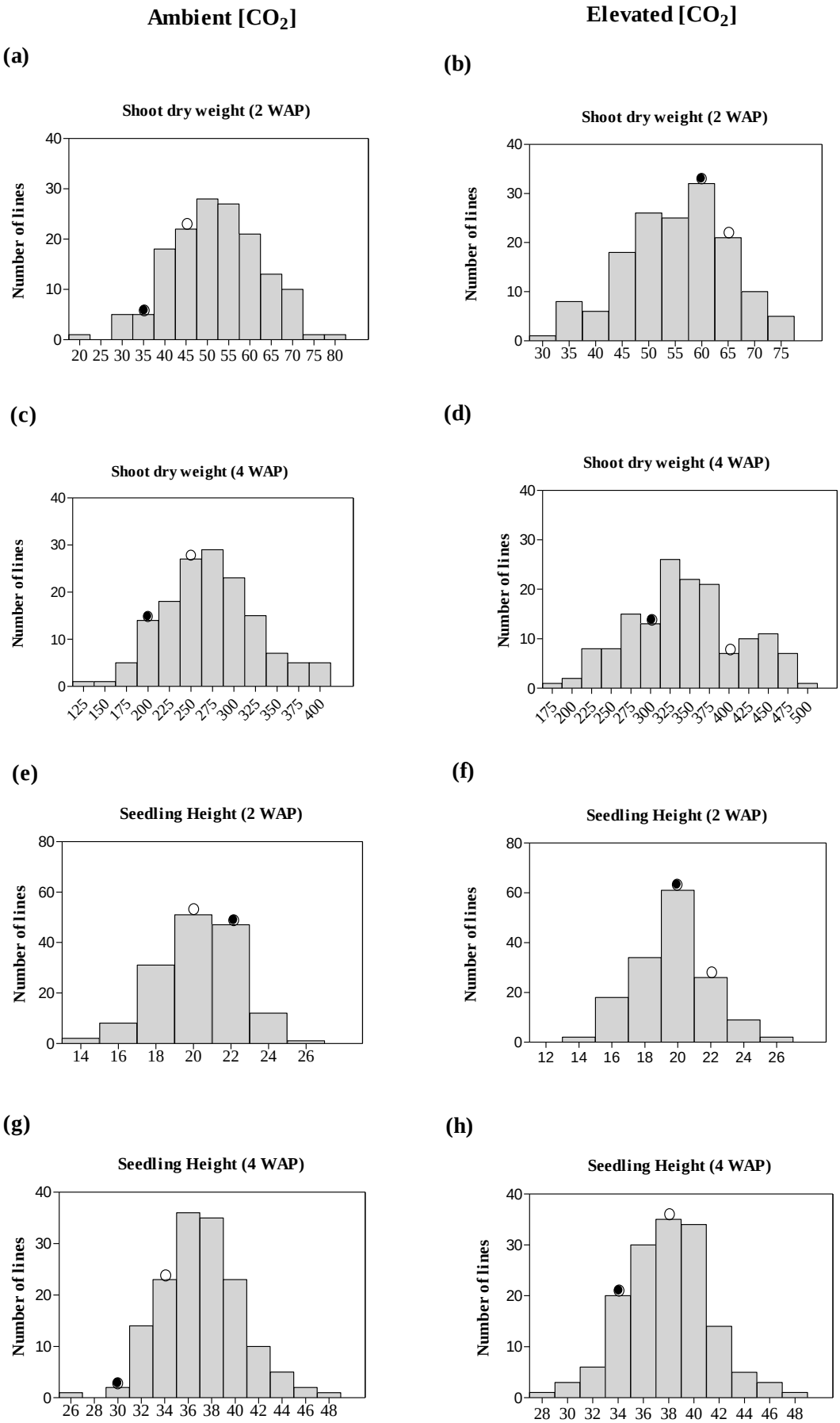

Rate of photosynthesis (µmol m-2s-1)
Kukri
RAC875
[CO2] - P<0.01, Cultivar - P<0.05,
[CO2] x Cul: P<0.01
Rate of photosynthesis (µmol m-2s-1)
Kukri
RAC875
[CO2] - P<0.01, Cultivar - P<0.05,
[CO2] x Cul: P<0.01
Vc.max (µmol CO2 m-2s-1)
Kukri
RAC875
[CO2]: ns, Cultivar: ns
[CO2] x Cul: ns
Jmax (µmol electron m-2s-1)
Kukri
RAC875
[CO2]: ns, Cultivar: P<0.01
[CO2] x Cul: P<0.01
TPU (µmol m-2s-1)
Kukri
RAC875
[CO2] - P<0.01, Cultivar - P<0.01,
[CO2] x Cul: ns
Transpiration rate (mmol H2O m-2s-1)
Kukri
RAC875
[CO2] - ns, Cultivar - P<0.01,
[CO2] x Cul: P<0.01
Ambient [CO2]
Elevated [CO2]

Figure 1


Ambient [CO2]
Elevated [CO2]
(a)
Shoot dry weight (2 WAP)
Number of lines
(b)
Shoot dry weight (2 WAP)
Number of lines
(c)
Shoot dry weight (4 WAP)
Number of lines
(d)
Shoot dry weight (4 WAP)
Number of lines
(e)
Seedling Height (2 WAP)
Number of lines
(f)
Seedling Height (2 WAP)
Number of lines
(g)
Seedling Height (4 WAP)
Number of lines
(h)
Seedling Height (4 WAP)
Number of lines

648

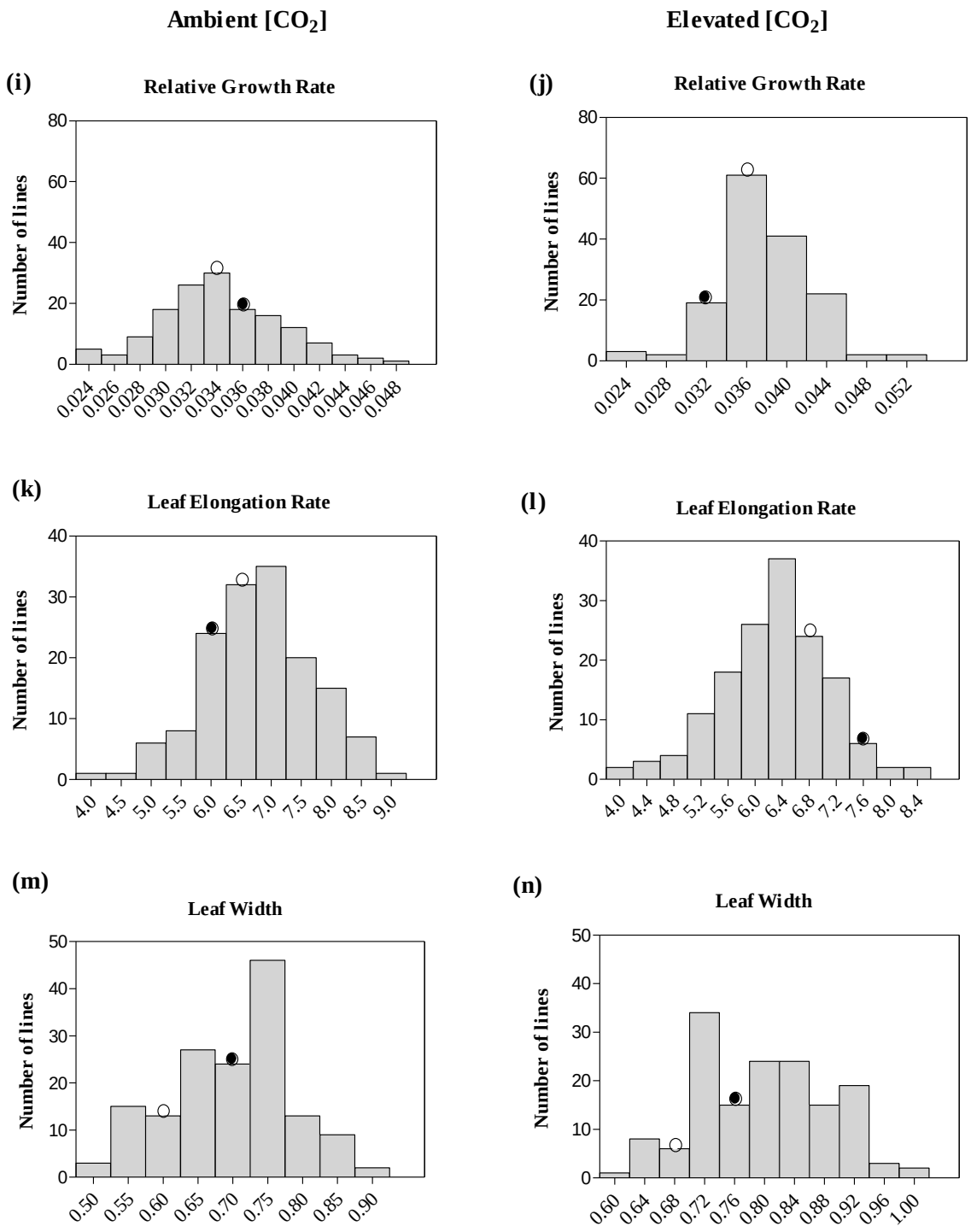
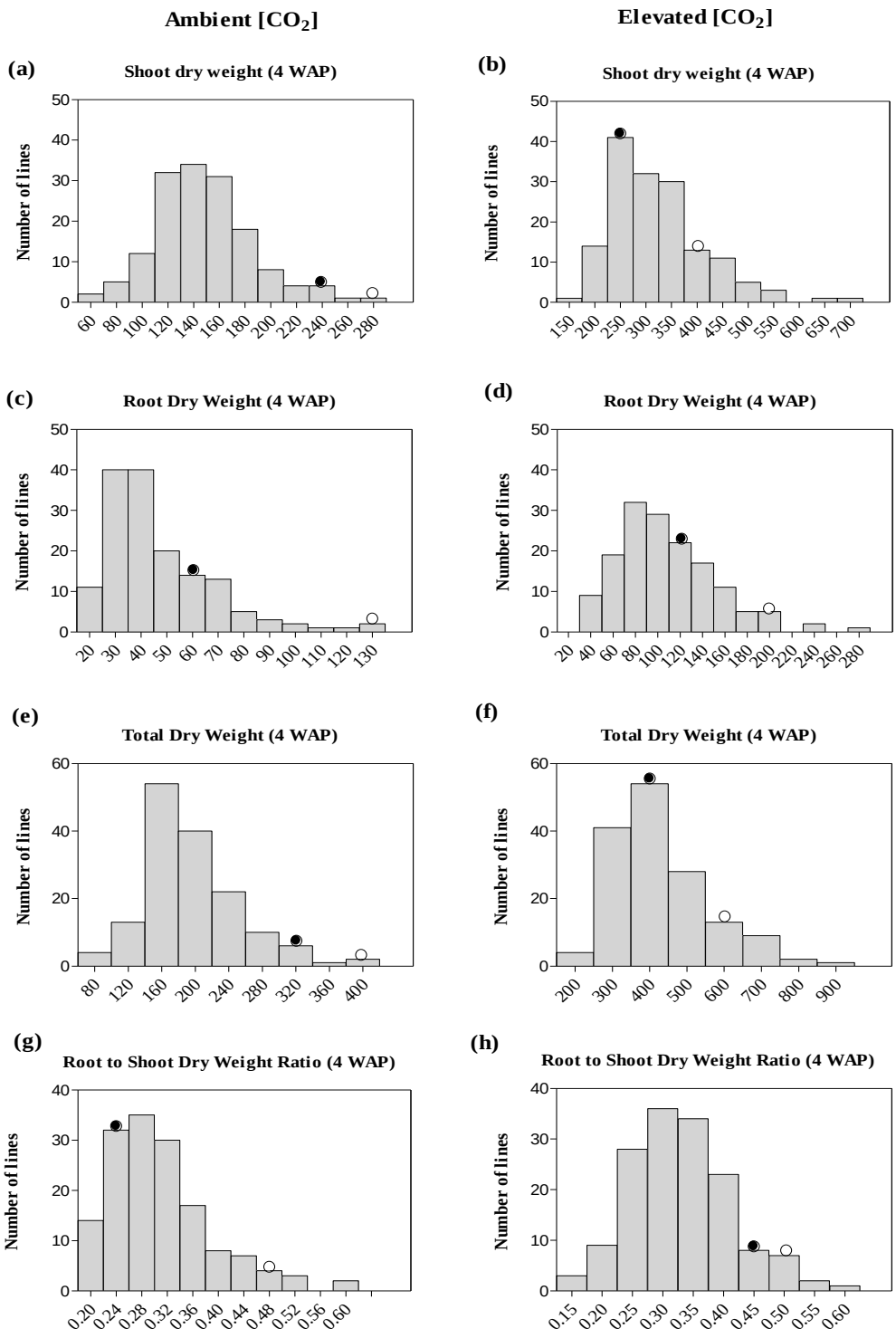
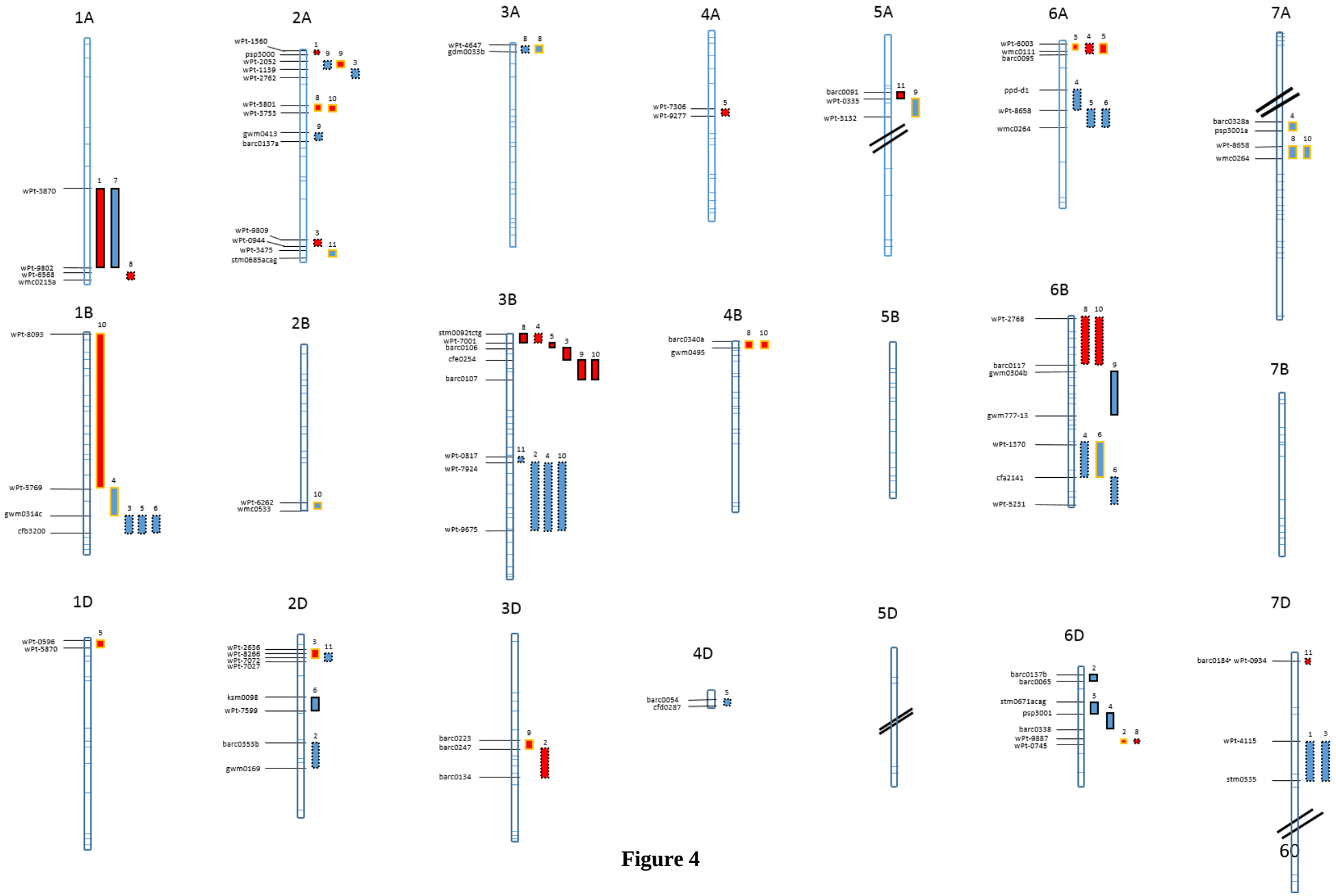

Ambient [CO2]
Elevated [CO2]
(i)
Relative Growth Rate
(j)
Relative Growth Rate
(k)
Leaf Elongation Rate
(l)
Leaf Elongation Rate
(m)
Leaf Width
(n)
Leaf Width
Number of lines

Figure 2


Ambient [CO2]
Elevated [CO2]
(a)
Shoot dry weight (4 WAP)
(b)
Shoot dry weight (4 WAP)
(c)
Root Dry Weight (4 WAP)
(d)
Root Dry Weight (4 WAP)
(e)
Total Dry Weight (4 WAP)
(f)
Total Dry Weight (4 WAP)
(g)
Root to Shoot Dry Weight Ratio (4 WAP)
(h)
Root to Shoot Dry Weight Ratio (4 WAP)
Number of lines

651 **Figure 3**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wPt-9802
wPt-6568
wmc0215a
2A
wPt-1560
psp3000
wPt-2052
wPt-1139
wPt-2762
wPt-5801
wPt-3753
gwm0413
barc0137a
wPt-9809
wPt-0944
wPt-3475
stm0685acag
3A
wPt-4647
gdm0033b
4A
wPt-7306
wPt-9277
5A
barc0091
wPt-0335
wPt-3132
6A
wPt-6003
wmc0111
barc0095
ppd-d1
wPt-8658
wmc0264
7A
barc0328a
psp3001a
wPt-8658
wmc0264
1B
wPt-8093
wPt-5769
gwm0314c
cfb3200
2B
wPt-6262
wmc0533
3B
stm0092tctg
wPt-7001
barc0106
cfe0254
barc0107
wPt-0817
wPt-7924
wPt-9675
4B
barc0340a
gwm0495
5B
6B
wPt-2768
barc0117
gwm0304b
gwm777-13
wPt-1370
cfa2141
wPt-5231
7B
1D
wPt-0596
wPt-5870
2D
wPt-2636
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wPt-7027
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wPt-7599
barc0353b
gwm0169
3D
barc0223
barc0247
barc0134
4D
barc0054
cfd0287
5D
6D
barc0137b
barc0065
stm0671acag
psp3001
barc0338
wPt-9887
wPt-0745
7D
barc0184* wPt-0934
wPt-4115
stm0535

Figure 4

Table 1. Growth parameters of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) and elevated [CO_2] (e[CO_2], 700 µmol mol^{-1}) at 4 weeks after planting (WAP) and at maturity. ns, not significant; *, P<0.05; **, P<0.01.

Trait	**Mean CO_2 effect**			**Mean cultivar effect**			**Interaction effect (ANOVA results)**
	a[CO_2]	**e[CO_2]**	**ANOVA result**	**Kukri**	**RAC875**	**ANOVA result**	**[CO_2]x Cultivar**
Growth-related traits at 4 WAP							
Shoot dry weight (mg/plant)	258.2	339.4	**	336	261.6	**	**
Root dry weight (mg/plant)	99.2	156.7	**	162.3	93.6	**	ns
Total dry weight (mg/plant)	357.4	496.1	**	498.3	355.2	**	**
Root to shoot ratio	0.38	0.46	**	0.484	0.352	**	**
Growth-related traits at maturity							
Total dry mass (g/plant)	11.7	14.9	**	13.3	13.3	ns	ns
Thousand grain weight (g/plant)	35.2	37.6	ns	37.6	35.1	ns	ns
Tiller number	7	9	**	8	8	ns	ns
Total grain yield (g/plant)	8.1	14	**	10.6	11.6	ns	ns

Table 2. Mean values of different physiological traits of parental lines and progenies of the DH population grown at ambient and elevated [CO_2] conditions. a[CO_2), ambient [CO_2]; e[CO_2], elevated [CO_2]; WAP, weeks after planting; Min, minimum value; Max, maximum value; *, P <0.05.

Traits	CO_2 level	Parents		DH population				
		Kukri	RAC875	Min	Max	Mean	Mean Squares	Broad-sense heritability
Experiment 02 – Glasshouse experiment								
Shoot dry weight (2WAP) (mg)	a[CO_2]	47.2	36.5	20.7	78.1	51.7 ± 0.9	784.7*	
	e[CO_2]	65.7	61.6	31	76.9	55.3 ± 0.8		0.79
Shoot dry weight (4WAP) (mg)	a[CO_2]	263.7	222.3	122.6	414	272.2 ± 4.5	380467.6*	
	e[CO_2]	413.5	294	166.3	495	345.4 ± 5.6		0.98
Seedling height (2WAP) (cm)	a[CO_2]	20.1	20.8	14.3	26.6	20.3 ± 0.2	31.1*	
	e[CO_2]	22.6	22.6	13.8	25.6	19.6 ± 0.2		0.77
Seedling height (4WAP) (cm)	a[CO_2]	35	30.4	26.5	47.6	37.1 ± 0.3	36.7*	
	e[CO_2]	37.6	34.9	28.8	48.1	37.9 ± 0.3		0.61
Relative growth rate (mg/day)	a[CO_2]	0.037	0.034	0.01	0.05	0.03 ± 0.0004	0.001*	
	e[CO_2]	0.038	0.032	0.02	0.05	0.04 ± 0.0004		0.96
Leaf elongation rate (mm/h)	a[CO_2]	6.3	6.1	4.1	8.4	6.3 ± 0.1	15.6*	
	e[CO_2]	6.8	7.6	1.4	8.8	6.7 ± 0.1		0.89
Leaf width (mm)	a[CO_2]	6.7	7.3	5	9	0.7 ± 0.01	0.7*	
	e[CO_2]	6.7	8	6	10	0.80 ± 0.01		0.98
Experiment 03 – Growth chamber experiment								
Shoot dry weight (4WAP) (mg)	a[CO_2]	271.6	247.2	61.6	251.2	146.1 ± 2.9	2293036*	0.99

	e[CO_2]	403.2	277.8	165.9	681.6	321 ± 7.5		
Root dry weight (4WAP) (mg)	a[CO_2]	133.6	63.6	16.5	131	46.3 ± 1.7	288182.2*	
	e[CO_2]	192.9	121.6	32	273.1	107.9 ± 3.6		0.99
Total dry weight (4WAP) (mg)	a[CO_2]	405.2	310.9	80.1	382.1	192.3 ± 4.4	4207025.2*	
	e[CO_2]	596	399.3	235.7	937.1	428.9 ± 10.5		0.99
Root to shoot ratio (4WAP)	a[CO_2]	0.49	0.26	0.15	0.85	0.33±0.008	0.048*	
	e[CO_2]	0.48	0.44	0.19	0.61	0.31±0.007		0.02

Table 3. QTL for growth traits of DH population detected in ambient [CO_2] conditions (400 µmol mol^{-1}). WAP, weeks after planting.

Trait	Chromosome	Position	Peak LOD	Explained variance (%)	Additive effect	Nearest marker interval
Experiment 02 - Glasshouse experiment						
Shoot dry weight (2WAP)	1A	174	2.407	7	8.06	wPt-3870 – wPt-9802
Shoot dry weight (4WAP)	6D	0	1.311	3.9	-11.34	barc0137b-barc0065
Seedling height (2WAP)	6D	22	3.087	8.9	-0.655	Stm0671acaq-psp3001
Seedling height (4WAP)	3B	20	3.404	9.8	1.15	Barc0106-cfe0254
	6D	26	2.949	8.5	-1.109	Psp3001-barc0338
Leaf elongation rate	3B	14	4.113	11.7	0.724	wPt-7001 – barc0106
Leaf width	2D	60	2.933	8.5	-0.028	Ksm0098 - wPt-7599
	6B	58	2.511	7.3	-0.024	Gwm0304b – wmm777-13
Relative growth rate	1A	166	3.673	10.5	-0.006	wPt-3870 – wPt-9802
Experiment 03 – Growth chamber experiment						
Shoot dry weight (4WAP)	3B	6	2.352	6.9	0.011	Stm0092tctg – wPt-7001
Root dry weight (4WAP)	3B	30	2.119	6.2	0.006	Cfe0254 – barc0170
Total dry weight (4WAP)	3B	30	2.479	7.2	0.016	Cfe0254 – barc0170

Root: Shoot dry weight ratio (4WAP)	5A	56	2.191	6.4	0.023	Barc0091 – wPt-0335

Table 4. QTL for growth traits of the DH population detected in elevated [CO_2] conditions (700 µmol mol^{-1}). WAP, weeks after planting.

Trait	Chromosome	Position	Peak LOD	Explained variance (%)	Additive effect	Nearest marker interval
Experiment 02 - Glasshouse experiment						
Shoot dry weight (2WAP)	3B	180	3.444	9.9	-3.238	wPt-7924 – wPt-9675
	2A	16	2.958	8.6	-2.884	wPt-2052 – wPt-1139
	7D	90	2.616	7.6	4.017	wPt-4115 – stm0535
Shoot dry weight (4WAP)	3D	108	2.622	7.6	21.269	Barc0247 – barc0134
Seedling height (2WAP)	3B	176	3.605	10.3	-0.896	wPt-7924 – wPt-9675
	6B	142	3.65	10.5	-0.739	wPt-1370 – cfa2141
	2A	168	2.545	7.4	0.618	wPt-9809 – wPt-0944
	7D	100	3.107	9	0.573	wPt-4115 – stm0535
	1B	170	2.588	7.5	-0.543	Gwm0314c – cfb3200
	2A	18	2.475	7.2	-0.531	wPt-1139 – wPt-2762
Seedling height (4WAP)	3B	8	4.512	12.8	1.138	Stm0092tctg – wPt-7001
	6A	2	2.853	8.3	1.026	wPt-6003 – barc0095
	6A	40	5.869	16.3	-1.937	Ppd-d1 – wPt-0330
	4A	88	2.582	7.5	0.201	wPt-7306 – wPt-9277

Leaf elongation rate	6A	62	3.18	9.2	-0.247	wPt-0330 – barc0328b
	1B	168	2.529	7.4	-0.209	Gwm0314c – cfb3200
Leaf width	6B	146	6.418	17.7	-0.033	Cfa2141 – wPt-5231
	2D	92	7.756	20.9	-0.042	Wmc0256a – barc0353b
	6A	78	4.974	14	-0.031	wPt-0330 – barc0328b
	1B	168	3.9	11.1	-0.026	Gwm0314c – cfb3200
Relative growth rate	4D	62	1.942	3.7	-0.001	Barc0054 – cfd0287
Experiment 03 – Growth chamber experiment						
Shoot dry weight (4WAP)	1A	212	3.322	9.6	0.029	wPt-6568 – wmc0215a
	6B	40	5.129	14.4	0.044	wPt-2768 – barc0117
	6D	52	3.23	9.3	0.025	wPt-9887 – wPt-0745
	3A	4	3.229	9.3	-0.024	wPt-4647 – gdm0033b
Root dry weight (4WAP)	2A	0	3.232	9.3	0.013	wPt-1560 – psp3000
	2A	76	4.469	12.7	-0.035	Gwm0413 – barc0137a
Total dry weight (4WAP)	3B	142	2.611	7.6	-0.106	wPt-7924 – wPt-9675
	6B	32	4.21	12	0.077	wPt - 2768 – barc0117
Root: Shoot dry weight ratio (4WAP)	3B	116	2.636	7.7	-0.024	wPt - 0817 – wPt - 7924
	2D	26	4.36	12.4	-0.057	wPt - 8266 – wPt - 6904
	7D	24	2.915	8.5	0.048	Barc0184 – wPt - 0934

Table 5. QTL for CO_2 responsiveness of growth traits of the DH population to elevated [CO_2] conditions (700 µmol mol^{-1}). WAP, weeks after planting.

Trait	Chromosome	Position	Peak LOD	Explained variance (%)	Additive effect	Nearest marker interval
Experiment 02 - Glasshouse experiment						
Shoot dry weight (2WAP)	6D	52	1.811	5.3	7	wPt-9887 – wPt-0745
Shoot dry weight (4WAP)	2D	96	2.585	7.5	-9.536	Barc0353b – gwm0169
	3D	98	3.508	10.1	13.613	Barc0223 – barc0247
Seedling height (2WAP)	2D	22	3.404	9.8	3.587	wPt-2636 – wPt-7027
	6A	0	2.515	7.3	3.408	wPt-6003 – wmc0111
	7A	72	2.494	7.3	-2.861	Barc0328a – psp3001a
Seedling height (4WAP)	1B	140	2.115	6.2	-2.917	wPt-5769 – gwm0314c
Leaf elongation rate	6A	2	1.632	4.8	10.516	wPt-6003 – barc0095
Leaf width	6B	144	2.018	5.9	-4.749	wPt-1370 – cfa2141
Relative growth rate	1D	4	1.888	5.6	5.563	wPt-0596 – wPt-5870
Experiment 02 - Growth chamber experiment						
	4B	0	2.506	7.3	16.69	Barc0340a – gwm0495

	2A	36	4.409	12.5	22.112	wPt-5801 – wPt-3753
Shoot dry weight (4WAP)	3A	0	2.941	8.5	-18.782	wPt-4647 – gdm0033b
	7A	98	4.761	13.4	-25.25	wPt-8658 – wmc0264
Root dry weight (4WAP)	5A	60	2.62	7.6	-28.261	wPt-0335 – wPt-3132
	2A	8	2.34	6.8	28.42	wPt-2052 – wPt-1139
	3D	104	2.442	7.1	27.287	Barc0223 – barc0247
Total dry weight (4WAP)	4B	0	2.511	7.3	17.968	Barc0340a – gwm0495
	2A	36	4.372	12.4	23.778	wPt-5801 – wPt-3753
	7A	98	4.957	13.9	-27.538	wPt-8658 – wmc0264
	1B	44	2.538	7.4	17.757	wPt-8093 – wPt-5769
	2B	184	3.042	8.8	-24.901	wPt-6262 – wmc0533
Root: Shoot dry weight ratio (4WAP)	2A	198	2.534	7.4	-9.068	wPt-3475 – stm0658acag

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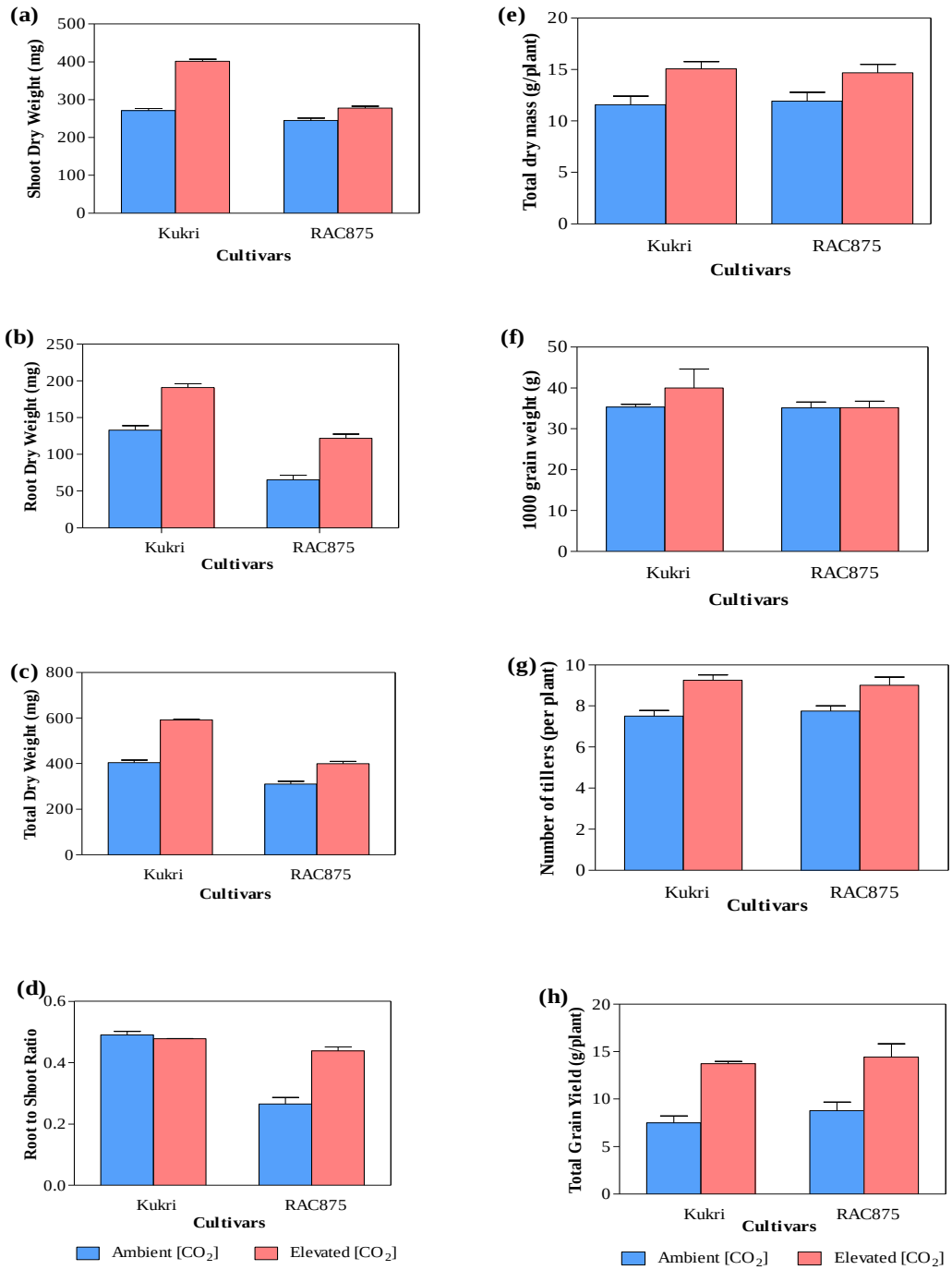

Supplementary Figure 1 Growth parameters of Kukri and RAC875 grown at ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}); (a) Shoot dry weight, (b) Root dry weight, (c) Total dry weight and (d) root to shoot ration at 4 weeks after planting (WAP) and (e) Total dry mass per plant, (f) 1000 grain weight per plant, (g) Number of tillers per plant and (f) Total grain yield per plant at maturity

Supplementary Table S1. Analysis of variance (ANOVA) results of gas exchange measurements of Kukri and RAC875 grown at ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}) at 4 weeks after planting (WAP) and at anthesis (DC65). a[CO_2), ambient [CO_2]; e[CO_2], elevated [CO_2]; G_s, rate of stomatal conductance; C_i, intracellular [CO_2]; V_{cmax}, Maximum carboxylation rate of Rubisco; J_{max}, Maximum rate of photosynthetic electron transport; *TPU*, rate of triose phosphate utilization; ns, not significant; *, P<0.05; **, P<0.01.

Trait	**Mean CO_2 effect**			**Mean cultivar effect**			**Interaction effect (ANOVA results)**
	a[CO_2]	**e[CO_2]**	**ANOVA result**	**Kukri**	**RAC875**	**ANOVA result**	**[CO_2]x Cultivar**
			Photosynthesis parameters at 4 WAP				
Photosynthesis rate (µmol CO_2 m^{-2} s^{-1})	23.6	29.1	**	27.8	24.9	**	ns
Gs (mol H_2O m^{-2} s^{-1})	0.35	0.311	ns	0.364	0.306	ns	ns
Ci (µmol CO_2 mol^{-1})	277.4	524.9	**	404.9	397.4	ns	ns
Transpiration	4.1	3.5	ns	3.9	3.7	*	ns
			Photosynthesis parameters at anthesis (DC65)				
Photosynthesis rate (µmol CO_2 m^{-2} s^{-1})	23.3	29.7	**	25.4	27.5	*	**
V_{cmax} (mol H_2O m^{-2} s^{-1})	44.1	43.6	ns	43.5	43.8	ns	ns
J_{max} (µmol CO_2 mol^{-1})	221.3	225.9	ns	218.3	228.3	**	**
TPU (µmol CO_2 m^{-2} s^{-1})	19.4	18.0	**	17.9	19.4	**	ns

Gs (mol H_2O m^{-2} s^{-1})	0.52	0.34	**	0.30	0.52	**	*
Ci (µmol CO_2 mol^{-1})	266.6	536.8	**	416.4	397.4	**	ns
Transpiration	4.47	4.46	ns	3.9	4.9	**	**

Supplementary Table S2. Analysis of variance (ANOVA) for different growth traits investigated in experiment 02 (glasshouse trial) and experiment 03 (growth chamber trial). WAP, weeks after planting; ns, not significant; *, P<0.05; **, P<0.01.

Variance component	**Experiment 02 – glasshouse trial**							**Experiment 03 – growth chamber trial**			
	Shoot dry weight (2WAP)	Shoot dry weight (4WAP)	Seedling height (2WAP)	Seedling height (4WAP)	Relative growth rate	Leaf elongation rate	Leaf width	Shoot dry weight (4WAP)	Root dry weight (4WAP)	Total dry weight (4WAP)	Root to shoot ratio (4WAP)
Genotype	**	**	**	**	*	**	**	**	**	**	**
[CO_2]	**	**	**	*	**	**	**	**	**	**	**
Genotype x [CO_2] interaction	**	ns	ns	ns	ns	**	**	**	**	**	**

Supplementary Table S3. Controlled environmental conditions provided to the Reach-in plant growth chambers throughout the experimental period.

Time Period	Temperature	Relative humidity	$[CO_2]$ concentration		Light intensity
			Ambient $[CO_2]$	Elevated $[CO_2]$	
06:00 hrs	15°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
08:00 hrs	20°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
11:00 hrs	23°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
15:00 hrs	20°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
18:00 hrs	15°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
20:00 hrs	13°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)

Supplementary Table S4. Correlation matrix of physiological traits of the DH population grown under different CO_2 concentrations in experiment 02 (glasshouse experiment). a[CO_2], ambient [CO_2); e[CO_2], elevated [CO_2]; WAP, weeks after planting; *, P<0.05; ** P<0.01.

Traits	**CO_2 level**	Shoot dry weight (2WAP)	Shoot dry weight (4WAP)	Seedling height (2WAP)	Seedling height (4WAP)	Relative growth rate	Leaf elongation rate	Leaf width
Shoot dry weight (2WAP)	e[CO_2]	1	0.430**	0.515**	0.147	-0.455**	0.368**	.334**
	a[CO_2]	1	0.337**	0.479**	0.088	-0.579**	0.295**	0.255**
Shoot dry weight (4WAP)	e[CO_2]		1	0.409**	0.151	0.597**	0.405**	0.36**
	a[CO_2]		1	.303**	0.174**	0.550**	0.272**	0.247**
Seedling height (2WAP)	e[CO_2]			1	0.338**	-0.051	0.701**	0.392
	a[CO_2]			1	0.207*	-0.165*	0.741**	0.412**
Seedling height (4WAP)	e[CO_2]				1	0.028	0.354**	0.090
	a[CO_2]				1	0.088	0.231**	0.356**
Relative growth rate	e[CO_2]					1	0.082	0.061
	a[CO_2]					1	-0.051	-0.005
Leaf elongation rate	e[CO_2]						1	0.277**
	a[CO_2]						1	0.375**
Leaf width	e[CO_2]							1
	a[CO_2]							1

Supplementary Table S5. Correlation matrix of physiological traits of the DH population grown under different CO_2 concentrations in experiment 03 (growth chamber experiment). a[CO_2]; ambient [CO_2]; e[CO_2], elevated [CO_2]; WAP, weeks after planting; *, P<0.05; **, P<0.01.

Traits	**CO_2 level**	Shoot dry weight (4WAP)	Root dry weight (4WAP)	Total dry weight (4WAP)	Root to shoot ratio (4WAP)
Shoot dry weight (4WAP)	e[CO_2]	1	.789**	.978**	.115
	a[CO_2]	1	.803**	.973**	.400**
Root dry weight (4 WAP)	e[CO_2]		1	.899**	.665**
	a[CO_2]		1	.919**	.851**
Total dry weight (4WAP)	e[CO_2]			1	.307**
	a[CO_2]			1	.594**
Root to shoot ratio (4WAP)	e[CO_2]				1
	a[CO_2]				1

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Chapter 4

Elevated carbon dioxide mediated early growth responses of wheat (*Triticum aestivum* L.): an analysis of source and sink interactions

This study was conducted to understand the role of key photosynthetic and post-photosynthetic metabolic processes in determining plant growth responses at elevated [CO_2]. A multidisciplinary approach was used to characterize photosynthesis, carbon metabolism, nitrogen metabolism and cell cycle functions using physiological and molecular tools. Experiments were conducted using selected wheat lines from the doubled haploid population based on their CO_2 responsiveness. Physiological characterization was carried out at the whole plant level whereas biochemical and molecular analyses were carried out at different organ levels. The last fully expanded leaf, expanding leaf, cell elongation region and shoot apex region were used in gene expression and sugar analyses to understand the source and sink interactions at elevated [CO_2]. This chapter has been prepared as a research article to be submitted to "Plant, Cell and Environment".

Gamage D, Thompson M, Dehigaspitiya P, Fukushima A, Sutherland M.W., Hirotsu N, Seneweera S, 'Elevated carbon dioxide mediated early growth responses of wheat (*Triticum aestivum* L.): an analysis of source and sink interactions' (Prepared for publication)

Article title:

Elevated carbon dioxide mediated early growth responses of wheat (*Triticum aestivum* L.): an analysis of source and sink interactions

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Abbreviations:

[CO_2] – Carbon dioxide concentration; Rubisco - Ribulose 1,5-bisphosphate carboxylase/oxygenase; LFELB - Last fully expanded leaf blade; ELB - Expanding leaf blade; CER – cell elongation region; LCEZ – leaf cell elongation zone; SAR – shoot apex region; *rbcL* - Ribulose 1,5 –bisphosphate carboxylase/oxygenase (large sub unit); *rbcS* - Ribulose 1,5 –bisphosphate carboxylase/oxygenase (small sub unit); *SPP1* - Sucrose Phosphate Phosphatase 1; *SPS1* - Sucrose Phosphate Synthase 1; *SUS1* - Sucrose Synthase type 1; *NiR* - Ferredoxin

[1] DG – Study design, acquisition, analysis and interpretation of data, drafting of manuscript, MT, PD, AF- Acquisition of data and revision of the manuscript, MWS – critical revision, NH analysis and interpretation of data and critical revision, SS- Study conception and design, analysis and interpretation of data, critical revision

Nitrite Reductase; *Fd-GOGAT* - Putative ferredoxin-dependent glutamate synthase; *GS2a* - Glutamine Synthetase (Plastidial) – GS2; *GS2b* - Glutamine Synthetase (Plastidial) – GS2; *NADH-GOGAT* - Putative NADH-dependent glutamate synthase; *GS1a* - Glutamine synthetase (cytosolic) – GS1; *GSr1* - Glutamine synthetase (cytosolic) – GS1; *TaEXPA1* - α – expansin A1; *TaEXPA3* - α – expansin A3; *TaEXPB1* - β – expansin B1; *TaEXPB2* - β – expansin B2; *TaEXPB3* - β – expansin B3; *TaEXPB6* - β – expansin B6; *TaEXPB23* - β – expansin B23; *TaXTH1* - Xyloglucan endotransglucosylase/hydrolase 1; *TaXTH2* - Xyloglucan endotransglucosylase/hydrolase 2; *TaXTH3* - Xyloglucan endotransglucosylase/hydrolase 3; *TaXTH4* - Xyloglucan endotransglucosylase/hydrolase 4; *TaXTH5* - Xyloglucan endotransglucosylase/hydrolase 5.

Summary statement

Soluble sugar accumulation at elevated [CO_2] varies between cultivar, CO_2 levels and the organ type. In response to the availability of different sugars, expression of key genes related to photosynthesis, carbon and nitrogen metabolism and cell wall metabolism have been upregulated.

Abstract

The physiological and molecular mechanisms of plant growth responses at elevated [CO_2] were investigated through developing an understanding of the source and sink interaction using wheat. Wheat plants were grown under ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (700 µmol mol^{-1}) for 6 weeks. Above ground and below ground biomass accumulation, total leaf area and rate of photosynthesis were measured at 42 days after planting. Total soluble carbohydrate concentration in different plant organs (last fully expanded leaf blade, expanding leaf blade and cell elongation region) was measured. Expression analysis of genes involved in photosynthesis (*rbcL*, *rbcS*), carbon metabolism (*SPP1*, *SPS1* and *SUS1*), nitrogen metabolism (*Fd-GOGAT*, *NADH-GOGAT*, *GS2a*, *GS2b*, *GSr1*, *GS1a*), cell wall metabolism (α and β – expansins, Xyloglucan endotransglucosylase/hydrolases) was performed using real-time quantitative-PCR. There were significant interactions between [CO_2] and cultivar for above ($P<0.01$) and below ground biomass ($P<0.01$), total leaf area ($P<0.01$), total soluble carbohydrate content ($P<0.01$) and leaf photosynthesis ($P<0.05$). Transcript abundance of key genes showed a marked difference at elevated [CO_2] showing a significant [CO_2] effect across all organ types and the cultivars ($P<0.05$). Genes involved in cell wall metabolism and sucrose synthesis were highly expressed in growing sink tissues. Significant correlations exist between sugar concentration and *SPP1* and some of the Xyloglucan endotransglucosylase/hydrolases encoding genes (*TaXTH3*, *TaXTH5*), implying that these metabolic activities may play a leading role in determining the growth response to elevated [CO_2].

Keywords: Carbon and nitrogen metabolism, Cell wall metabolism, Elevated [CO_2], Organ-specific, Post-photosynthetic processes, Transcript abundance

1. Introduction

By 2100, the carbon dioxide concentration ([CO_2]) in the atmosphere is predicted to reach ~730 to ~1020 µmol mol^{-1}, which is approximately double the [CO_2] of the atmosphere today (IPCC 2014). According to Tans and Keeling (2018), the current [CO_2] has already reached up to 400 µmolmol^{-1} and has significantly impacted precipitation and temperature patterns. Despite these negative effects of elevated [CO_2], it also has a positive impact on plant growth and development through improvements in photosynthesis capacities of crops, especially of C_3 origin (Ainsworth et al. 2008; Gamage et al. 2018). Overarching research studies have shown that stimulation of photosynthesis and growth of C_3 plants at elevated [CO_2] is primarily due to the CO_2 to O_2 ratio at the site of CO_2 fixation. The current atmospheric [CO_2] is insufficient to saturate primary carboxylation, the rate-limiting step of photosynthesis by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and thus, the majority of C_3 plants are not photosynthetically saturated at current [CO_2] (Bowes 1993; Aranjuelo et al. 2015; Bloom 2015). Therefore, high [CO_2] at the site of fixation leads to increased carboxylation efficiency of Rubisco and thereby increases the photosynthesis capacity of the plant. This process, substantially reduces a competing O_2 fixing reaction commonly known as photorespiration and indirectly facilitates the primary CO_2 fixation at elevated [CO_2] (Bloom 2015). However, the initial stimulation of photosynthesis at elevated [CO_2] gradually decreases and settles in the latter part of the plant development. This process is known as photosynthetic acclimation and depend on species, nutritional status and other environmental conditions (Gutiérrez et al. 2009; Alonso, Pérez, and Martínez-Carrasco 2009). Consistent with this proposition, it has been demonstrated that the highest plant growth response occurs at early growth stages, which will eventually lead to structural changes, greater biomass accumulation and yield of crops (Makino et al. 1997; Seneweera et al. 2002; Thilakarathne et al. 2015; Kimball 2016; Jitla et al. 1997). Therefore, understanding the underlying physiological and molecular mechanism of plant growth responses to elevated [CO_2] at early growth stages is highly beneficial for incorporating CO_2 responsive traits into current breeding programs in order to improve crop productivity. (Gamage et al. 2018).

Photosynthesis and its subsequent events associated with plant growth and morphogenesis, are closely linked with the carbohydrate metabolism of the plant (Masle 2000; Paul and Pellny 2003). Photosynthesis is a two-way process, including carbohydrate synthesis and utilization; however, these processes are subjected to a feedback regulation process, which highly controls

the source and sink interaction (Paul and Foyer 2001; Paul and Pellny 2003; Körner 2015). It has been identified that one of the key factors that determine plant growth is the conversion of Triose Phosphate (the first stable photosynthesis intermediate) to other types of sugars. In addition, efficient loading/unloading of these sugars into phloem and transport into growing sink tissues also play a role in determining the growth response to elevated [CO_2] (Masle 2000; Yang et al. 2016). High carbohydrate availability at elevated [CO_2] will therefore be used by the plants to develop additional sinks through tiller and secondary shoot production which will significantly influence plant growth habits and thereby the final yield (Jitla et al. 1997; Makino and Mae 1999; Seneweera et al. 2002). This growth stimulation is then involved with meristem initiation, cell division, expansion and differentiation and integrates these processes to develop new sink tissues in order to utilize Triose Phosphate in an efficient manner (Masle 2000; Gamage et al. 2018).

The increased carbon supply at elevated [CO_2] not only influences the source-sink integration of plants, but also has a profound effect on the plant transcriptome, which determines the mechanism for adjusting to a particular environmental change (Vicente et al. 2015; Ainsworth et al. 2006). The transcript level changes in key genes associated with different metabolic pathways have been previously reported in wheat grown at elevated [CO_2]. It has been well established that a number of genes, including carbon and nitrogen metabolism-related genes, function partly through a sugar sensing mechanism. These genes differentially expressed between the organs and highly cross-regulate with resource allocation and utilization among plant tissues (Koch 1996; Koch 2004). For example, plants grown at elevated [CO_2] showed no repression of photosynthetic genes and photosynthetic capacity with enhanced carbon supply, provided that nitrogen and sink capacity of the plants are not limited (Geiger et al. 1999; Martin, Oswald, and Graham 2002; Ainsworth et al. 2004). This efficient carbohydrate generation and utilization can be observed especially in the early stages of plant growth at elevated [CO_2] when the sink strength of the plant is generally high (Ainsworth and Bush 2011).

At elevated [CO_2], plants produce excess sugars, which includes different metabolically active sugars such as sucrose, glucose and fructose. Sugar concentrations in different plant tissues vary in a significant manner, facilitating a broader range of signals which may ultimately contribute to adaptive changes to different environmental conditions (Koch 1996; Gibson 2005; Smeekens et al. 2010). For example, photosynthesis genes in carbon exporting cells or

in source tissues are typically upregulated, as the generated sugars are constantly translocated to sink tissues in which genes related to sucrose formation and amino acids synthesis are upregulated (Paul and Pellny 2003; Stitt, Lunn, and Usadel 2010). This suggests that the roles performed by each gene and their related proteins can be tissue/organ-specific and their roles may be cross-linked with the sugar availability of a particular tissue or an organ. Despite the importance of this phenomena, tissue/organ-specific gene expression and its role in determining plant growth and development at elevated [CO_2] have not been studied in detail (Gamage et al. 2018; Vicente et al. 2015). The high sugar supply will result in changes in metabolism, enzyme activity and development activity, but this information has not been evaluated in an extensive manner with relation to CO_2 enriched conditions (Gamage et al. 2018; Koch 1996; Aranjuelo et al. 2015; Thompson et al. 2017). Therefore, the role of sugar-responsive genes in specific tissues, crosstalk with carbon and nitrogen metabolism, together with plant growth responses, deserves further exploration.

In this study, we investigated various aspects related to the underlying physiological and molecular mechanisms of plant growth responses at elevated [CO_2]. Here we tested the following hypotheses: (i) plant early growth response to elevated [CO_2] varies between cultivars; (ii) plant growth responses are primarily mediated through a variation in net photosynthesis capacity; (iii) response to elevated [CO_2] is partially controlled by post-photosynthetic processes, such as carbon and nitrogen metabolism, and cell cycle functions, such as cell elongation and expansion, and; (iv) both photosynthetic and post-photosynthetic processes are mediated through sugar supply and source-sink integration under elevated [CO_2]. This is the first step towards dissecting the mechanism of early growth of wheat in response to elevated [CO_2], focusing on post-photosynthesis metabolism.

2. Materials and methods

2.1 Plant materials

This study used plant material from a mapping population of wheat derived from the two parental genotypes Kukri and RAC875, which have shown contrasting growth habits, nitrogen use efficiencies and has shown a large variation of growth response to elevated [CO_2] (Izanloo et al. 2008, our unpublished data; Bennett, Izanloo, et al. 2012; Bennett, Reynolds, et al. 2012). The two parental lines have been previously screened for their growth parameters and CO_2

responsiveness at elevated [CO_2] (unpublished data). Seeds of the mapping population were sourced from Australian Centre for Plant Functional Genomics, University of Adelaide, Australia. Based on the results of this screening trial, four high responsive and four less CO_2-responsive wheat lines were selected and progressed for further analysis in this study (Supplementary Table S1). For the clarity in interpretation of results, high responsive lines will be named henceforth H_1, H_2, H_3 and H_4 while less responsive lines will be named as L_1, L_2, L_3 and L_4.

2.2 Growth Conditions

The ten selected wheat lines (four high responsive, four less responsive and two parental lines) were grown in two identical growth chambers (Reach in growth chambers, PGC-105, Percival, USA) at the University of Southern Queensland, Australia. Plants were grown with a 14-hour photoperiod and a day and night temperature of 23ºC and 13ºC, respectively (Supplementary Table S2). The relative humidity of both chambers was maintained at 70% throughout the day while light intensity varied. A light intensity of 1000 $\mu molm^{-2}s^{-1}$ was maintained during mid-day throughout the growing period. Elevated [CO_2] conditions were maintained at ~700 $\mu molmol^{-1}$ and ambient [CO_2] conditions were maintained at ~400 $\mu molmol^{-1}$ throughout the growing season.

Seeds from all the ten lines were surface sterilized for 1 min in 2.6% NaClO and washed thoroughly using reverse osmosis water. Then, sterilized seeds were placed on moistened filter papers (Whatmann, Sigma-Aldrich, USA) in Petri dishes and allowed for germination. After one week of germination, seedlings were transplanted on 400 mm plastic pots filled with 500 g of brown top soil. Then, these pots were either placed in elevated [CO_2] or ambient [CO_2]. The experiment was comprised of a completely randomized design at the three-replication level and was maintained for six weeks after transplantation. Pots were randomized every four days within each chamber and swapped between chambers weekly to minimize the chamber effect. Throughout the growing period plants were carefully monitored and all the standard management practices were carried out to ensure crop health.

2.3 Biomass and leaf area determination

At the end of the six weeks growth period (42 days after planting [DAP]), a set of seedlings from all ten lines were harvested and separated to last fully expanded leaf blades, other leaf

blades and roots to determine above-ground and below-ground biomass, total leaf area of the young wheat seedlings. Before determining biomass, leaf area of the last fully expanded leaves and other leaf blades were measured using a leaf area meter. Then, to determine the biomass, leaf blades and root samples of all the ten wheat lines were oven dried for 48 hours at 60ºC.

2.4 Tissue carbohydrate analysis

2.4.1 Sample Collection

After six weeks of transplanting, four seedlings of each replicate were carefully sampled for carbohydrate analysis. Seedlings were separated into last fully expanded leaf (LFELB), immediate growing leaf and roots. Then, the stem was cut from the base of the main plant, and the leaf sheaths removed to clear the expanding leaf blade (ELB). From the expanding leaf, a 2 cm section was cut from the base of the seedling and this section was considered as the "shoot apex region" (SAR) of the young seedling. From the remaining expanding leaf , another section of 5 cm from the remaining leaf blade was cut and used for further analysis as the "leaf cell elongation zone" (LCEZ). These tissues were carefully sampled and separately stored in 1.5 mL Eppendorf tubes for further analysis.

2.4.2 Determination of Carbohydrates

Samples collected; LFELB, ELB and pooled samples representing LCEZ and SAR (henceforth named as cell elongation region [CER]) of each line, were oven dried at 65ºC for 72 hours. All tissue types were then ground to fine powder using a ball mill (Tissue Lyser II, QIAGEN, Australia) and 50 mg of each sample was used to analyze the glucose, fructose and sucrose in each tissue type at both elevated [CO_2] and ambient [CO_2]. Analysis of these sugars was assayed enzymatically using a Boehringer Mannheim kit (Catalogue No – 10716260035) according to the manufacturer's instructions (R-Biopharm, Germany).

2.5 Gas exchange and chlorophyll fluorescence measurements

Six weeks after planting, gas exchange measurements of the last fully expanded leaf were carried out as described by Seneweera et al. (2002). Gas exchange measurements were carried out using a portable photosynthesis system (IRGA, LI-6400, LI-COR, USA) from 9.00 am to 2.00 pm. Leaf chamber temperature and air flow rate of the LI-COR system was maintained at

25ºC and 500 µmol s^{-1}, respectively. Leaf chamber [CO_2] was controlled by the [CO_2] mixer and reference [CO_2] in the chamber was maintained at either 400 µmol mol^{-1} or 700 µmol mol^{-1} under ambient [CO_2] and elevated [CO_2], respectively. Chamber irradiance was supplied by a red and blue light source and light intensity was maintained at 1500 µmol quanta m^{-2} s^{-1}. Relative humidity of the leaf chamber was maintained between 50–70%. Prior to starting gas exchange measurements, the leaf was allowed to reach a steady state of photosynthesis. The steady state of photosynthesis was achieved between 10–15 min and then spot measurements of the rate of photosynthesis, stomatal conductance, intracellular [CO_2] and rate of transpiration were recorded in each genotype.

Chlorophyll fluorescence parameters were estimated using the leaf chamber fluorometer attached to an LI-6400XT portable photosynthesis system (LI-6400, LI-COR, USA). After plants reached a steady state photosynthesis rate, PSII operating efficiency (ϕPSII), electron transport rate (ETR), photochemical quenching (q_P), non-photochemical quenching (NPQ) and maximum PSII efficiency (Fv´/Fm´) were measured for the two parental cultivars Kukri and RAC875.

2.6 Gene expression analysis

2.6.1 Sample collection

Another set of samples were used for gene expression analysis. From one wheat line, four different plant organs, as described in the earlier section, were used for gene expression analysis (LFELB, ELB, LCEZ and SAR). Four plants from each replicate were used for this analysis. Samples were harvested between 10:00 to 13:00 hours and, as soon as they were harvested, were carefully transferred into liquid nitrogen and stored at -80ºC until total RNA extraction was completed.

2.6.2 RNA extraction and quantification

Total RNA was extracted from 300 mg (fresh weight basis) of frozen plant materials. Plant materials were ground with liquid nitrogen using a chilled mortar and pestle to obtain a fine powder. Then, 700 µl of TPS buffer (100 mM Tris-HCl (pH 8), 10 mM EDTA and 1 M KCl) and 500 µl (per 300 mg of plant tissues) of Trizol® Reagent (Invitrogen by Thermo Fisher Scientific, USA) were added to the ground plant materials and further ground until the sample

was completely homogenized. Then, RNA extraction for these homogenized samples was carried out according to the manufacturer's instructions. The quantity of extracted RNA was determined using a Qubit 3 Fluorimeter (Invitrogen by Thermo Fisher Scientific, USA) as per the manufacturer's instructions. Finally, total RNA concentration was adjusted to a constant concentration (1µg/µl) per sample for subsequent reactions.

2.6.3 cDNA Synthesis

All the samples of RNA were treated with DNase I, Amplification Grade (Invitrogen by Thermo Fisher Scientific, USA), according to the manufacturer's instructions, to avoid possible contaminations of genomic DNA in the samples. Then, cDNA synthesis was carried out by reverse transcription reactions performed using a SensiFAST^{TM} cDNA synthesis kit (Catalogue No – Bio-65054, Bioline, UK) in a total volume of 20 µl according to manufacturer's instructions.

2.6.4 Identification of gene-specific primers

Gene-specific primers associated with photosynthesis, carbon metabolism, nitrogen metabolism and cell cycle were sourced from previously published literature and verified for their specificity with *Triticum aestivum* L. using the Primer-Blast option in National Centre for Biotechnology Information (NCBI). Details of the genes used in the study along with their primer sequences and relevant literature are listed in Table 1 and Supplementary Table S2. All the primers were sourced from Integrated DNA Technologies, Inc. USA.

2.6.5 *Real-time Quantitative PCR (qRT-PCR)*

The qRT-PCR assays were carried out in an optical 96 well plate with the QuantStudio 3 Real-Time PCR system (Applied Biosystems from Thermo Fisher Scientific, USA), using SYBR green to monitor double-stranded DNA synthesis. The assay reaction mixture of 20 µl contained 4 µl of diluted cDNA (1:10), 2 µl 10 mM gene-specific primer, 4 µl of DNAse/RNase free water and 10 µl of 2X-SYBR Green Master Mix reagent (Applied Biosystems from Thermo Fisher Scientific, USA). The programme for the thermal cycler was 95°C for 10 minutes, 40 cycles of 95°C for 15 sec and 60°C for 1 minute. The melt curve protocol followed with 15 seconds at 95°C and then 60 seconds each at 1.6°C increments between 60°C and 95°C. Samples from LFELB, ELB, LCEZ and SAR of two parental lines (Kukri and RAC0875) were

used in three biological replicates per treatment in the qRT-PCR assay. The efficiency of the qRT-PCR was determined using raw fluorescence data as input in LinRegPCR v2012.3 (Ruijter et al. 2009). Determination of relative expression was done using the comparative threshold cycle (C_t) method $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008; Pfaffl 2001) and presented as the $\log_2$ fold change of elevated and ambient [CO_2].

2.7 Statistical Analysis

Treatment effects and interactions were determined by Analysis of Variance (ANOVA) through general linear modeling. Standard errors of differences and the Tukey's test were used to determine the differences between treatments. Differences were considered significant at $P<0.05$. Pearson product-moment correlation test was conducted to analyse the potential correlation between sugar content and the relative gene expression at elevated [CO_2]. All statistical analyses were performed using SPSS statistical software version 23 (IBM, Armonk, NY, USA). All graphical representations were carried out using GraphPad Prism scientific software version 5.01 (GraphPad Software, San Diego, CA).

3. Results

3.1 Genetic variation in growth response to elevated [CO_2]

Growth characteristics of two parental lines of the mapping population showed a significant difference when they were grown at elevated [CO_2]. There were significant interactions between [CO_2] and cultivar for biomass accumulation (Figure 1(a), $P<0.05$) and total leaf area (Figure 1(b), $P<0.05$, Details of the ANOVA results are listed in Supplementary Table S4). Above ground biomass accumulation and total leaf area of Kukri were increased by 11.5% and 10.2% at elevated [CO_2]. Similarly, in RAC875, total plant dry mass and total leaf area were increased by 86% and 34.5%, respectively at elevated [CO_2].

In the selected wheat lines, there were significant interactions between [CO_2] and cultivar for above ($P<0.01$) and below ground biomass ($P<0.01$) and total leaf area ($P<0.01$, Table 2). The mean CO_2 responsiveness for above and below ground biomass accumulation were 225.4% and 175.7%, respectively, for high responsive lines. Of them, the highest CO_2-responsiveness was recorded for the H_2 wheat line for both above and below ground dry mass accumulation.

Increase in above and below ground dry mass of less responsive wheat lines showed a less response to elevated [CO_2] and showed an increase of 31.2% and 34.7%, respectively, under elevated [CO_2]. The overall increase in leaf area production at elevated [CO_2] was higher at elevated [CO_2], however, there was a marked decrease in CO_2 responsiveness of total leaf area (-17.99%) of the less responsive lines (Table 2).

3.2 Net photosynthesis and chlorophyll fluorescence activity at elevated [CO_2]

The rate of photosynthesis of the parental cultivars, Kukri and RAC875, was significantly stimulated when grown under elevated [CO_2] ($P<0.05$) (Figure 2(a)). However, RAC875 showed comparatively higher photosynthetic stimulation when compared to Kukri. In RAC875, the net photosynthetic rate has been increased by 70.46% at elevated [CO_2]. With regards to the chlorophyll fluorescence parameters, the maximum efficiency of PSII and non-photochemical quenching of these two cultivars were not significantly different at elevated [CO_2]. However, the operating efficiency of PSII (Figure 2(b)), the rate of electron transport in PSII (Figure 2(c)) and photochemical quenching of PSII (Figure 2(d)) were significantly higher ($P<0.05$) in RAC875 under elevated [CO_2].

The photosynthesis capacity of the other wheat lines was also substantially increased at elevated [CO_2]. There was a significant interaction between [CO_2] and cultivar for leaf photosynthesis of these wheat lines ($P<0.05$, Table 2). A higher response for photosynthesis at elevated [CO_2] was demonstrated in high responsive wheat lines with the H_2 wheat line showing the highest percentage increase. The increases in photosynthesis capacity of less responsive lines were less under high [CO_2] and showed a marked decrease in CO_2 responsiveness for rates of photosynthesis (-12.16%, Table 2).

3.3 Carbohydrate biosynthesis and organ-specific carbohydrate accumulation at elevated [CO_2]

Large differences in sugar accumulation between two parental cultivars were observed under elevated [CO_2]. The soluble sugar concentrations of two parental cultivars significantly varied between genotypes ($P<0.05$) and different organs ($P<0.05$) under high CO_2 conditions (Figure 1(c)). The highest total sugar content was found in the CER at elevated [CO_2] which showed an increase of 64.4% in Kukri and 117.3% in RAC875 ($P<0.01$, Figure 3). In ELB, the highest total soluble sugar content was observed in RAC875 with an increase of 67% at elevated [CO_2]

(Figure 3). In general, the highest sucrose concentration was detected in the LFELB and the contents were increased by 177.9% and 398.6% in Kukri and RAC875, respectively, at elevated [CO_2] (Supplementary Figure S1). Among all the sugars, glucose and fructose were the prominent soluble sugars in CER of the wheat seedlings. At elevated [CO_2], glucose and fructose concentrations were increased by 145.6% and 33.8%, respectively, in Kukri and 227.8% and 177.2%, respectively, in RAC0875 (Supplementary Figure S1). Overall, RAC875 showed the highest response for carbohydrate biosynthesis and accumulation under elevated [CO_2] across all the organ types.

Among the other eight selected wheat lines, the total soluble carbohydrate content of high responsive lines showed higher percentage increase at elevated [CO_2], when compared with the less responsive lines (Table 2). The highest CO_2 responsiveness for total soluble carbohydrates accumulation was observed in wheat lines H_1 and H_2, with a 31.5% and 25.1% increase in soluble carbohydrate concentration at elevated [CO_2] respectively (Table 3). The least response for soluble carbohydrate accumulation was observed in the wheat line L_1, which showed a – 5.5% responsiveness to elevated [CO_2] (Table 3). There were significant interactions among [CO_2], cultivar and organ for sucrose ($P<0.01$), glucose ($P<0.01$) and fructose ($P<0.01$) contents across the different wheat lines tested in this study (Table 3). The sucrose and fructose contents of LFELB, ELB and CER were higher in plants grown at elevated [CO_2]. However, the glucose concentrations were lower at elevated [CO_2] across all organs when compared to ambient [CO_2] grown plants. The highest CO_2 responsiveness for total soluble carbohydrate accumulation was observed in wheat lines H_1 and H_2, with a respective increase of 33.4% and 42.8% in last fully expanded leaves,14.2% and 40.2% in ELB and 62.36% and 35.18% in CER of the plants grown at elevated [CO_2].

3.4 Effect of elevated [CO_2] on the expression of key photosynthesis-related genes

Genes encoding Rubisco, *rbcL* and *rbcS*, were tested to understand the variation in photosynthesis at the molecular level. Relative expression of *rbcL* and *rbcS* varied significantly among different organs ($P<0.01$) in two CO_2 levels ($P<0.01$) (Supplementary Table S5). Expression of *rbcL* did not differ between the genotypes, however, expression of *rbcS* showed a significant difference between the two genotypes ($P<0.05$). In general, *rbcL* expression was higher in photosynthetic tissues (LFELB and ELB) while *rbcS* expression was higher in non-photosynthetic tissues (SAR) (Table 4). Expression of both genes was upregulated in elevated

[CO_2] across all plant organs. More importantly, expression of *rbcL* and *rbcS* in LFELB, was greater in RAC875 than Kukri at elevated [CO_2]. The expression of *rbcL* and *rbcS* in the LFELB of RAC875 showed a 1.7 and 1.9-fold increase, respectively at elevated [CO_2] when compared to the expression of these genes in Kukri.

3.5 Effect of elevated [CO_2] on carbon metabolism

Distinctive organ-specific expression patterns were observed for *SPP1*, *SPS1* and *SUS1* across the genotypes tested. These genes showed a significant increase in expression at elevated [CO_2] when compared to plants grown at ambient [CO_2] ($P<0.01$, Supplementary Table S5). However, a significant genotypic difference was only observed for *SPS1* gene expression at elevated [CO_2] (Supplementary Table S5, $P<0.01$). Expression of all these genes significantly differed in different organs ($P<0.01$, Supplementary Table S5). In the last fully expanded leaf and expanding leaf, relative expression of *SPP1* was the highest, while *SUS1* showed the highest expression in cell elongation and shoot apex regions (Table 5).

The expression of *SPP1*, *SPS1* and *SUS1* in the LFELB of RAC875 grown at elevated [CO_2] showed a significant increase in expression compared to ambient [CO_2] grown plants, with a 5.4, 4.8, 2.0-fold increase, respectively. The expression of *SPS1* was upregulated at elevated [CO_2] across all organ types, where the highest fold increase was observed in the LFELB of both cultivars. In contrast, the *SPP1* gene was upregulated only in photosynthetic tissues, while it showed a lower transcript abundance in the non-photosynthetic tissues. This scenario was comparatively less at elevated [CO_2] demonstrating an indirect upregulation of this gene. Expression of *SUS1* gene was higher in the cell elongation and shoot apex regions of RAC875, with a fold increase of 2.19 and 2.64 at elevated [CO_2].

3.6 Effect of elevated [CO_2] on genes associated with plant nitrogen metabolism

Since the leaf is the predominant site for nitrogen assimilation in plants, key genes involved in nitrogen metabolism were examined in the LFELB and ELB of Kukri and RAC875. The expression of *NiR*, *GS1a*, *GSr1*, *NADH-GOGAT*, *GS2a*, *GS2b* and *Fd-GOGAT* at elevated [CO_2] significantly differed from the expression levels observed at ambient [CO_2] ($P<0.01$, Supplementary Table S5). Further, expression of all these genes was significantly varied between LFELB and ELB organs ($P<0.05$, Supplementary Table S5) except for *NADH-GOGAT* and *NiR* expression. Highest transcript abundance for *GS2a* and *Fd-GOGAT* was

observed in ELB than the LFELB under both ambient and elevated [CO_2] (Table 6). However, the CO_2 effect on expression of these genes was higher in LFELB. For example, Kukri showed 5.9 and 4.1 fold change increase and RAC875 showed 1.63 and 2.53 fold change increase, respectively, for *Fd-GOGAT* and *GS2a* expression at elevated [CO_2]. Similarly, expression of *GS1a* was comparatively higher in LFELB, of which RAC875 showed the highest expression with a fold increase of 8.6 when compared to ambient [CO_2]. In contrast, *GSr1* and *NADH-GOGAT* expressions were comparatively less in both LFELB and ELB. A marked upregulation of these two genes was observed in the LFELB of RAC875 at elevated [CO_2] with a fold increase of 21 and 9.6, respectively.

3.7 Effect of elevated [CO_2] on the expression of key genes related to cell elongation and expansion

A majority of genes associated with α and β-expansins were highly expressed in meristematic tissues in LCEZ and SAR of both Kukri and RAC875. With reference to α-expansins, expression of *TaEXPA3* varied significantly between organ types and genotypes at two different CO_2 treatments ($P<0.05$, Supplementary Table S5). In the SAR, there was a higher upregulation of this gene in both cultivars at elevated [CO_2], of which Kukri showed the highest expression. Although the transcript abundance for *TaEXPA1* was high in the LCEZ and SAR, no significant differences were found between the two CO_2 treatments.

The expression of β-expansins also differed significantly between two CO_2 treatments except for *TaEXPB1* ($P<0.01$, Supplementary Table S5). In general, expression of *TaEXPB2*, *TaEXPB3*, *TaEXPB6* and *TaEXPB23* were highly expressed at elevated [CO_2] in both varieties (Table 7). In the LCEZ of Kukri, *TaEXPB2*, *TaEXPB6* and *TaEXPB23* showed a marked upregulation, while all tested genes, except for *TaEXPB1*, were highly expressed in RAC875 at elevated [CO_2] (Table 7). In contrast, the expression of *TaEXPB1* was only upregulated in the SAR of both cultivars ($P<0.05$, Supplementary Table S5). The genotype differences in expression of *TaEXPB3* and *TaEXPB6* were significant, of which RAC875 showed a higher response to elevated [CO_2].

The detection of several α and β-expansins was very low in LFELB and ELB, and therefore, those results are not reported in this study. In leaf tissues, *TaEXPA3*, *TaEXPB6* and *TaEXPB23* were the only three genes in expansins category that showed a detectable relative expression at

both CO_2 levels and significantly varied between last fully expanded and expanding leaves ($P<0.05$, Supplementary Table S5). These three genes showed a down-regulation in LFELB while they showed an upregulation in ELB (Table 7). Further, *TaEXPA3* and *TaEXPB6* expression varied significantly between Kukri and RAC875 at elevated [CO_2].

There effect of [CO_2] ($P<0.01$) and genotype ($P<0.05$) on XTH gene expression were significant except for *TaXTH4* (Supplementary Table S5). However, the expression of all XTH members was varied significantly between different organs ($P<0.01$, Supplementary Table S5). and organ type on the expression of different *XTH* genes tested in this study. The expression of *TaXTH1*, *TAXTH2* and *TaTH3* were highest in the ELB of both cultivars (Table 8). At elevated [CO_2], expression of these genes together with *TaXTH5* was upregulated in both cultivars (Table 8). In RAC875, expression of *TaXTH1*, *TaXTH2*, *TaXTH3* and *TaXTH5* showed 2.88, 2.01, 8.39 and 2.59-fold increases, respectively, when compared to ambient [CO_2]. Conversely, the LFELB of the two genotypes showed the least transcript abundance for *XTH* gene members compared with the other organ types, both at ambient and elevated [CO_2] (Table 8).The expression of *TaXTH2*, *TaXTH3*, and *TaXTH4* in LFELB of Kukri was down-regulated while *XTH5* showed substantial upregulation at elevated [CO_2]. In RAC875, *TaXTH1* and *TaXTH4* were down-regulated at elevated [CO_2] while *TaXTH2*, *TaXTH3* and *TaXTH4* were upregulated.

In general, expression of all the *XTH* gene members was high in SAR and LCEZ ($P<0.01$, Supplementary Table S5), of which *TaXTH5* showed the highest expression (Table 8). At elevated [CO_2], a significant genotypic difference was only observed for *TaXTH1*, *TaXTH2* and *TaXTH3* expression in the SAR ($P<0.05$, Supplementary Table S5), with 1.4, 0.7 and 0.8 fold increases, respectively, in Kukri and 6.1, 3.4 and 3.7 fold increase, respectively, in RAC875. There was a significant upregulation of *TaXTH5* at elevated [CO_2], although there was no genotypic difference. The expression of *XTH* genes in the LCEZ was also substantially higher than leaf tissues. In Kukri, *TaXTH1*, *TaXTH2* and *TaXTH5* showed the highest upregulation with fold increases of 1.6, 1.4 and 2.3, respectively, at elevated [CO_2] (Table 8). The expression of, *TaXTH3* and *TaXTH5* was highest in RAC875, with a fold increase of 3.7 and 2.6, respectively, at high CO_2 conditions (Table 8).

4. Discussion

Faster growth at elevated atmospheric [CO_2] during early ontogeny appears to be a major contributor to accelerated growth for many crops (Makino and Mae 1999; Jitla et al. 1997). Thus, this enhanced early growth and vigor is crucial in determining the final grain yield of a crop (Thilakarathne et al. 2015; Seneweera and Conroy 2005). The genetic variation in plant responses to elevated [CO_2] is well documented (Thilakarathne et al. 2013; Thilakarathne et al. 2015; Tausz et al. 2013), but it has been suggested that this growth variation cannot only be explained through increases in photosynthetic capacity at elevated [CO_2] (Taylor et al. 1994; Gamage et al. 2018). A strong carbon and nitrogen relationship between source and sink tissues at elevated [CO_2] has been established in previous studies (Paul and Foyer 2001; Paul and Pellny 2003; Seneweera et al. 2002), but the underpinning mechanisms that drive the genetic variation in plant response to elevated [CO_2] are not yet established (Gamage et al. 2018).

4.1 Biomass accumulation and enhanced growth stimulation at elevated [CO_2]

The increased photosynthetic capacity and improved water use efficiency at elevated [CO_2] have paved the way for improvements in biomass accumulation in many crops (Ainsworth and Rogers 2007), including wheat (Thilakarathne et al. 2013; Thilakarathne et al. 2015). This has been further confirmed in this current study as both the parental lines (Kukri and RAC875) showed a higher carbon assimilation rate and a higher biomass accumulation at elevated [CO_2] (Figure 1 and 2). Consistent with this, the total soluble sugar content of these two cultivars was also higher, indicating the greater availability of carbohydrate substrates for other post-photosynthetic key metabolic activities of the plant (Figure 1 and 3). Of the two parental lines, RAC875 consistently showed higher photosynthetic rates, soluble sugar accumulation and biomass accumulation at elevated [CO_2] when compared to Kukri. On the other hand, the effect of [CO_2] on photosynthesis of selected wheat lines was not statistically significant, while the interaction effect between [CO_2] and genotype was significant in our study (Table 2). This supports our hypothesis that increases in rates of photosynthesis alone cannot explain the growth enhancement observed at elevated [CO_2]. Thus it is suggested that along with photosynthesis, modifications in key post-photosynthetic metabolic processes such as sucrose metabolism, nitrogen metabolism and cell wall metabolism, are required (Gamage et al. 2018) to maintain optimum growth rates at elevated [CO_2], especially at the early vegetative growth stage. The selected wheat lines were different to their parental lines were selected on the based

on their CO_2 responsiveness. The lines H_1, H_2, H_3, and H_4 showed a highest growth response to elevated [CO_2] while L_1, L_2, L_3 and L_4 showed the lowest response to elevated [CO_2] compared to parental lines. For example, the highest above biomass response to elevated [CO_2] was observed in wheat line H_3 with 257.5% increase in biomass, which was higher than the both parental lines. Similarly, lowest response of above ground biomass at elevated [CO_2] was observed in wheat line L_3 with 14.9% which was below both parental lines. Further, the high responsive lines showed a mean increase of below ground biomass accumulation by 176% in response to elevated [CO_2] while less responsive lines showed an average increase of 34.8% at elevated [CO_2]. The increase in leaf area at elevated [CO_2] was higher in high responsive lines with an average increase of 27.6%. However, there was a substantial decrease in leaf area increment (-18.0%) in less responsive lines when exposed to elevated [CO_2]. These results clearly showed that the selected progeny lines perform differently to the parental lines and to each other when exposed to high levels of [CO_2].

4.2 Soluble sugar contents vary among plant organs, genotypes and CO_2 concentrations

Glucose, fructose and sucrose are the main soluble sugars that play a key role in maintaining overall plant growth and development (Rosa et al. 2009). The abundance of these sugars differ in different organ types and their accumulation largely varied during plant ontogeny. In particular, glucose and fructose are actively involved in cell division (Koch 2004), hence the supply of these sugars to growing tissues is critically important for accelerated growth (Eveland and Jackson 2011). Consistent with this proposition, our study demonstrated that glucose and fructose contents in the growing tissues such as young leaves, cell elongation and the shoot apex region were significantly higher in plants at elevated [CO_2] (Supplementary Figure S1 and Table 3). Perhaps, sucrose hydrolysis is accelerated at elevated [CO_2] as a result of increased metabolic demand.On the other hand, sucrose availability at elevated [CO_2] was high in mature tissues such as LFELB indicating its role in cell differentiation and maturation (Koch 2004). As soon as sucrose is produced in the source tissues, they are rapidly translocated to sink tissues to be used for growth or to store as reserves. It has also been established that soluble sugars play a major role in controlling the proliferation of plant organs (Gibson 2005). This could be a possible reason for the observed higher leaf area stimulation of the wheat seedlings grown at elevated [CO_2] in our study.

A significant genotypic variation for soluble carbohydrate production was observed under elevated [CO_2]. High responsive wheat lines showed a higher soluble sugar content at elevated [CO_2] while less responsive wheat lines did not show a notable increase despite the increases in CO_2 assimilation rates at elevated [CO_2]. This suggests that carbohydrate production at elevated [CO_2] not only depends on the increased photosynthetic capacity, but also varies based on the genotype and their genetic capacity to utilize these carbohydrates in different metabolic activities. For example, plants that can utilize these carbohydrates to generate more tillers or secondary shoots and increase their sink capacity are likely to produce more soluble sugar at elevated [CO_2] (Ainsworth et al. 2004). Therefore, plants' genetic capacity to utilize photo-assimilates efficiently is a critical factor to sustain photosynthesis and thereby regulate their growth and development.

4.3 Light-dependent and light independent reactions of photosynthesis are genetically varied at elevated [CO_2]

The light reaction of photosynthesis plays a key role in increasing photosynthetic capacity of plants at elevated [CO_2] (Gamage et al. 2018). The activity of Photosystem II (PSII) in the light reaction is quite sensitive to a wide range of biotic and abiotic factors making it a good indicator of how plants generally respond to climate change and adapt to ecological variations (Murchie and Lawson 2013). Chlorophyll fluorescence is one of the best tools to obtain detailed information on the state of PSII in response to an environmental change (Sekhar, Sreeharsha, and Reddy 2015). Both Kukri and RAC875 showed no significant difference in maximum PSII efficiency under light, when all the reaction centers are open (Fv´/Fm´), and non-photochemical quenching (NPQ), suggesting that plants do not experience any stressful condition at elevated [CO_2] (Figure 2). However, the effective quantum yield (ϕPSII) of both the parental lines was significantly higher in elevated [CO_2] suggesting that the proportion of absorbed light used in PSII photochemistry increased under rising [CO_2] (Figure 2, Supplementary Table S4). This may be because of the increased number of PSII reaction centers opened at PSII allowing reaction centers to accept further electrons. This was further confirmed by the increased photochemical quenching (q_p) of parental cultivars at elevated [CO_2]. The increased electron transport rate (ETR) observed at elevated [CO_2] may have facilitated higher photochemical quenching (q_p) as electrons are transported away from PSII

due to the enzymes involved in CO_2 fixation. Overall, chlorophyll fluorescence measurements suggested that the light utilization efficiency of PSII system is higher at elevated [CO_2] and consequently the electron flow between the photosystems is increased. This correlates well with the increased photosynthesis rates of the two parental lines, which further indicates that the light reaction of photosynthesis tends to produce more ATP and NADPH to support increased rates of light independent reaction at elevated [CO_2]. A good correlation between PSII efficiency and CO_2 assimilation has been reported in previous studies (Sekhar, Sreeharsha, and Reddy 2015; Rasineni, Guha, and Reddy 2011; Brestic et al. 2012) suggesting that chlorophyll fluorescence measurements can be used to screen photochemically efficient genotypes for better carbon assimilation in a changing climate.

The light-independent reaction of photosynthesis commences with catalysis by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which can carboxylate in the presence of CO_2 or oxygenate with O_2 (Makino and Mae 1999; Drake, Gonzàlez-Meler, and Long 1997). Rubisco has a higher affinity for CO_2 than O_2, and therefore, with the higher partial pressure of CO_2 at elevated [CO_2], the rate of Rubisco carboxylation tends to increase, enhancing photosynthesis rates. This stromal protein contains eight small subunits and eight large subunits encoded by nuclear multigene family (*rbcS*) and single gene in the chloroplast genome (*rbcL*) (Suzuki et al. 2010; Suzuki et al. 2009). Changes in Rubisco synthesis is primarily explained by the changes to transcript abundance of the Rubisco encoding genes (Suzuki et al. 2010). At elevated [CO_2] conditions, expression of these two photosynthetic genes varies between the two genotypes. In general, expression of *rbcL* and *rbcS* were higher in plants grown at elevated [CO_2] (Table 4). The highest expression for *rbcL* was observed in fully expanded leaves of RAC875 along with higher maximum net photosynthesis rates than Kukri at elevated [CO_2]. Moreover, expression of *rbcS* in fully expanded leaves was also higher in elevated [CO_2] than in ambient [CO_2]. Since the expression of these two genes correlates with the synthesis of Rubisco, these results suggest that more Rubisco synthesis has taken place in fully the expanded leaves than in other organs. This could have determined the increased photosynthesis capacity of the plants observed at high CO_2 conditions. In the expanding leaf, *rbcL* and *rbcS* expression were higher in Kukri than in RAC875 at elevated [CO_2] suggesting further that transcript abundance is subject to genotypic variations. We could observe comparatively higher expression in *rbcL* in leaf tissues when compared with *rbcL* expression in non-photosynthetic tissues. For example, the expression of the *rbcL* gene was less while *rbcS* expression was high in cell elongation and shoot apex regions when compared to other photosynthetic tissues. This

could be due to lower abundance of the chloroplast genome to code for *rbcL* expression. A similar pattern of expression in *rbcL* and *rbcS* was also observed in previous studies conducted in amaranth (Nikolau and Klessig 1987), pea (Sasaki, Nakamura, and Matsuno 1987) and rice (Suzuki, Makino, and Mae 2001). Overall, expression of photosynthetic genes tends to change with the plants' genotype, growth stage, tissue type and the environmental conditions in which the plants are grown.

4.4 Expression of key genes associated with sucrose metabolism was influenced by genotype, organ type and elevated [CO_2]

Sucrose plays a critical role in plant growth, development, storage, signal transduction and support for plant acclimation to different environmental stresses (Jiang et al. 2015). Transfer of sucrose from its source tissues to sink tissues is the main factor contributing towards the source and sink integration of the plant which finally determines plant growth and yield (Bihmidine et al. 2013). In this study, there was a marked difference in expression of the genes, *SPP1*, *SPS1* and *SUS1* in different organs and CO_2 conditions (Table 5). Moreover, expression of these genes was varied with wheat genotype. The correlation analysis between the change of soluble sugar contents and the relative expression of sucrose metabolism genes showed that there was a strong positive correlation between *SPP1* and fructose concentration ($r=0.783$, $P<0.05$, Supplementary Table S6). This indicates that regulation of sucrose metabolism genes at elevated [CO_2] may be regulated through a sugar sensing mechanisms.

Sucrose Phosphate Synthase (*SPS1*) catalyzes the conversion of fructose-6-phosphate and UDP-Glucose into Sucrose-6-Phosphate and is considered to be the major rate-limiting enzyme of sucrose biosynthesis in plants (Lunn and MacRae 2003). This also plays a key role in partitioning carbon between sucrose and starch in both photosynthetic and non-photosynthetic tissues (MacRae and Lunn 2006; Hashida et al. 2016). In this study, it has been clearly demonstrated that transcript abundance of *SPS1* was increased at elevated [CO_2] across all the organ types. Higher expression of SPS reflects the increased sucrose synthesis capacity of plants under elevated [CO_2] leading to increased plant development (Pessarakli 1996). This was further confirmed by the increased biomass accumulation and photosynthetic rates observed for the RAC875 parental line and the same cultivar showed a higher expression for *SPS1* (Table 5). The higher expression of *SPS1* under elevated [CO_2] may be due to the allosteric nature of this enzyme (Yonekura et al. 2013), which is activated by glucose-6-

phosphate and inhibited by inorganic phosphate (Hashida et al. 2016). As the rate of inorganic phosphate used by photosynthesis is higher at elevated [CO_2], the availability of inorganic phosphate as an inhibitor of *SPS1* gene is less under high CO_2 conditions. Thus, increased availability of Glucose-6-Phosphate at elevated [CO_2] allosterically increases the activity of SPS. The concentration of sucrose at elevated [CO_2] was also higher in RAC875 suggesting that *SPS1* could also facilitate carbon partitioning to sucrose over starch in leaf tissues (Huber and Israel 1982; Huber 1983). In addition, SPS activity was found to be high in growing sink tissues and similar results were reported in our study, particularly in growing leaves, the cell elongation region and shoot meristem tissues (Table 5). High expression of SPS in major sink tissues may be involved in re-synthesis of sucrose imported via apoplastic cleavage and may help in redistribution within the sink tissue (Sturm and Tang 1999; Geigenberger et al. 1999). The enzyme SPP catalyzes the final step of sucrose biosynthesis, irreversibly hydrolizing Sucrose-6-Phosphate, produced by SPS, to sucrose (Jiang et al. 2015) and is usually expressed in significant quantities in both vascular and non-vascular plants (Lunn 2003). In the current study, a higher expression of *SPP1* compared to *SPS1* and *SUS1* was observed across all tissue types in the two CO_2 treatments. It was suggested that maximum potential activity of SPS and SPP are similar to each other (Lunn 2003) in wheat, and that a multienzyme complex may form between these two enzymes (Echeverria et al. 1997), suggesting that both *SPS1* and *SPP1* may have worked together in the synthesis of sucrose under elevated [CO_2].

Channeling sucrose into other metabolic activities, such as respiration, biosynthesis of cell wall polysaccharides and storage reserve formation, require its cleavage by different enzymes (Sturm and Tang 1999). The expression of *SUS1* was examined since SUS is one of the main sucrose-cleaving enzymes and activity of *SUS1* was higher in cell elongation and shoot meristem tissues when compared to the last fully expanded leaf (Table 5). The enzyme SUS cleaves sucrose into UDP-Glucose and fructose (Jiang et al. 2015), where UDP-Glucose then acts as the primary building block for cell wall synthesis (Verbančič et al. 2017). Since only low levels of cell wall synthesis are required in the LFELB, the majority of the UDP-Glucose produced will be directed to the synthesis of sucrose (Stitt, Lunn, and Usadel 2010) and then transported to sink tissues (Sauer 2007) suggesting SUS gene mainly expressed in primary sink tissues. Consistent with this proposition, results outlined in Table 5 showed that *SUS1* expression was higher in meristematic tissues under both CO_2 treatments. However, these expression patterns were even pronounced in plants grown at elevated [CO_2]. As elevated [CO_2] induces high cell division and expansion rates in meristematic tissues (Gamage et al.

2018), most of the sucrose produced will be translocated to these tissues to be used in different anabolic activities (Sturm and Tang 1999) and thus help to produce more tillers or secondary shoots and increase leaf area development, consequently leading to high biomass and yield production.

4.5 Organ-specific expression of nitrogen assimilation genes varied at elevated [CO_2]

Plant cells require both carbon and nitrogen for growth and development. Carbon is essential to fuel metabolic processes while nitrogen availability is a key factor in maintaining protein turnover of cells (White et al. 2015). Glutamine Synthetase (GS) catalyzes the conversion of glutamate to glutamine using ammonia as the substrate during nitrogen assimilation. The two classes of GS (GS1 and GS2) have organ-specific roles (Cren and Hirel 1999; Masclaux-Daubresse et al. 2010). The enzyme GS2 is predominantly found in leaves and catalyses the primary nitrogen assimilation of ammonia resulting from the reduction of nitrite in chloroplasts and reassimilation of respiratory ammonia (Foyer and Zhang 2011). Consistent with this, the expression of *GS2a*, encoding one of the isoenzymes of the GS2 class, was found to be significantly expressed in both LFELB and ELB of our study (Table 6). Further, this *GS2a* was upregulated in both LFELB and ELB at elevated [CO_2] suggesting that increased nitrogen assimilation occurs at elevated [CO_2], especially in the early development stages. The expression of GS1 enzyme related genes, *GS1a* and *GSr1* in leaf tissues was comparatively lower than the *GS2a* expression. However, the expression of *GS1a* was significantly higher in RAC875 at elevated [CO_2]. It has been previously reported that GS1 supports the remobilization of nitrogen into the developing sink organs (Masclaux-Daubresse et al. 2006; Pageau et al. 2005; Tabuchi, Abiko, and Yamaya 2007). These GS enzymes function together with two distinct forms of GOGAT (glutamate synthase) to incorporate amino groups into amino acid precursors required for protein synthesis (Masclaux-Daubresse et al. 2010). In our results, the expression of *Fd-GOGAT* was significantly higher compared to *NADH-GOGAT* in the LFELB and ELB. These findings are consistent with Yamaya et al. (1992) as *NADH-GOGAT* is predominantly present in non-photosynthetic tissues of the plants while *Fd-GOGAT* is primarily located in the chloroplasts of photosynthetic tissues (Suzuki and Knaff 2005). The expression of *Fd-GOGAT* showed a substantial upregulation at elevated [CO_2] across both parental cultivars while a notable upregulation of *NADH-GOGAT* was only observed in the LFELB of RAC875. This might indicate that transcriptional regulation of these genes is subjected to intra-specific variation, which may have contributed towards the different nitrogen

use efficiencies of these two cultivars. On the other hand, Bernard et al. (2008), also found that expression of these genes increases as the leaf ages while *GS2a*, *GS2b* and *Fd-GOGAT* expression decreases. For example, in RAC875, the expression of *GS2a* and *Fd-GOGAT* increased in the ELB, while expression of *GS1a*, *GSr1* and *NADH-GOGAT* was higher in the LFELB (Table 6). As carbon and nitrogen status is critically balanced in the plants, up-regulation of *GS2a*, *GS1a*, *Fd-GOGAT* and *NADH-GOGAT* at elevated [CO_2] suggests that high sugar supply indirectly stimulates production and mobilization of glutamine and glutamate, which can donate amino groups to produce amino acids essential for cellular activities (Zheng 2009). Further, increased amino acid biosynthesis facilitates production of different structural and metabolic proteins that are critical for optimum plant growth in a high CO_2 environment.

4.6 Cell expansion and cell wall loosening were influenced by elevated [CO_2]

At elevated [CO_2], increased shoot elongation is more likely to depend on cell expansion rather than cell proliferation (Taylor et al. 1994; Ranasinghe and Taylor 1996; Liu et al. 2007). Expansins and Xyloglucan endotransglucosylase/hydrolases are extracellular proteins that play a key role in relaxing the cell walls during cell division and elongation (Liu et al. 2007). Our expression analysis revealed that the expression of *TaEXPA3* was prominent in the shoot apex region at elevated [CO_2] in both cultivars (Table 7). Alpha-expansins have a distinct role in leaf primordia formation (Reinhardt et al. 1998; Shin et al. 2005), and thus upregulation of *TaEXPA3* is consistent with the growth stimulation at elevated [CO_2]. Several previous studies revealed that β-expansin expression is generally higher at early vegetative stages of grasses (Wu, Meeley, and Cosgrove 2001; Liu et al. 2007; McQueen-Mason and Rochange 1999). Consistent with this, our study's results demonstrated a higher transcript abundance of β-expansin genes across different organs when compared to α-expansin expression (Table 7). In addition, most of the β-expansin genes tested in our study were upregulated at elevated [CO_2], especially in the shoot apex and cell elongation regions of both parental cultivars. With high sugar supply to these regions at elevated [CO_2], the activity of β-expansins may have been upregulated and may positively contribute towards determining the internode elongation and plant height (Lee and Kende 2001; Liu et al. 2007). A noteworthy expression was observed for *TaEXPB23* among other β-expansins in both cultivars, with a higher upregulation at elevated

[CO_2] implying a possible significant role played by this gene at elevated [CO_2]. It has been previously found that higher expression of *TaEXPB23* in plants may result in accelerated leaf and internode growth at early development stages (Xing et al. 2009).

With reference to XTH genes, the highest expressions of *TaXTH1*, *TaXTH2* and *TaXTH3* was observed in ELB indicating the significant role played by the XTH enzymes in cell wall loosening and expansion (Table 8). Similarly, Taylor et al. (1994) reported that XTH enzyme activity is increased at elevated [CO_2], enabling cell walls to expand rapidly in response to increased carbon supply. However, the expression level of *XTH* genes was less in the LFELB which is consistent with the relative maturity of these tissues. Further, higher expression of *XTH* genes was reported in LCEZ and SAR at elevated [CO_2] (Table 8) presumably due to a more rapid cell wall synthesis in these tissues which may have supported the increased cell division and growth. According to our expression analysis, *XTH* expression showed a distinct tissue/organ-specific expression pattern, implying that the expression of these genes is influenced by plant development stage. These results were consistent with Liu et al. (2007) who also demonstrated that XTH gene family perform tissue/organ-specific developmental roles that are not interchangeable in plant growth and development.

Overall, our results clearly showed that β-expansin genes showed higher expression than the α-expansins at elevated [CO_2]. A similar pattern of expression of these genes was observed by Liu et al. (2007) in wheat. This differential expression further suggests functional differences in two groups of expansins: α-expansins are involved mainly in cell-specific functions whereas β-expansins play a role in expansion-related functions, mainly the maintenance of cell wall structure (Li, Jones, and McQueen-Mason 2003; Wu, Meeley, and Cosgrove 2001). In addition, XTH genes had an even higher expression, which may be due to their nonsubstitutable specific function in cell wall loosening (Liu et al. 2007; Yokoyama and Nishitani 2000). Most of the β-expansins and XTH genes tested in our study have been upregulated at elevated [CO_2], implying the increased expression of these enzymes is required to support maximum growth at elevated [CO_2]. The correlation analysis between the change of soluble sugar contents and the relative expression of genes indicates that cell wall metabolism-related genes tend to show a significant correlation with the soluble sugar concentration at elevated [CO_2]. For example, *TaXTH5* and *TaEXPA1* showed a strong positive correlation with glucose content at elevated [CO_2] ($r=0.816$, $P<0.05$; $r=0.994$, $P<0.01$, respectively, Supplementary Table 5). Also, *TaXTH3* showed a strong positive correlation with total sugar content at elevated [CO_2]

(r=0.74, P<0.05). These results further suggest that the expression of these genes is influenced by changes to the soluble sugar content of a particular tissue type and thus, is potentially regulated by sugars. However, apart from these key genes, there are many gene regulatory factors that influence on expression of genes associated with cell expansion and cell wall extension. For example, in Arabidopsis, expression of GCCR motif is necessary for the expression of cell cycle genes such as Cyclin (*CYCB1;1*) which is responsible for cell division in shoot meristem (Li *et al.*, 2005). Therefore, it is also necessary to study gene regulatory factors for key genes tested in this study, as this will provide additional information on how gene regualtory network modifies at elevated [CO_2].

4.7 Impact of expression of key genes related to photosynthesis, sucrose metabolism, nitrogen metabolism and cell wall expansion on source-sink interaction of wheat

The key results of this study and their impact on plant source and sink interactions is summarized in Figure 4. Elevated [CO_2] increases the CO_2/O_2 ratio at the site of CO_2 fixation (Bowes, 1991). Therefore, the carboxylation efficiency of Rubisco is improved through the lowering of photorespiration. In the same time, the genes encoding for Rubisco large and small subunits, *rbcL* and *rbcS* substantially upregulated in LFELB, the main source organ. These findings support our previous results that increased assimilations rates requires optimum Rubisco to support the photosynthesis machinery. The chlorophyll fluorescence data supported the higher efficiency of PSII, electron transport rate and photochemical quenching at elevated [CO_2] which may have involved in producing more ATP and NADPH, to fulfil the higher energy requirement to maintain photosynthesis at an optimum rate. This increased photosynthesis at elevated [CO_2] observed in all the wheat lines, and also likely to associated with increased levels of sucrose. A higher expression of *SPS1*, *SPP1* and *SUS1*, the key genes in sucrose metabolism were observed in those lines at elevated [CO_2]. This increased level of sucrose together with other photosynthetic products provide energy and carbon skeletons for amino acid biosynthesis. Respiratory breakdown of sucrose generates 2-oxoglutarate (2-OG) and this serves as the carbon skeleton for the synthesis of glutamate. In the process of NO_3^- photo assimilation, conversion of NO_2^- to NH_4^+ by *NiR* showed an upregulation at elevated [CO_2]. Consequently, the expression of *GS2a*, *GS1a* and *GSr1* was significantly higher in LFELB at elevated [CO_2]. Further genes encoding enzyme Fd-GOGAT and NADH-GOGAT were substantially higher at elevated [CO_2] suggesting that nitrogen assimilation is efficient at early stages of plant development.

In the early vegetative phase, ELB and the SAR can be considered as the major sink organs. In SAR and ELB, sucrose translocated from LFELB is cleaved by sucrose synthase (*SUS1*), which showed a strong upregulation in growing tissues at elevated [CO_2]. The resulting UDP-Glucose plays an important role as a substrate for the re-synthesis of sucrose from available glucose and fructose (Koch, 2004). Also, UDP-Glucose is an important component in cell wall biosynthesis of dividing cells (Verbančič *et al.*, 2017). Increased activity of cell wall metabolism was evident from the higher expression of genes encoding β-expansins and Xyloglucan endotransglucosylase/hydrolases. This implied that cell wall loosening, and expansion has been promoted at elevated [CO_2]. High availability of glucose and fructose of SAR and ELB positively correlates with cell division (Koch, 2004). Therefore, in the SAR and ELB, active utilization of carbohydrates and increased production and expansion of cells, may have facilitated increased plant growth rates at elevated [CO_2].

5. Conclusion

In this study, we investigated the early growth response of wheat under elevated [CO_2] with the aim of understanding the underlying physiological and molecular mechanisms of plant growth. Here we hypothesized that increased carbon assimilation at elevated [CO_2] may alter the carbohydrate pools of plants and thereby influence the key metabolic processes that control the growth of source and sink tissues of plants. We conclude that elevated [CO_2] stimulates the early growth of wheat in a significant manner, but the magnitude of these responses is largely varied between the two parental cultivars. The increased photosynthetic capacity was a key contributor towards this growth response, however, photosynthetic data alone could not explain the growth response to elevated [CO_2]. Elevated [CO_2] always promotes the soluble carbohydrate accumulation and allocation in an organ-specific manner. Consistently, a large amount of carbohydrates was allocated to the sink tissues, particularly in expanding leaf, apical meristem and the cell elongation zone of the expanding leaf. These increased carbon pools significantly influence other post-photosynthetic key metabolic processes, which include sucrose metabolism, nitrogen assimilation and cell elongation and expansion under elevated [CO_2]. The expression of key genes associated with these metabolic processes showed significant variation in different genotypes in an organ-specific manner. The strong positive correlation between sucrose and *SPP1* (sucrose biosynthesis) *TaXTH5* and *TaEXPA1* (cell wall expansion) at elevated [CO_2] further indicated that sugar plays a significant role in altering transcript level responses associated with sucrose metabolism and cell elongation and expansion, and thereby facilitate enhanced plant growth under a CO_2 rich environment. However, these relationships among soluble sugar concentrations and transcript abundance, and their influence on plant growth at elevated [CO_2], are complex and varied even within the same species, making it challenging to establish a generalized holistic mechanism for plant growth responses to elevated [CO_2] across all plant species.

List of Figures and Supplementary Figures

Figure 1 The effect of [CO_2] on **(a)** above ground biomass accumulation , **(b)** leaf area development and **(c)** total soluble carbohydrate content of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from 3 replicates. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Figure 2 The effect of [CO_2] on **(a)** rate of photosynthesis, **(b)** PSII operating efficiency (ϕPSII), **(c)** electron transport rate (ETR), **(d)** photochemical quenching (q_P), **(e)** Non-photochemical quenching (NPQ), **(f)** Maximum PSII efficiency (Fv´/Fm´) of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from 6 replicates. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Figure 3 The effect of [CO_2] on total soluble sugar content of **(a)** last fully expanded leaf blade (LFELB) , **(b)** expanding leaf blade (ELB) and **(c)** cell elongation region (CER) of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from 3 replicates. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Figure 4 Schematic diagram of key gene expression of source (last fully expanded leaf) and sink (expanding leaf and shoot apex region) integration of wheat at elevated [CO_2] in early vegetative stage of wheat. *A*: photosynthesis rate, PSII: photosystem II, ETR: electron transport rate, q_P- photochemical quenching, Glc: glucose, Fru: fructose, Suc: sucrose, F6P- fructose 6 phosphate, UDP-Glc: UDP glucose, Suc6P: sucrose 6 phosphate, G1P: glucose 1 phosohate, UTP: uridine triphosphate, AA- amino acids synthesis & metabolism, *rbcL* - ribulose 1,5 – bisphosphate carboxylase/oxygenase (large sub unit); *rbcS* - ribulose 1,5 –bisphosphate carboxylase/oxygenase (small sub unit); *SPP*- Sucrose Phosphate Phosphatase; *SPS*- Sucrose Phosphate Synthase; *SUS* - Sucrose Synthase type 1; *NiR* - Ferredoxin Nitrite Reductase; *Fd-GOGAT* - Putative ferredoxin-dependent glutamate synthase; *GS2a* - Glutamine Synthetase (Plastidial), *NADH-GOGAT* - Putative NADH-dependent glutamate synthase; *GS1a* -

Glutamine synthetase (cytosolic); *GSr1* - Glutamine synthetase (cytosolic) *TaEXPB* - β – expansins *TaXTH* - Xyloglucan endotransglucosylase/hydrolases

Supplementary Figure S1. The effect of [CO_2] on sucrose content of **(a)** last fully expanded leaf blade (LFELB), **(b)** expanding leaf blade (ELB) and **(c)** cell elongation region (CER); glucose content of **(d)** LFELB, (e) ELB, **(f)** CER; fructose content of **(g)** LFELB, (h) ELB, **(f)** CER of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from 3 replicates. Abbreviations: ns, not significant; *, $P<0.05$; **, $P<0.01$.

List of Tables and Supplementary Tables

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Table 3. The effect of [CO_2] on organ-specific sucrose, glucose and fructose concentrations of different wheat lines selected from a doubled haploid mapping population of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], 700±10 µmol mol^{-1}). Summary of the ANOVA results is shown. Data presented are the mean from n=3 replicates. Abbreviations: LFELB, last fully expanded leaf blade; ELB, expanding leaf blade; CER- cell elongation region; ns, not significant; *, P<0.05; **, P<0.01.

Table 4. Heatmap for transcript abundance of *rbcL and rbcS* genes related to photosynthesis regulation in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Table 5. Heatmap for transcript abundance of *SPP1*, *SPS1* and *SUS1* genes of sucrose metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Table 6. Heatmap for transcript abundance of *NiR, GS2a, GS2b, Fd-GOGAT* genes of nitrogen metabolism in last fully expanded leaf (LFELB) and expanding leaf (ELB) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Table 7. Heatmap for transcript abundance of α and β expansin genes of cell wall metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Table 8. Heatmap for transcript abundance of Xyloglucan endotransglucosylase/hydrolase genes of cell wall metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Supplementary Table S1. CO_2 responsiveness of the selected wheat lines of the double haploid mapping population in the previous experimental trials.

Supplementary Table S2. Controlled environmental conditions provided to the Reach-in plant growth chambers throughout the experimental period.

Supplementary Table S3. List of primer sequences of selected genes for the RT-PCR analysis.

Supplementary Table S4. The effect of [CO_2] on growth-related traits, photosynthetic parameters and chlorophyll fluorescence parameters of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], 700±10 µmol mol^{-1}). Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from n=3 and 6 replicates. Abbreviations: ns, not significant; *, $P<0.05$; **, $P<0.01$.

Supplementary Table S5. Summary of the Analysis of Variance (ANOVA) results for the effect of [CO_2] on different genes tested for two parental lines, Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Supplementary Table S6. Coefficients of correlation (*r*) between the response of sugar content and gene expression to elevated [CO_2]. Data used were the relative change of sugar content between ambient and elevated [CO_2] and the relative expression for each gene under elevated [CO_2]. Measurements were collected from Kukri and RAC875 42 DAP. Significant data are indicated in bold; *, *P* <0.05; **, P<0.01.

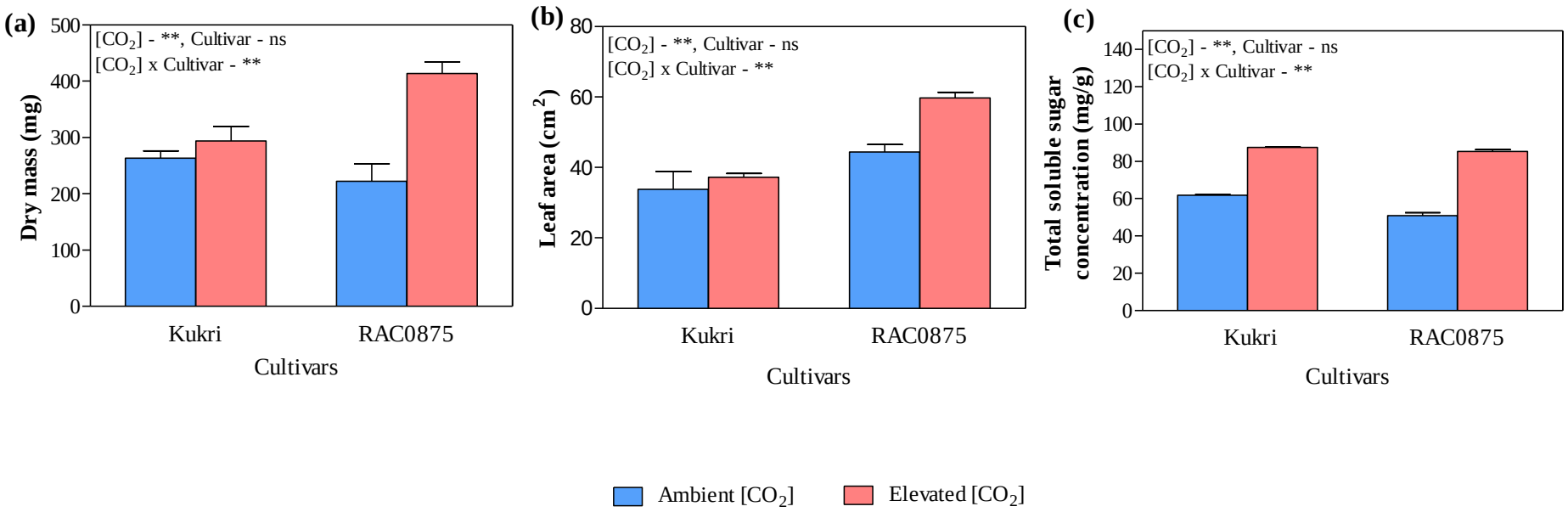
(a)
[CO2] - **, Cultivar - ns
[CO2] x Cultivar - **
Dry mass (mg)
0
100
200
300
400
500
Kukri
RAC0875
Cultivars
(b)
[CO2] - **, Cultivar - ns
[CO2] x Cultivar - **
Leaf area (cm2)
0
20
40
60
80
Kukri
RAC0875
Cultivars
(c)
[CO2] - **, Cultivar - ns
[CO2] x Cultivar - **
Total soluble sugar concentration (mg/g)
0
20
40
60
80
100
120
140
Kukri
RAC0875
Cultivars
Ambient [CO2]
Elevated [CO2]

Figure 1

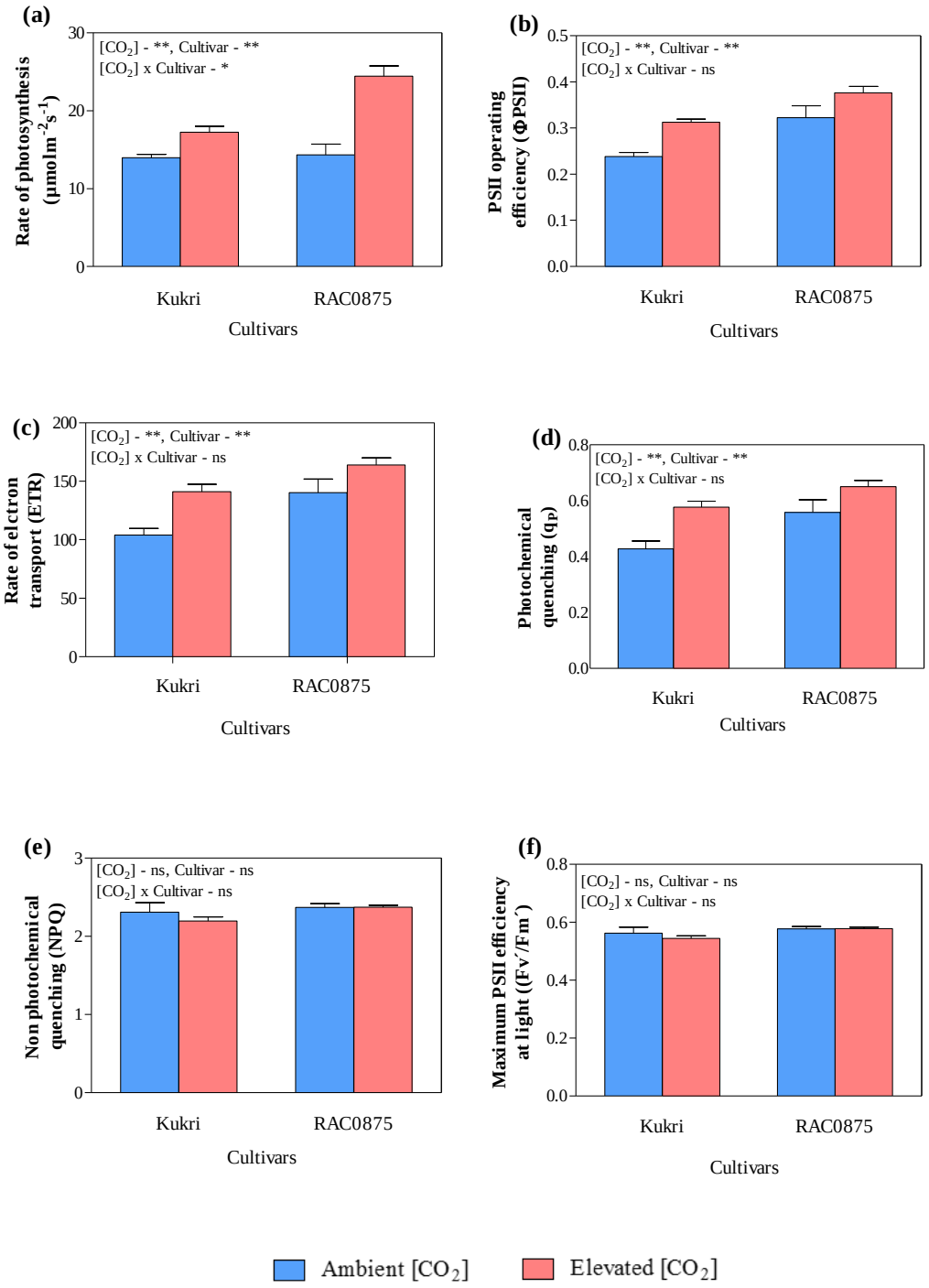
(a)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - *
Rate of photosynthesis (µmolm-2s-1)
Kukri
RAC0875
Cultivars
(b)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - ns
PSII operating efficiency (ΦPSII)
Kukri
RAC0875
Cultivars
(c)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - ns
Rate of elctron transport (ETR)
Kukri
RAC0875
Cultivars
(d)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - ns
Photochemical quenching (qP)
Kukri
RAC0875
Cultivars
(e)
[CO2] - ns, Cultivar - ns
[CO2] x Cultivar - ns
Non photochemical quenching (NPQ)
Kukri
RAC0875
Cultivars
(f)
[CO2] - ns, Cultivar - ns
[CO2] x Cultivar - ns
Maximum PSII efficiency at light ((Fv´/Fm´)
Kukri
RAC0875
Cultivars
Ambient [CO2]
Elevated [CO2]

Figure 2

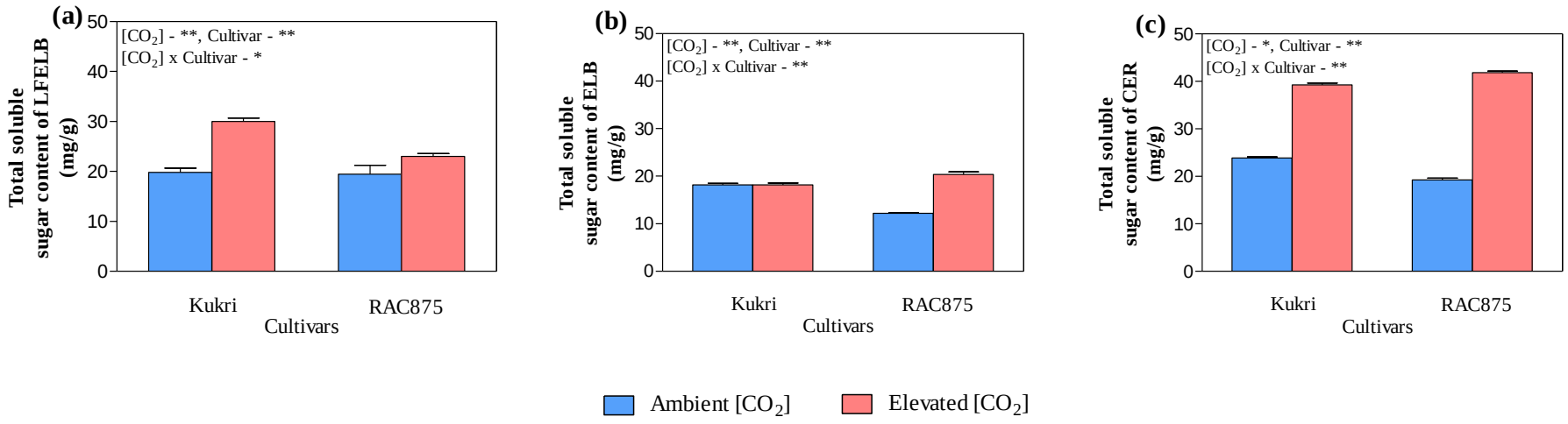
(a)
Total soluble sugar content of LFELB (mg/g)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - *
Kukri
RAC875
Cultivars
(b)
Total soluble sugar content of ELB (mg/g)
[CO2] - **, Cultivar - **
[CO2] x Cultivar - **
Kukri
RAC875
Cultivars
(c)
Total soluble sugar content of CER (mg/g)
[CO2] - *, Cultivar - **
[CO2] x Cultivar - **
Kukri
RAC875
Cultivars
Ambient [CO2]
Elevated [CO2]

Figure 3

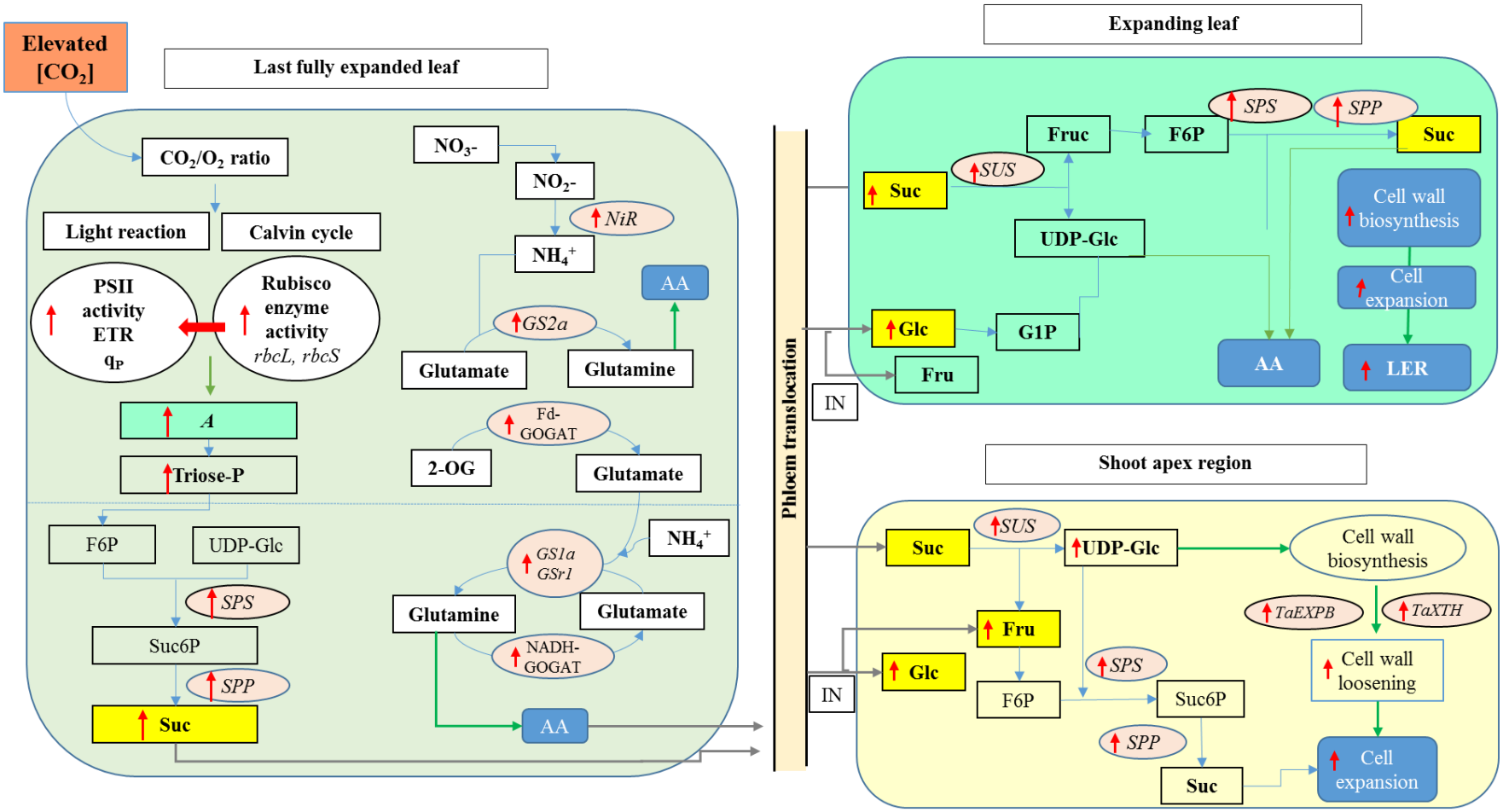
Elevated [CO2]
Last fully expanded leaf
CO2/O2 ratio
Light reaction
Calvin cycle
PSII activity ETR qP
Rubisco enzyme activity rbcL, rbcS
A
Triose-P
F6P
UDP-Glc
SPS
Suc6P
SPP
Suc
NO3-
NO2-
NiR
NH4+
AA
GS2a
Glutamate
Glutamine
Fd-GOGAT
2-OG
Glutamate
NH4+
GS1a GSr1
Glutamine
Glutamate
NADH-GOGAT
AA
Phloem translocation
IN
Expanding leaf
Suc
SUS
Fruc
F6P
SPS
SPP
Suc
UDP-Glc
Cell wall biosynthesis
Cell expansion
Glc
G1P
Fru
AA
LER
IN
Shoot apex region
Suc
SUS
UDP-Glc
Cell wall biosynthesis
TaEXPB
TaXTH
Fru
Glc
SPS
Cell wall loosening
F6P
Suc6P
SPP
Suc
Cell expansion

Figure 4

Table 1. List of selected genes and primer sequences used for the RT-qPCR based gene expression analysis (LFELB: Last fully expanded leaf; ELB: expanding leaf; LCEZ: cell elongation region; SAR: shoot apex region).

Metabolism	Accession No.	Name of the gene	Gene abbreviation	Tissue
Photosynthesis	AY328025	Ribulose 1,5 –bisphosphate carboxylase/oxygenase (large sub unit)	*rbcL*	LFELB, ELB, LCEZ, SAR
	AB020957	Ribulose 1,5 –bisphosphate carboxylase/oxygenase (small sub unit)	*rbcS*	
Carbon metabolism	AF321556	Sucrose Phosphate Phosphatase 1	*SPP1*	LFELB, ELB, LCEZ, SAR
	AF310160	Sucrose Phosphate Synthase 1	*SPS1*	
	AF321556	Sucrose Synthase type 1	*SUS1*	
Nitrogen metabolism	FJ527909	Ferredoxin Nitrite Reductase	*NiR*	LFELB, ELB
	TC394038	Putative ferredoxin-dependent glutamate synthase	*Fd-GOGAT*	
	DQ124212	Glutamine Synthetase (Plastidial) – GS2	*GS2a*	
	DQ124213	Glutamine Synthetase (Plastidial) – GS2	*GS2b*	
	TC387834	Putative NADH-dependent glutamate synthase	*NADH-GOGAT*	
	DQ124209	Glutamine synthetase (cytosolic) – GS1	*GS1a*	
	AY491968	Glutamine synthetase (cytosolic) – GS1	*GSr1*	
Cell wall metabolism	AY589583	α – expansin A1	*TaEXPA1*	LCEZ, SAR
	AY692477	α – expansin A3	*TaEXPA3*	LFELB, ELB, LCEZ, SAR
	AY589578	β – expansin B1	*TaEXPB1*	LCEZ, SAR
	AY589579	β – expansin B2	*TaEXPB2*	
	AY589580	β – expansin B3	*TaEXPB3*	
	AY692478	β – expansin B6	*TaEXPB6*	LFELB, ELB, LCEZ, SAR
	AY260547	β – expansin B23	*TaEXPB23*	
	AY589585	Xyloglucan endotransglucosylase/hydrolase 1	*TaXTH1*	
	AY589586	Xyloglucan endotransglucosylase/hydrolase 2	*TaXTH2*	
	AY589586	Xyloglucan endotransglucosylase/hydrolase 3	*TaXTH3*	
	AY589586	Xyloglucan endotransglucosylase/hydrolase 4	*TaXTH4*	
	AY589586	Xyloglucan endotransglucosylase/hydrolase 5	*TaXTH5*	

Table 2. The effect of [CO_2] on aboveground biomass accumulation, below ground biomass accumulation, total soluble sugar content, total leaf area per plant and photosynthesis rate of different wheat lines selected from doubled haploid mapping population of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2],400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], 700±10 µmol mol^{-1}). Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from n=3 replicates. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Trait	**Mean CO_2 effect**			**Mean genotype effect**	**Interaction effect [CO_2]x Cultivar**	**Mean Responsiveness to e[CO_2] (%)**	
	a[CO_2]	**e[CO_2]**	**ANOVA result**	**ANOVA result**	**ANOVA result**	**High responsive lines**	**Low responsive lines**
Above ground biomass (mg/plant)	142.80	294.13	**	**	**	225.38	31.30
Below ground biomass (mg/plant)	44.73	81.69	**	**	**	175.67	34.74
Total soluble sugar content (mg/g)	71.88	84.56	**	**	**	19.75	14.96
Total leaf area (cm^2/plant)	43.37	44.19	ns	**	**	27.58	-17.99
Photosynthesis rate (µmol CO_2 $m^{-2}s^{-1}$)	14.97	15.25	ns	**	*	8.82	-12.16

Table 3. The effect of [CO_2] on organ-specific sucrose, glucose and fructose concentrations of different wheat lines selected from a doubled haploid mapping population of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], 700±10 µmol mol^{-1}). Summary of the ANOVA results is shown. Data presented are the mean from n=3 replicates. Abbreviations: LFELB, last fully expanded leaf blade; ELB, expanding leaf blade; CER- cell elongation region; ns, not significant; *, P<0.05; **, P<0.01.

		Mean CO_2 effect							**ANOVA results**					
		a[CO_2]			**e[CO_2]**				**[CO_2] effect**	**Genotype effect**	**Organ effect**	**[CO_2]x Cultivar x Organ**		
Line	**Plant organ**	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose	**Responsiveness to e[CO_2] (%)**	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose
H_1	LFELB	5.88	16.26	3.94	3.57	13.53	2.45	31.50	**	**	**	**	**	**
	ELB	0.77	12.42	6.64	2.88	9.28	10.49							
	CER	6.59	13.17	11.32	8.54	27.22	14.72							
H_2	LFELB	5.10	16.31	3.51	15.74	10.64	9.20	25.05	**	**	**	**	**	**
	ELB	0.79	14.75	2.78	5.97	10.57	9.12							
	CER	9.62	19.04	5.69	4.38	33.02	9.02							
H_3	LFELB	3.11	14.99	2.50	11.62	11.59	7.10	3.74	**	**	**	**	**	**
	ELB	1.54	15.60	3.63	1.81	9.23	9.85							
	CER	5.55	12.97	9.65	5.01	20.88	4.78							
H_4	LFELB	6.54	13.06	3.55	6.74	9.46	4.15	1.77	**	**	**	**	**	**
	ELB	2.93	11.67	4.67	3.98	10.77	3.96							
	CER	5.15	18.42	6.51	6.27	18.00	7.66							
L_1	LFELB	3.08	14.52	1.74	16.75	9.35	9.07	-5.48	**	**	**	**	**	**
	ELB	2.33	13.87	5.02	2.57	7.21	10.85							
	CER	7.61	12.67	12.17	3.53	21.00	4.53							

L_2	LFELB	3.14	13.24	2.60	11.70	11.17	6.27	2.90	**	**	**	**	**	**
	ELB	2.09	13.55	5.24	3.02	9.40	10.40							
	CER	6.14	15.93	9.10	5.73	22.02	3.54							
L_3	LFELB	0.58	13.58	1.07	10.64	9.16	6.23	0.90	**	**	**	**	**	**
	ELE	2.30	13.18	6.28	3.24	8.76	10.04							
	CER	3.95	20.83	4.40	3.90	23.01	2.58							
L_4	LFELB	3.17	14.02	2.53	8.49	9.58	4.82	4.63	**	**	**	**	**	**
	ELB	3.16	13.03	5.62	5.88	10.67	8.61							
	CER	3.22	22.60	2.40	3.07	22.85	2.18							

Table 4. Heatmap for transcript abundance of *rbcL and rbcS* genes related to photosynthesis regulation in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Organ type	**Gene Name**	**Gene expression level**				**Log_2 fold change**	
		Kukri		**RAC875**		**Kukri**	**RAC875**
		a[CO_2]	**e[CO_2]**	**a[CO_2]**	**e[CO_2]**	**e[CO_2]/a[CO_2]**	
LFELB	*rbcL*	1.73	3.62	4.25	6.21	1.06	0.55
	rbcS	0.26	2.38	1.88	4.61	3.22	1.29
ELB	*rbcL*	2.70	3.69	1.40	3.12	0.45	1.16
	rbcS	1.26	2.65	1.42	2.00	1.07	0.49
LCEZ	*rbcL*	3.67	2.79	1.87	2.78	-0.40	0.57
	rbcS	2.01	2.41	1.44	2.75	0.26	0.94
SAR	*rbcL*	0.73	1.53	0.55	1.69	1.06	1.61
	rbcS	3.68	3.77	4.35	9.09	0.04	1.06

Table 5. Heatmap for transcript abundance of *SPP1*, *SPS1* and *SUS1* genes of sucrose metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Organ type	Gene Name	Gene expression level				Log_2 fold change	
		Kukri		RAC875		Kukri	RAC875
		a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	e[CO_2]/a[CO_2]	
LFELB	*SPP1*	6.84	5.78	8.42	45.78	-0.24	2.44
	SPS1	1.51	5.57	3.70	17.76	1.88	2.26
	SUS1	0.32	0.82	1.09	2.18	1.36	1.01
ELB	*SPP1*	4.47	4.48	3.48	10.32	0.00	1.57
	SPS1	0.95	1.50	1.26	1.27	0.65	0.02
	SUS1	0.43	1.81	0.56	1.50	2.07	1.43
LCEZ	*SPP1*	0.34	0.27	0.12	0.62	-0.32	2.38
	SPS1	1.86	2.22	2.61	6.06	0.25	1.21
	SUS1	4.27	3.91	1.66	3.63	-0.13	1.13
SAR	*SPP1*	0.05	0.23	0.07	0.22	2.18	1.58
	SPS1	0.91	1.69	0.71	1.72	0.89	1.27
	SUS1	5.05	14.08	3.53	9.31	1.48	1.40

Table 6. Heatmap for transcript abundance of *NiR*, *GS2a*, *GS2b*, *Fd-GOGAT* genes of nitrogen metabolism in last fully expanded leaf (LFELB) and expanding leaf (ELB) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Organ type	Gene Name	Gene expression level				Log_2 fold change	
		Kukri		RAC875		Kukri	RAC875
		a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	e[CO_2]/a[CO_2]	
LFELB	*NiR*	0.07	0.65	0.21	0.60	3.30	1.54
	Fd-GOGAT	2.91	11.92	8.45	21.39	2.04	1.34
	GS2a	0.89	5.23	1.69	2.76	2.56	0.71
	GS2b	0.00	0.02	0.02	0.05	2.44	1.73
	GS1a	3.96	3.56	4.84	41.50	-0.15	3.10
	GSr1	0.14	0.57	0.16	3.37	2.05	4.39
	NADH-GOGAT	0.10	0.15	0.19	1.79	0.56	3.26
ELB	*NiR*	0.30	0.56	0.41	1.19	0.89	1.55
	Fd-GOGAT	13.24	29.92	15.61	28.57	1.18	0.87
	GS2a	24.98	46.56	14.65	26.80	0.90	0.87
	GS2b	0.09	0.15	0.11	0.32	0.70	1.49
	GS1a	3.36	4.28	3.15	4.26	0.35	0.44
	GSr1	0.33	0.68	0.25	0.27	1.04	0.12
	NADH-GOGAT	0.29	0.71	0.26	0.26	1.31	0.04

Table 7. Heatmap for transcript abundance of α and β expansin genes of cell wall metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Organ type	Gene Name	Gene expression level				Log_2 fold change	
		Kukri		RAC875		Kukri	RAC875
		a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	e[CO_2]/a[CO_2]	
LFELB	*TaEXPA3*	0.01	0.03	0.46	0.95	1.31	1.05
	TaEXPB6	0.05	0.05	0.76	0.74	0.17	-0.03
	TAEXPB23	0.01	0.05	0.11	0.38	2.48	1.82
ELB	*TaEXPA3*	1.32	1.23	1.77	5.48	-0.10	1.63
	TaEXPB6	6.11	7.51	7.46	17.14	0.30	1.20
	TAEXPB23	11.55	28.49	13.23	13.55	1.30	0.03
LCEZ	*TaEXPA1*	0.03	0.02	0.04	0.06	-0.80	0.57
	TaEXPA3	0.06	0.39	0.38	0.44	2.71	0.20
	TAEXPB1	0.10	0.13	0.17	0.23	0.36	0.41
	TAEXPB2	0.76	2.43	0.84	1.74	1.68	1.04
	TAEXPB3	0.46	0.73	0.60	1.37	0.68	1.20
	TAEXPB6	0.64	1.82	3.15	6.19	1.51	0.98
	TAEXPB23	2.65	10.46	3.58	8.43	1.98	1.24
SAR	*TaEXPA1*	0.05	0.03	0.02	0.03	-0.63	0.69
	TaEXPA3	1.19	2.65	2.94	4.91	1.16	0.74
	TAEXPB1	4.99	3.32	1.19	5.58	-0.59	2.23
	TAEXPB2	0.52	2.94	0.88	3.94	2.50	2.16
	TAEXPB3	0.28	1.57	0.65	2.30	2.50	1.83

	TAEXPB6	4.10	5.12	6.25	9.33	0.32	0.58
	TAEXPB23	3.40	9.94	3.98	14.82	1.55	1.90

Table 8. Heatmap for transcript abundance of Xyloglucan endotransglucosylase/hydrolase genes of cell wall metabolism in last fully expanded leaf (LFELB), expanding leaf (ELB), cell elongation region (LCEZ) and shoot apex region (SAR) of Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript relative abundance at elevated [CO_2] are presented in Log_2 scale.

Organ type	Gene Name	Gene expression level				Log_2 fold change	
		Kukri		RAC875		Kukri	RAC875
		a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	e[CO_2]/a[CO_2]	
LFELB	*TaXTH1*	0.63	0.65	5.83	5.33	0.04	-0.13
	TaXTH2	2.26	2.10	4.32	5.90	-0.11	0.45
	TaXTH3	0.78	0.40	1.71	2.04	-0.96	0.25
	TaXTH4	0.01	0.01	0.16	0.03	-0.60	-2.64
	TaXTH5	0.05	2.12	0.12	0.45	5.42	1.96
ELB	*TaXTH1*	39.54	46.23	23.16	66.77	0.23	1.53
	TaXTH2	36.34	65.60	46.83	94.22	0.85	1.01
	TaXTH3	26.76	8.70	4.31	36.13	-1.62	3.07
	TaXTH4	0.61	0.40	0.88	1.92	-0.60	1.12
	TaXTH5	1.94	2.58	0.94	2.44	0.42	1.37
LCEZ	*TaXTH1*	10.53	16.27	30.98	36.44	0.63	0.23
	TaXTH2	4.75	6.75	9.68	17.91	0.51	0.89
	TaXTH3	0.91	1.30	1.82	6.77	0.52	1.90
	TaXTH4	0.17	1.12	3.19	2.67	2.75	-0.26
	TaXTH5	2.28	5.29	2.49	6.38	1.22	1.36
SAR	*TaXTH1*	5.00	6.91	3.46	21.07	0.47	2.60
	TaXTH2	6.60	4.49	3.90	13.09	-0.55	1.75
	TaXTH3	2.40	1.87	0.87	3.24	-0.36	1.89
	TaXTH4	2.04	1.87	9.46	17.23	-0.12	0.86
	TaXTH5	46.11	46.87	17.38	61.97	0.02	1.83

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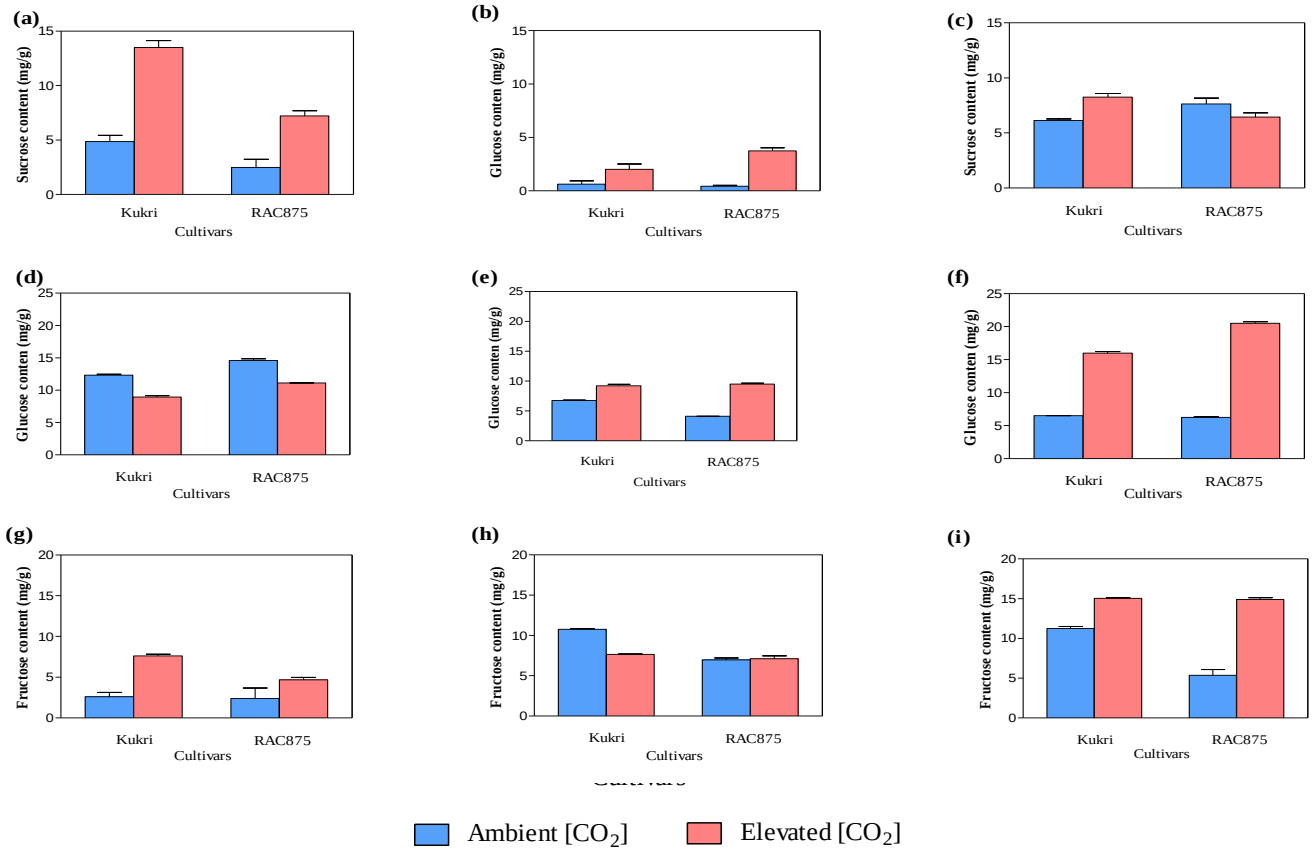
Supplementary Data



Supplementary Figure S1. The effect of [CO_2] on sucrose content of **(a)** last fully expanded leaf blade (LFELB), **(b)** expanding leaf blade (ELB) and **(c)** cell elongation region (CER); glucose content of **(d)** LFELB, (e) ELB, **(f)** CER; fructose content of **(g)** LFELB, (h) ELB, **(f)** CER of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from 3 replicates. Abbreviations: ns, not significant; *, $P<0.05$; **, $P<0.01$.

Supplementary Table S1. CO_2 responsiveness of the selected wheat lines of the double haploid mapping population in the previous experimental trials.

	Reference name	**Double haploid ID**	**CO_2 responsiveness of aboveground biomass accumulation**	**CO_2 responsiveness of below ground biomass accumulation**
High responsive wheat lines	H_1	DH_R241	260.53%	305.94%
	H_2	DH_R281	281.58%	335.77%
	H_3	DH_R181	364.09%	284.60%
	H_4	DH_R186	281.98%	352.34%
Less responsive wheat lines	L_1	DH_R092	55.87%	55.19%
	L_2	DH_R009	40.86%	41.26%
	L_3	DH_R215	30.39%	-4.73%
	L_4	DH_R220	52.75%	51.67%

Supplementary Table S2. Controlled environmental conditions provided to the Reach-in plant growth chambers throughout the experimental period.

Time Period	Temperature	Relative humidity	$[CO_2]$ concentration		Light intensity
			Ambient $[CO_2]$	Elevated $[CO_2]$	
06:00 hrs	15°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
08:00 hrs	20°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
11:00 hrs	23°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
15:00 hrs	20°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
18:00 hrs	15°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)
20:00 hrs	13°C	70%	400 µmolmol^{-1}	700 µmolmol^{-1}	Partial (40%)

Supplementary Table S3. List of primer sequences of selected genes for the RT-PCR analysis.

Metabolism	Accession No.	Gene abbreviation	Forward primer sequence (5'-3' sequence)	Reverse primer sequence (5'-3' sequence)	Reference
Photosynthesis	AY328025	*rbcL*	GGCTGCAGTAGCTGCCGAATCT	TCCCCAGCAACAGGCTCGATGT	*Vicente et al, 2015*
	AB020957	*rbcS*	*AGCCTCAGCAGCGTCAGCAAT*	*CGTGGATAGGGGTGGCAGGTAAGA*	
Carbon metabolism	AF321556	*SPP1*	*GCGCACGGGAAGGAGTTTTTCTTCT*	*GACCTCCGTAGACATCATCCAGCCC*	*Vicente et al, 2015*
	AF310160	*SPS1*	*AGAAGGCTCTGCCTCCCATTTGGTC*	*AGGATCATCGGCTTGTGCGGGTT*	
	AF321556	*SUS1*	*GTATGTTCACCAGGGCAAGGGCA*	*GGCGTCAAACTCAGCAAGCAGC*	
Nitrogen metabolism	FJ527909	*NiR*	*AACCTCCTCTCCTCCTACATCA*	*CCTAGGAAGGTTGGTGATGGC*	*Vicente et al, 2015*
	TC394038	*Fd-GOGAT*	*CGGCAATGGAGGCTGAGCAACA*	*TGAGCCTGCTCGATGGTCACTGT*	
	DQ124212	*GS2a*	*CTCATGGTGTGTTGCGAACC*	*GGTCCTCCAGGTATCCTTTGC*	
	DQ124213	*GS2b*	*TGAAGGAAACGAGCGGAGAC*	*CTCGCCCCACACGAATAGAG*	
	TC387834	*NADH-GOGAT*	*GCCATTGAATCAGTTCCAGGGCCAC*	*GCCAGCACCTGAGCTTTCCTGATG*	
	DQ124209	*GS1a*	*AGGTCATCGTGGATGCCGTGGA*	*TTTGCGACGCCCCAGCTGAA*	
	AY491968	*GSr1*	*AAGGGCTACTTCGAGGACCGCA*	*ATGATCTGGCGGCGGTAGGCAT*	*Liu et al, 2007*
Cell wall metabolism	AY589583	*TaEXPA1*	*CCACCAACAAGCAGTTCTCTTAATT*	*CATACATCCCCACAAAAAAAGGAC*	
	AY692477	*TaEXPA3*	*GTCTGTGTTGGTGTGTTTTTCCC*	*AACCATCACCTCTTTACCCTAATCA*	
	AY589578	*TaEXPB1*	*TACAGATCCCTGGTCCAGTTCG*	*CATCATAGGTAGAACAAGACGACGA*	
	AY589579	*TaEXPB2*	*CTCCATCGTCCAGTACAGCTGA*	*CATGATTCCAAATGATGAGTTCG*	
	AY589580	*TaEXPB3*	*CTGCTGGCTACGATTAATTGCTC*	*CCCACGCAAAATACAAAAGATAAA*	
	AY692478	*TaEXPB6*	*TGCCTTATGTAGCAGGGTGAGAC*	*GTACACGATGGACGACGACACTA*	
	AY260547	*TaEXPB23*	*CATGCGCATCACCAACGAGT*	*TGGACGATGGAGCGGTAGAAG*	*Yang Han et al 2012*
	AY589585	*TaXTH1*	*CGAGAGCAAGTACATGTCCTACGA*	*GAAAGGAAGAAACTGATGGACGAT*	*Liu et al, 2007*
	AY589586	*TaXTH2*	*ATCCAGCGTCAATTCCTTCCTT*	*TCGAATCGAATGGACAAACAAG*	
	AY589586	*TaXTH3*	*CGAGAGCAAGTACATGTCCTACGA*	*AAGAAAGGAAGAATTTGATGATGGA*	
	AY589586	*TaXTH4*	*TCATTGATTAATTTCTTCCGTTGCT*	*AAGATGGGATGAACAAGAAGAACAA*	
	AY589586	*TaXTH5*	*CCGTCATCTGAGATGTGTTTTGTT*	*CATTTACACCATCCGACAGAGCT*	

Reference genes		*ADP-ribosylation factor*	*GCTCTCCAACAACATTGCCAAC*	*GCTTCTGCCTGTCACATACGC*	*Vicente et al, 2015*
		α – Tubulin	*TCTCATGTACCGTGGTGATGTTG*	*AACTGAATAGTGCGCTTGGTCTT*	*Liu et al, 2007*

Supplementary Table S4. The effect of [CO_2] on growth-related traits, photosynthetic parameters and chlorophyll fluorescence parameters of Kukri and RAC875 grown at ambient [CO_2] (a[CO_2], 400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], 700±10 µmol mol^{-1}). Measurements were taken at 42 DAP. Summary of the ANOVA results is shown. Data presented are the mean from n=3 and 6 replicates. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Trait	**Mean CO_2 effect**			**Mean cultivar effect**			**Interaction effect (ANOVA results)**
	a[CO_2]	**e[CO_2]**	**ANOVA result**	**Kukri**	**RAC875**	**ANOVA result**	**[CO_2]x Cultivar**
Growth-related traits							
Above ground biomass (mg/plant)	242.96	353.70	**	278.8	317.86	ns	**
Total soluble sugar content (mg/g)	56.38	86.38	**	74.68	68.09	**	**
Total leaf area (cm^2/plant)	39.08	48.49	**	35.48	52.09	**	ns
Photosynthesis parameters							
Photosynthesis rate (µmol CO_2 m^{-2} s^{-1})	14.15	20.85	**	15.61	19.38	**	*
g_s (mol H_2O m^{-2} s^{-1})	0.31	0.22	ns	0.26	0.27	ns	*
C_i (µmol CO_2 mol^{-1})	295.50	524.33	**	411.33	408.5	ns	ns
Transpiration rate (mmol H_2O m^{-2} s^{-1})	4.46	3.75	ns	3.89	4.33	ns	*
Chlorophyll fluorescence parameters							

PSII operating efficiency (ϕPSII)	0.28	0.34	**	0.27	0.34	**	ns
Electron transport rate (ETR)	122.27	152.72	**	122.70	152.29	**	ns
Photo-chemical quenching (q_P)	0.49	0.61	**	0.50	0.60	**	ns
Non-photochemical quenching (NPQ)	2.34	2.28	ns	2.25	2.37	ns	ns
Maximum PSII efficiency (Fv´/Fm´)	0.56	0.56	ns	0.55	0.57	ns	ns

Supplementary Table S5. Summary of the Analysis of Variance (ANOVA) results for the effect of [CO_2] on different genes tested for two parental lines, Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Abbreviations: ns, not significant; *, P<0.05; **, P<0.01.

Metabolism	Name of the gene	Cultivar effect	[CO_2] effect	Organ effect	Cultivar x [CO_2]	Cultivar x [CO_2] x Organ
Photosynthesis	*rbcL*	ns	**	**	ns	ns
	rbcS	**	**	**	ns	**
Carbon metabolism	*SPP1*	ns	**	**	ns	ns
	SPS1	**	**	**	ns	ns
	SUS1	ns	**	**	ns	ns
Nitrogen metabolism	*NiR*	*	**	*	ns	ns
	Fd-GOGAT	*	**	*	ns	ns
	GS2a	ns	**	*	*	**
	GS2b	*	**	*	ns	ns
	NADH-GOGAT	*	**	ns	ns	ns
	GS1a	*	**	*	*	**
	GSr1	ns	**	ns	ns	ns
Cell wall metabolism	*TaEXPA1*	ns	ns	ns	ns	ns
	TaEXPA3	*	**	**	ns	ns
	TaEXPB1	ns	ns	**	*	*
	TaEXPB2	ns	**	ns	ns	ns
	TaEXPB3	*	**	ns	ns	ns
	TaEXPB6	*	**	**	ns	ns
	TaEXPB23	*	**	**	ns	ns
	TaXTH1	*	**	**	ns	ns
	TaXTH2	*	**	**	*	ns
	TaXTH3	*	**	**	*	
	TaXTH4	ns	ns	**	ns	*
	TaXTH5	*	**	**	ns	*

Supplementary Table S6. Coefficients of correlation (*r*) between the response of sugar content and gene expression to elevated [CO_2]. Data used were the relative change of sugar content between ambient and elevated [CO_2] and the relative expression for each gene under elevated [CO_2]. Measurements were collected from Kukri and RAC875 42 DAP. Significant data are indicated in bold; *, *P* <0.05; **, P<0.01.

	SPP1	***TaEXPA1***	***TaEXPB1***	***TaEXPB2***	***TaEXPB3***	***TaEXPB6***	***TaEXPB23***	***TaXTH1***	***TaXTH2***	***TaXTH3***	***TaXTH4***	***TaXTH5***
Sucrose	-0.354	0.994*	0.701	-0.444	-0.55	0.90	-0.74*	0.72	0.142	0.156	-0.043	0.044
Glucose	-1.38	0.994*	0.701	-0.444	-0.55	0.625	-0.307	0.606	0.578	0.512	-0.207	0.816*
Fructose	0.783*	0.994*	0.701	-0.444	-0.55	-0.382	0.571	-0.45	-0.71	0.243	0.186	-0.472
Total sugar	0.093	0.994*	0.701	-0.444	-0.55	-0.45	0.553	0.553	0.500	0.740*	-0.109	0.420

Chapter 5

Leaf proteome responses of wheat (*Triticum aestivum* L.) to elevated atmospheric carbon dioxide during early vegetative growth

In this study, a comparative proteomics analysis was performed to identify differentially expressed proteins under elevated [CO_2]. This chapter aims to provide information on the most active metabolic proteins, regulatory enzymes and other biomolecules involved in key metabolic processes and thus, will be beneficial in dissecting the growth mechanism of plants in a high CO_2 environment. The experiments in this chapter were conducted with the parental lines of the doubled haploid mapping population used in the previous chapters. Comparative proteomics analysis was conducted for the expanding leaf blades of young wheat seedlings to develop an understanding of which metabolic processes are more active in the growing tissues when exposed to elevated [CO_2]. Protein expression at elevated [CO_2] was compared with protein expression at ambient [CO_2] to determine the effect of elevated [CO_2] on the regulation of protein levels. Proteins of key metabolic processes that showed significant change at elevated [CO_2] are reported in this chapter. This chapter has been prepared as a research article to be submitted to "Journal of Experimental Botany".

Gamage D, Thompson M, Dehigaspitiya P, Faou P, Rajapaksha KH, Downs R, Sutherland M.W., Hirotsu N, Seneweera S, Leaf proteome responses of wheat (*Triticum aestivum* L.) to elevated atmospheric carbon dioxide during early vegetative growth (Prepared for publication)

Article title:

Leaf proteome responses of wheat (*Triticum aestivum* L.) to elevated atmospheric carbon dioxide during early growth

Running title: Leaf proteome responses to elevated [CO_2]

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Abbreviations:

[CO_2] – Carbon dioxide concentration

Rubisco - Ribulose 1,5-bisphosphate carboxylase/oxygenase

LFELB - Last fully expanded leaf blade

ELB - Expanding leaf blade

CE – cell elongation region

Highlight:

Elevated [CO_2] alters protein expression related to synthesis and respiratory breakdown of carbohydrates, protein synthesis, cell division and cell elongation and thereby influence plant growth at early vegetative stages.

Abstract

The impact of rising atmospheric carbon dioxide [CO_2] on the proteome of the growing leaf blade was investigated using two winter wheat cultivars: Kukri and RAC875, focusing on the early vegetative stage. Two wheat genotypes were grown at ambient (400 µmol mol^{-1}) and elevated (700 µmol mol^{-1}) [CO_2] in controlled environmental conditions. Elevated [CO_2] increased the rate of photosynthesis and biomass production in Kukri by 16.4 and 32.6%, respectively, when compared with 20 and 48% in RAC875. The nitrogen and protein concentrations in the expanding leaf blades of both cultivars were high; however, RAC875 showed the highest nitrogen percentage (56.3%). Results of the comparative proteomics analysis showed that leaf proteome responses at elevated [CO_2] were genotype dependent and the proteome composition was altered at elevated [CO_2]. Most of the differentially expressed proteins at elevated [CO_2] belonged to carbon metabolism, energy pathways, protein synthesis and cell cycle functions. Additionally, several proteolytic enzymes involved in post-translational modifications of proteins, antioxidant enzymes and molecular chaperones showed a noteworthy upregulation at elevated [CO_2] in both cultivars. These findings suggest that photosynthetic stimulation and lower stomatal conductance are not the only factors governing plant growth at elevated [CO_2]. In response to increased sugar supply to developing leaves at elevated [CO_2], other key regulatory processes such as cell cycle function, protein and cell redox homeostasis tend to be modified, significantly altering growth responses at the whole plant level.

Keywords: Carbon metabolism, Expanding leaf blade, Leaf proteome, Plant growth, Protein

Introduction

The rapid increase in industrialization and population growth has led to significant increases in carbon dioxide concentrations ([CO_2]) in the atmosphere. The current atmospheric [CO_2] has already exceeded 400 µmol mol^{-1} (Tans and Keeling, 2018) and is predicted to double by the end of this century (IPCC, 2014). As one of the prominent greenhouse gases, this increase in [CO_2] leads to changes in the global climate, causing significant changes in temperature and precipitation patterns and subsequently influencing agricultural productivity (Ainsworth *et al.*, 2008; Solomon *et al.*, 2007). On the other hand, increasing [CO_2] is beneficial for plants as it is the primary substrate for photosynthesis. Therefore, elevated [CO_2] could potentially offset losses in agricultural production caused by increased drought and high-temperature incidences (Ainsworth *et al.*, 2008). However, it will be highly challenging to capitalize CO_2 enrichment for crop improvement because of the complex relationships among elevated [CO_2], photosynthesis, plant growth and yield responses (Gamage *et al.*, 2018; Thompson *et al.*, 2017). This has led researchers to investigate the underlying physiological and molecular mechanisms of plant growth responses to elevated [CO_2] as the first step in gaining a better understanding of maximizing future crop production in a changing climate.

The effect of elevated [CO_2] is more pronounced in C_3 crops, including critical cereal crops such as rice and wheat, stimulating their growth and yield (Seneweera, 2011; Seneweera and Conroy, 2005; Thilakarathne *et al.*, 2013). Generally, plants respond to elevated [CO_2] through increased photosynthesis and reduced stomatal conductance (Ainsworth and Rogers, 2007). These two primary processes are central to all the other effects observed in plant communities in response to high CO_2 conditions. The increase in photosynthesis of C_3 crops is mainly due to the increased carboxylation of Ribulose 1,5- bisphosphate carboxylase/oxygenase (Rubisco), which is not saturated under current CO_2 partial pressure (Lorimer, 1981; Makino and Mae, 1999). The higher availability of CO_2 at the site of fixation also suppresses photorespiration, which facilitates Rubisco carboxylation (Makino and Mae, 1999). Theoretically, photosynthesis of a mature sunlit leaf will increase by approximately 38% at elevated [CO_2] (Long *et al.*, 2004). Conversely, stomatal conductance of both C_3 and C_4 crops tend to decrease at elevated [CO_2], which significantly reduces plant evapotranspiration and leads to improvements in plant water use efficiency (Ainsworth and Long, 2005; Leakey *et al.*, 2009).

The direct effects of elevated [CO_2], mainly the stimulation of photosynthesis, provides the fundamental basis for modifications to key post-photosynthetic metabolic pathways associated with plant growth and development (Pritchard *et al.*, 1999; Taylor *et al.*, 1994). Carbon/nitrogen metabolism, cell cycle and cell wall metabolism, along with hormonal regulation, are the main metabolic networks involved in plant growth responses at elevated [CO_2] (Gamage *et al.*, 2018). For any organism to maximize its fitness in response to different environmental conditions, precise regulation and coordination of these metabolic networks to a particular environment is necessary (Gaudinier *et al.*, 2015). Thus, in response to environmental stimuli, such as elevated [CO_2], plants tend to change their transcriptional and translational regulation, which thereby affects protein synthesis that governs plant growth and development. This protein network governing plant growth at elevated [CO_2] involves a complex interplay of different regulatory enzymes, components of signal transduction mechanisms and other important biomolecules, such as plant growth regulators, which collectively function in response to external environmental stimulation (Bokhari *et al.*, 2007).

The CO_2 responsiveness of C_3 plants is generally higher during the early growth stages and is affected by the plants' sink capacity and nutritional status (Li *et al.*, 2008; Makino and Mae, 1999; Seneweera *et al.*, 2002). In the early growth stages, plants are able to generate more sink organs to utilize the increased carbon supply at elevated [CO_2] (Jitla *et al.*, 1997; Thilakarathne *et al.*, 2015). Higher relative growth rates and higher net assimilation rates have been observed for rice (Jitla *et al.*, 1997; Makino and Mae, 1999) and wheat (Hikosaka *et al.*, 2005; Neales and Nicholls, 1978) in the early growth stages at elevated [CO_2]. In general, it has been reported that even a 10% increase in the RGR of plants grown at elevated [CO_2] can translate into an absolute growth increment of up to 50% during the exponential growth phase of plants (Kirschbaum, 2010). Therefore, understanding how plants respond to elevated [CO_2], particularly during the early growth stages, is essential for breeding crops for a future CO_2 rich atmosphere.

A number of research studies have investigated the underlying physiological (For a review: Poorter and Nagel, 2000; Poorter and Navas, 2003) and molecular mechanisms (For a review: Gamage *et al.*, 2018) of plant growth responses to elevated [CO_2]. The physiological responses of crops, including wheat (Seneweera, 2011; Seneweera and Conroy, 2005; Thilakarathne *et al.*, 2015; Thilakarathne *et al.*, 2013), have been extensively studied to gain an understanding of how plants adapt to the high CO_2 environment. Despite the importance of understanding the

regulatory mechanisms of plant growth response to elevated CO_2, the activity of different regulatory enzymes and other proteins involved in these physiological processes have not been thoroughly investigated (Bokhari *et al.*, 2007). Without this information, it is very difficult to develop a complete understanding of the interplay of protein networks among key metabolic pathways and how they determine plant growth responses to external environmental signals such as elevated [CO_2]. Therefore, a fundamental understanding of proteome-wide responses of plants to elevated [CO_2] will provide a unique opportunity to unravel the mechanisms of how plants respond to elevated [CO_2] (Hashiguchi *et al.*, 2010; Hossain *et al.*, 2011).

The array of proteins within a cell or an organ, their interactions and modifications, hold the key to understanding the responses of a particular biological system under stressed or non-stressed conditions (Salekdeh and Komatsu, 2007). With recent technological advances in proteomic science, it is relatively easy to identify proteins, expression profiles, post-translational modifications and protein-protein interactions under different environmental conditions (Hashiguchi *et al.*, 2010). Further, proteomic analysis highly beneficial in identifying the possible candidate genes that can be used as genetic markers in breeding crops for future climate (Ahmad *et al.*, 2016).

In this study, we investigated the proteome-wide changes in young wheat leaves (*Triticum aestivum* L.) in order to understand the underlying molecular mechanisms of plant growth responses to elevated [CO_2]. To the best of our knowledge, there has been no published report to date using a comparative proteomic approach to study the growth responses of wheat to elevated [CO_2] focusing on the early vegetative stage. Our study mainly focused on understanding the functions of sink tissues of plants at elevated [CO_2] during the early growth stages. Further, the expression patterns of the differentially expressed proteins identified at both elevated and ambient [CO_2], and their physiological implications, are discussed with a mechanistic perspective in this paper.

Materials and methods

Plant materials and environmental conditions

A solution culture experiment was carried out using two wheat (*Triticum aestivum* L.) cultivars, Kukri and RAC875, under ambient (400 µmol mol^{-1}) and elevated [CO_2] (700±10 µmol mol^{-1}) in two identical controlled environment growth chambers (Reach in growth chambers, PGC-105, Percival, USA) at the University of Southern Queensland, Australia. These two cultivars are the parental lines of a doubled haploid mapping population with contrasting water use efficiencies, nitrogen use efficiencies and growth characteristics to elevated [CO_2]. Plants were provided with a 14-hour photoperiod with a day and night temperature of 23ºC and 13ºC, respectively. The relative humidity of both chambers was maintained at 70% throughout the experiment and the light intensity was maintained at 1000 µmolm^{-2}s^{-1} at midday throughout the growing period.

Seedling growth

Seeds from wheat (*Triticum aestivum* L.) cultivars, Kukri and RAC875, were surface sterilized using 2.6% NaClO for 1 minute and washed thoroughly using Milli-Q water. The sterilized seeds were then allowed to germinate on moistened filter papers (Whatmann, Sigma Aldrich, USA) in Petri dishes. After four days of germination, seedlings were transferred to floating nets placed on 40L opaque basins filled with an aerated nutrient solution as described in Fernando *et al.* (2017) and grown at either ambient [CO_2] (400 µmol mol^{-1}) or elevated [CO_2] (700±10 µmol mol^{-1}) for 42 days. Macro and micronutrients included in the solution culture were adapted from (Makino and Osmond, 1991) and Fernando *et al.* (2017) (Supplementary Table S1). The nutrient solution was replenished once a week maintaining the pH at 6-6.5. Basins were re-randomized weekly within each chamber and swapped between chambers fortnightly to minimize location and chamber effect.

Physiological trait measurements and sample collection

At 42 days after planting (DAP), one set of wheat seedlings was carefully harvested to determine the total dry mass of the two cultivars from both CO_2 treatments. Another set of plants was harvested and separated into last fully expanded leaf blades (LFELB) and expanding

leaf blade (ELB), cell elongation and shoot apex region (CE) in order to determine the carbon and nitrogen concentrations of each cultivar in two different CO_2 conditions.

To determine total dry mass, harvested plants were oven dried for 48 hours at 65ºC and measurements were taken when a constant weight was reached. Samples were oven dried and ground in a ball mill (Tissue Lyser II, QIAGEN, Australia) to a fine powder of ~100 µm to determine carbon and nitrogen concentration. Finely ground samples of LFELB, ELB and CE of the two cultivars were analyzed for total carbon and nitrogen content using a CN analyzer (Leco CN628, USA).

Gas exchange measurements for Kukri and RAC875 were carried out at 42 DAP using a portable photosynthesis system (LI-6400XT, LI-COR, USA), as described in Seneweera *et al.* (2002). The net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular [CO_2] (C_i) and rate of transpiration were measured between 10:00 to 13:00 hours for each cultivar in each CO_2 treatment. A red and blue light source was used to supply chamber irradiance and the light intensity was maintained at 1500 µmol quanta $m^{-2}s^{-1}$. Relative humidity was maintained between 50-70%. Measurements were taken using LFELB of each cultivar and leaves were allowed to reach a steady state photosynthesis before taking spot measurements.

For comparative proteomics analysis, the ELB of seedlings of each cultivar grown at both ambient (control) and elevated [CO_2] (treated) was collected at the same time each day. Upon collection, samples were securely packed and immediately immersed in liquid N_2 and stored at -80ºC for subsequent protein extraction.

Leaf protein extraction

(i) Total soluble protein extraction

For the extraction of total leaf protein, 300mg of the sample was ground to a fine powder using liquid N_2. This leaf powder was homogenated in 500 µl of modified RIPA solubilizing buffer [10mM Tris-HCl (pH 8.0, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 140mM NaCl and 1mM PMSF]. The homogenate was then alternatively vortexed and sonicated three times and allowed to incubate at 37ºC with shaking for 30 minutes. The samples were then centrifuged at 18,000*g* for 10 minutes and the supernatant was carefully transferred to a clean Eppendorf tube. Extracted total proteins were quantified using the microplate bicinchoninic

acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

(ii) Rubisco precipitation and protein purification

Since Rubisco comprises a large percentage of total proteins, it tends to hinder the detection of other important low abundance signaling and regulatory proteins (Gupta *et al.*, 2015). As the roles and fates of most of the high abundance proteins such as Rubisco are well established to date, in this study, we attempted to focus more on identifying low abundance proteins which may play a significant role in plant growth at elevated [CO_2] (Gupta *et al.*, 2015). Therefore, to the supernatant fraction of total proteins, Protamine Sulfate (PS) solution was added to a final concentration of 0.1 %, as described in Gupta and Kim (2015) and Kim *et al.* (2013). This was incubated in ice for 30 minutes allowing sufficient time to precipitate Rubisco. The samples were centrifuged at 12,000*g* for 5 min at 4°C to pellet the precipitated Rubisco. Immediately, the supernatant was transferred to a clean Eppendorf tube and the samples were further purified using Zeba spin desalting columns to desalt the protein samples.

(iii) Protein precipitation

To precipitate protein, 400 µl of methanol was added to 100 µl of a soluble protein fraction and mixed thoroughly followed by centrifuging for 10 seconds at 9000 g. Then, 100 µl of chloroform was added to 100 µl of protein samples and centrifuged for 10 s at 9000 g. Afterward, 300 µl of double-distilled H_2O was added to 100 µl of sample, mixed thoroughly and again centrifuged at 9000 g for 1 minute. After this step, three clear phases were obtained: an upper H_2O-methanol phase, a protein interphase and a lower chloroform phase. The upper phase was carefully removed without disturbing or touching the protein interphase. Then 300 µl of methanol was added to the remaining phases, mixed thoroughly and centrifuged for 2 minutes at 9000 g to pellet the proteins. The resultant supernatant was removed carefully and the protein pellet was allowed to dry until no chloroform smell was detectable. Protein pellets at room temperature were sent to Comprehensive Proteomics Platform, La Trobe Institute for Molecular Science, La Trobe University, Australia for mass spectrometry analysis.

Leaf proteome analysis

Leaf proteome analysis was carried out using liquid chromatography-mass spectrometry (LC-MS/MS) as described in Lowe *et al.* (2015). Comparative proteomics analysis was carried out at Comprehensive Proteomics Platform, La Trobe Institute for Molecular Science, La Trobe University, Australia. The procedure for analysis is outlined below.

(i) Sample preparation - Trypsin digestion

Each protein sample (50 µg) was first resuspended in 100 µl of 8 M urea (pH=8.3). The protein solution was then reduced for 5 hours with 1 µl of 200 mM tris (2-carboxyethyl) phosphine (TCEP). Samples were then alkylated for 1 hour at 25°C in the dark with 4 µl of 1 M iodoacetamide (IAA). Digestions were performed overnight (37°C) by adding 1 µg of trypsin (Promega, Madison WI, USA) and 900 µL of 50 mM Tris (pH=8.3) followed by a second digestion step with 1 µg trypsin and an additional incubation of 4 hours at 37°C.

(ii) Solid phase extraction clean-up of tryptic peptides

The digested solution was collected and dried using SpeedVac centrifugation. Digested proteins were resuspended in 100 µl of 1% (v/v) formic acid and centrifuged at 14,000 rpm for 2 minutes. The solid phase extraction was performed with Empore reversed-phase extraction disks (SDB-XC reversed-phase material, 3M) according to Ishihama et al. (Ishihama *et al.*, 2006) with the following modifications: the membrane was conditioned with 50 µl of 80% (v/v) acetonitrile (ACN), 0.1% (w/v) trifluoroacetic acid (TFA), then washed with 50 µl of 0.1% TFA before the tryptic peptides were bound to the membrane. The bound peptides were eluted by 50 µL 80% (v/v) ACN, 0.1% (w/v) TFA, and dried in a SpeedVac centrifuge.

(iii) LC-MS/MS analysis

Peptides (2 µg) were reconstituted in a final volume of 10 µl 0.1% TFA and 2% ACN (buffer A), then loaded and washed onto a trap column (C18 PepMap 100 µm i.d. × 2 cm trapping column, Thermo-Fisher Scientific) at 5 µl/min for 6 min before switching the precolumn in line with the analytical column (Easy-Spray 75 µm i.d. × 50 cm, Thermo-Fisher Scientific). The separation of peptides was performed at 250 nl/min using a linear ACN gradient of two

buffers: buffer A (0.1% formic acid, 2% ACN) and buffer B (0.1% formic acid, 80% ACN), starting from 5% buffer B and increasing to 60% over 300 minutes.

Data were collected on an Orbitrap Elite (Thermo-Fisher Scientific) in Data-Dependent Acquisition mode using m/z 300–1500 as MS scan range. C*ollision-induced dissociation* (CID) MS/MS spectra were collected for the 20 most intense ions. The dynamic exclusion parameters used were: repeat count 1, duration 90 s and the exclusion list size was set at 500 with early expiration disabled. Other instrument parameters for the Orbitrap were as follows: MS scan at 120 000 resolution, maximum injection time 150 ms, automatic gain control (AGC) target 1×10^6, CID at 35% energy for a maximum injection time of 150 ms with AGT target of 5000. The Orbitrap Elite was operated in dual analyzer mode with the Orbitrap analyzer being used for MS and the linear trap being used for MS/MS.

(iv) Database search and quantitation

Spectra analysis was performed using the MaxQuant software (version 1.6). Proteins were identified and quantified using the Andromeda peptide search engine integrated into the MaxQuant environment using the *Triticum aestivum* proteome downloaded from the UniProt database. A total of three biological replicates were used for each of the two wheat genotypes to determine the proteome response to elevated [CO_2].

Statistical analysis

Treatment and interaction effects of growth and photosynthesis parameters were determined through ANOVA and standard errors of differences. Differences were considered significant at $P<0.05$. All the data were analyzed using SPSS statistical software version 23 (IBM, Armonk, NY, USA). Statistical analysis for proteomics data was carried out using the statistical programming language R. All the graphical representations were carried out using GraphPad Prism scientific software version 5.01 (GraphPad Software, San Diego, CA).

Results

Effect of elevated [CO_2] on biomass accumulation, the rate of photosynthesis and carbon/nitrogen concentrations of two cultivars

The effects of [CO_2] was significant for above ground biomass accumulation ($P \leq 0.01$) and rate of photosynthesis ($P \leq 0.01$) of the two wheat cultivars tested (Figure 1a & 1c). Biomass accumulation ($P < 0.05$) and photosynthesis ($P < 0.01$) also varied significantly between the two cultivars (Figure 1). Elevated [CO_2] increased photosynthesis rate and above-ground dry mass by 16.4 and 32.6% respectively for Kukri compared with 20 and 48% for RAC875. Stomatal conductance of both varieties showed a considerable reduction at elevated [CO_2] (Figure 1b).

Carbon and nitrogen analysis of LFELB, ELB and CE region of the two cultivars showed significant interactions between [CO_2] and cultivar for nitrogen ($P \leq 0.01$, Figure 2) and protein concentration ($P \leq 0.01$, Figure 2). Nitrogen and protein concentrations of LFELB were greatly reduced at elevated [CO_2] (Figure 2a & 2b). In contrast, nitrogen concentration of ELB significantly increased at elevated [CO_2], with 30.9% and 56.3% for Kukri and RAC875, respectively (Figure 2c & 2d). In addition, the nitrogen concentration of the CE region was also significantly higher at elevated [CO_2], with Kukri showing the highest response (Figure 3e & 3f). The effect of [CO_2] on carbon concentration was significant ($P < 0.01$) and significantly varied among the organ types ($P < 0.01$) of the two cultivars. At elevated [CO_2], there was a reduction in the carbon concentration of the different organ types and ELB showed the highest decline in both cultivars (Supplementary Figure S2).

Responses of expanding leaf proteome to elevated [CO_2]

Overall, 1123 and 982 proteins were identified to be differentially expressed in Kukri and RAC875, respectively, at elevated [CO_2]. However, significant changes in protein expression at elevated [CO_2] were only observed for 61 and 76 proteins in Kukri and RAC875, respectively. Among these proteins, many (67.2% in Kukri and 67.9% in RAC875) were classified as uncharacterized proteins, as their structure and functions are not yet confirmed. The rest of the characterized proteins belonged to different key metabolic processes, that are essential for maintaining healthy growth and development of plants (Figure 3). The major proteins that showed a statistical significance from Kukri and RAC875 are listed in Table 1 and Table 2, respectively.

In Kukri (Table 1), three main enzymes involved in carbohydrate metabolism were differentially expressed at elevated [CO_2], with UDP-glucose 6-hydrogenase showing a fold increase of 4.68 ($P \leq 0.01$). The expression of glucose-6-phosphate isomerase and Beta-amylase enzymes were significantly reduced in the ELB of Kukri at elevated [CO_2] ($P \leq 0.05$). Further, it was observed that specific proteins and enzymes related to protein synthesis, processing and transport were significantly positively changed at elevated [CO_2]. Among them, 40S ribosomal protein SA, heat shock protein 90 and clathrin heavy chain proteins were prominent at elevated [CO_2] with fold increases of 4.02 ($P \leq 0.05$), 8.43 ($P \leq 0.01$) and 5.43 ($P \leq 0.05$), respectively. In addition, proteins related to cell proliferation and differentiation, such as Tubulin alpha chain (fold increase of 5.97, $P \leq 0.05$) and Tubulin beta chain (fold increase of 4.48, $P \leq 0.05$), were upregulated at elevated [CO_2]. Along with these proteins, it was observed that several key proteins related to the energy generation pathway, were also upregulated. For example, ATP synthase subunit D (mitochondrial) and pyruvate kinase showed the highest fold increases of 6 ($P \leq 0.01$) and 3.9 ($P \leq 0.05$), respectively at elevated [CO_2].

In RAC875 (Table 2), significant upregulation of the sucrose synthase enzyme was observed with a fold increase of 2.42 at elevated [CO_2] ($P \leq 0.01$). Several proteins related to protein synthesis, such as elongation factor G (mitochondrial, $P \leq 0.05$), 40S ribosomal protein S15a ($P \leq 0.01$) and S12 ($P \leq 0.05$), were also upregulated at elevated [CO_2]. Further, significant upregulation of several proteolysis related proteins involved in post-translational modifications of proteins were identified in RAC875. For example, ATP-dependent Clp protease proteolytic subunit (fold increase of 2.02, $P \leq 0.01$) and beta-type proteasome subunit (fold increase of 2.65, $P \leq 0.05$) were highly expressed at elevated [CO_2]. Cell proliferation and differentiation-related proteins, such as translationally controlled tumor protein homolog (fold increase of 1.01, $P \leq 0.05$) and alpha-type proteasome subunit (fold increase of 2.05, $P \leq 0.05$), showed higher fold increases at elevated [CO_2]. Several proteins related to photosynthesis such as oxygen-evolving enhancer protein 1 (chloroplastic, $P \leq 0.05$) and uroporphyrinogen decarboxylase ($P \leq 0.05$), involved in chlorophyll synthesis, were also upregulated at elevated [CO_2].

In both cultivars, several anti-oxidant enzymes, such as peroxidases, catalases and superoxide dismutases (Cu-Zn), were differentially expressed at elevated [CO_2] (Table 1 and 2). Among them, cell wall peroxidases; W5ANF5 in Kukri and A0A1D6BEM4 in RAC875 showed a 2.6 and 1 fold increase at elevated [CO_2]. In addition, catalase was upregulated by 4.4-fold increase in Kukri (Table 1, $P<0.05$), while all the superoxide dismutases (Cu-Zn) were down-regulated

at elevated [CO_2] in RAC875 (Table 2, P≤0.05). In addition, some of the important proteins related to signal transduction and cell homeostasis maintenance, namely calmodulin TaCaM1-1 (Ca^{2+} signaling) and calmodulin (P≤0.05), were upregulated in RAC875 at elevated [CO_2] (Table 2).

Genotypic differences in the expanding leaf blade proteome responses to elevated [CO_2]

There was a noteworthy difference between the protein expression in the ELB of the two cultivars both at ambient and elevated [CO_2] conditions. Overall, 3359 proteins were identified as differentially expressed proteins between RAC875 and Kukri at elevated [CO_2]. Out of them, only 44 proteins showed a significant difference between the two cultivars. Among them, 59% were uncharacterized proteins and other significant characterized proteins are listed in Table 3. There was a significant increase in UT-glucose-1-phosphate uridylyltransferase in RAC875 compared to Kukri at elevated [CO_2], with a fold increase of 4.2 (P≤0.01). Along with this, proteins related to energy generation, ATP synthase subunit D (mitochondrial) and fructose bi-phosphate aldolase, increased by 6.35 (P≤0.01) and 2.17 (P≤0.05), respectively, at elevated [CO_2]. Further, tubulin alpha chain (5.34, P≤0.05), TSK1 protein (1.5, P≤0.05) and phospholipase (2.07, (P≤0.05) proteins related to cell cycle functions were also more highly expressed in RAC875 than in Kukri. Protein translation and processing related proteins were highly expressed in RAC875 with elongation factor 1-alpha and heat shock protein 90 showing the highest fold increase (7.85:P≤0.01 and 7.29;P≤0.01, respectively) when compared to Kukri. In addition, expression of proteolytic enzymes such as aminopeptidase and carboxypeptidase were also higher in RAC875 at elevated [CO_2] (Table 3).

In addition, the protein expression of two cultivars also differed under ambient [CO_2]. Under ambient [CO_2] , carbohydrate metabolism of RAC875 was comparatively higher than Kukri (Supplementary Table S2). For example, RAC875 showed a substantial increase in key enzymes involved in carbohydrate metabolism such as sucrose synthase and UTP-glucose-1-phosphate uridylyltransferase by fold increases of 2.86 (P≤0.01) and 3.28 (P≤0.01), than that of Kukri. Consistent with this, Rubisco large subunit protein expression was also higher in RAC875 than in Kukri (P≤0.01). Furthermore, proteins related to cell proliferation and differentiation such as alpha type proteasome subunit and beta-glucanase in RAC875 were 2.4 (P≤0.01) and 1.5 (P≤0.05) fold higher than Kukri (Supplementary Table S2).

Discussion

Physiological mechanisms regulating growth responses at elevated [CO_2]

The stimulated growth response of C_3 species, including wheat, to elevated [CO_2] during the early developmental stages is mainly characterized by the dynamics of leaf growth. Accelerated leaf area production at elevated [CO_2] results from a faster leaf elongation rate (LER) and is strongly correlated with higher relative growth rates, total biomass and final grain yield production (Jitla *et al.*, 1997; Seneweera and Conroy, 2005). Therefore, understanding the underlying mechanisms of plant growth responses to elevated [CO_2] is essential to adapt to rising atmospheric CO_2 concentration.

Enhanced rates of photosynthesis and lower stomatal conductance are the key strategies that support plant growth at elevated [CO_2] (Ainsworth and Rogers, 2007). Improving photosynthesis rate per given leaf area has been identified as one of the main targets for improving crop productivity in the 21st century (Parry *et al.*, 2010). Therefore, elevated atmospheric [CO_2] provides a natural way to increase the photosynthesis and thereby the crop productivity. For this task, a fundamental understanding of crop responses to elevated [CO_2] is essential. In our study, elevated [CO_2] increased photosynthesis rates in both the cultivars studied. Of the two cultivars, RAC875 showed the highest response to elevated [CO_2]. The photosynthetic response of RAC875 was 22% higher than Kukri at elevated [CO_2] (Figure 1). Stomatal conductance of Kukri and RAC875 was reduced by 20 and 15% at elevated [CO_2] (Figure 1), indicating that both cultivars benefitted from the improved water use efficiency at elevated [CO_2]. Similar intraspecific variations in photosynthesis rates at elevated [CO_2] have been observed in many studies (Thilakarathne *et al.*, 2015; Thilakarathne *et al.*, 2013). Such changes in photosynthetic rates and stomatal conductance is likely to contributed towards the variability in biomass accumulation observed in the tested cultivars. The above ground biomass accumulation was significantly higher in both Kukri and RAC875, but this response was genotype dependent, suggesting that there is a fine genetic controlling mechanism for biomass accumulation in response to elevated [CO_2].

Plant growth is tightly coordinated by plant carbon and nitrogen metabolism; leaf level investments of these nutrients is central to whole plant growth (Zheng, 2009). Our study clearly indicated that growth at elevated [CO_2] alters the dynamics of carbon and nitrogen at the plant

organ level. We observed a reduction in nitrogen concentration in the LFELB of both Kukri and RAC875 at elevated [CO_2], with Kukri showing the highest reduction (35%, Figure 2). Similarly, the protein concentration of Kukri was less than RAC875 at elevated [CO_2]. The reduction in leaf nitrogen concentration may be due to the dilution of nitrogen by the increased biomass observed at early growth stages (Thilakarathne *et al.*, 2015). Further, this could have been due to the changes in the concentrations of enzymes that contain high nitrogen concentrations such as Rubisco (Seneweera *et al.*, 2011). However, the nitrogen and protein concentrations of the ELB and the CE region were significantly higher at elevated [CO_2]. This may be due to the increased partitioning of nitrogen into the growing sink tissues such as expanding leaf blade and shoot apex region where more nitrogen is needed to produce proteins required for the production of new cells (Seneweera and Conroy, 2005). In a previous study by Thilakarathne *et al.* (2015) , it was reported that higher nitrogen concentrations in ELB at elevated [CO_2]. Perhaps, such increased nitrogen in ELB could be linked to rapid protein synthesis, due to increases in cell division, synthesis of photosynthetic proteins and regulation of cell pressure potential that govern cell expansion (Radin and Boyer, 1982).

Together with nitrogen, carbon is also required for amino acid biosynthesis. At elevated [CO_2], a reduction in carbon concentration was observed across all the organ types with the highest reduction observed in the ELB of RAC875. Such reductions could be due to the breakdown of carbohydrates to provide biochemical energy for leaf expansion and growth at elevated [CO_2] (Ainsworth *et al.*, 2006). On the other hand, it has also been demonstrated that sugar accumulation at elevated [CO_2] depends on the species and growth conditions (Nakano *et al.*, 1997; Rogers *et al.*, 1996). Carbohydrate analysis of RAC875 and Kukri (unpublished data) showed that total soluble carbohydrate concentration at elevated [CO_2] was higher across both the cultivars, however RAC875 showing a higher soluble sugar accumulation in expanding leaves than Kukri at elevated [CO_2] (Supplementary Figure S3(a)). The sucrose content of the ELB in Kukri and RAC875 showed a fold increase of 3.2 and 8.7, respectively, at elevated [CO_2] (Supplementary Figure S1(b)). Similarly, glucose content of the two cultivars was also significantly increased at elevated [CO_2] with a fold increase of 1.4 for Kukri and 2.3 for RAC875 (Supplementary Figure S1(c)). Therefore, high sucrose availability, together with increased nitrogen partitioning to ELB, may have contributed positively towards accelerated LER observed under elevated [CO_2]. The higher nitrogen content in ELB was observed in a study conducted by Thilakarathne *et al.* (2015) at elevated [CO_2]. An increase in nitrogen may have supported rapid rates of protein synthesis required for cell division, synthesis of

photosynthetic proteins and regulation of cell pressure potential that govern cell expansion (Radin and Boyer, 1982). Together with the carbon skeletons provided by sucrose and glucose, and ammonium provided by nitrogen partitioning, synthesis of amino acids takes place in the expanding leaf blades. These amino acids and resultant proteins govern the growth and development of the leaf blades.

Leaf proteome responses to elevated [CO_2] is genotypically varied

This study is the first attempt in investigating changes in the leaf proteome of wheat in response to elevated [CO_2]. Protein expression of RAC875 and Kukri varied under high [CO_2] with RAC875 showing a higher number of significantly affected proteins than Kukri. These findings suggest that the proteome changes observed in leaves under elevated [CO_2], also depend on the genotype. Similarly, in a study conducted by Arachchige *et al.* (2017), grain proteome responses of wheat to elevated [CO_2] also genotypically varied. Overall, characterized proteins could be categorized into those associated with photosynthesis, carbohydrate metabolism, cell division, cell wall metabolism, energy pathways, protein synthesis and processing, post-translational modifications (PTM), key anti-oxidant enzymes, signal transduction, nitrogen and amino acid metabolism, lipid metabolism, hormonal metabolism and other intermediate metabolic proteins that are involved in several metabolic activities (Figure 3). A similar expression of proteins was observed using rice seedlings grown at elevated [CO_2] in a study conducted by Bokhari *et al.* (2007).

Elevated [CO_2] shuffles the carbohydrate metabolism

Increased photosynthesis capacity at elevated [CO_2] contributes to the greater accumulation of soluble carbohydrates and starch, which causes a major shift in plant carbon metabolism (Makino and Mae, 1999). Despite a large number of proteins being differentially expressed at elevated [CO_2], oxygen-evolving enhancer protein 1 (chloroplastic) was the only protein showing a significant upregulation (Table 2). This protein is responsible for photosystem II assembly and stabilization (Ali *et al.*, 2018), and hence, upregulation of this protein may contribute towards improving the efficiency of photosynthesis light-dependent reactions. At elevated [CO_2], upregulation of Uroporphyrinogen decarboxylase, a key enzyme in chlorophyll biosynthesis was also observed (Table 2). Generally, higher chlorophyll production at elevated [CO_2] contributes to improved efficiency in harvesting light and subsequent energy conversion in the light reaction of photosynthesis.

In RAC875, increased abundance of sucrose synthase at elevated [CO_2], one of the key enzymes involved in sucrose metabolism, was observed (Table 2). Sucrose synthase is capable of cleaving sucrose into UDP-Glucose and fructose and channeling them into multiple pathways involved in metabolic, structural and storage functions (Jiang *et al.*, 2015; Sturm and Tang, 1999). Sucrose synthase did not only increase at the protein level, the transcript abundance of *SUS1* (sucrose synthase 1) gene in ELB of RAC875 also showed a fold increase of 1.43 at elevated [CO_2] when compared to ambient [CO_2] (unpublished data, Table 4). Similarly, in RAC875, UTP-glucose-1-phosphate uridylyltransferase enzyme was upregulated by 4 folds higher than Kukri at elevated [CO_2] (Table 3). This is the key enzyme that synthesizes UDP-glucose from glucose-1-phosphate and Uridine Triphosphate (UTP) (Kleczkowski *et al.*, 2010). UDP-glucose is one of the key substrates to produce sucrose-6-phosphate, from which sucrose is produced in plant cells (Jiang *et al.*, 2015; Koch, 2004). High sucrose availability in RAC875 at elevated [CO_2] could be due to the increased expression of UTP-glucose-1-phosphate uridylyltransferase which provides more UDP-glucose as a substrate to produce sucrose. On the other hand, UDP-glucose is the primary building block for cell wall synthesis (Wai *et al.*, 2017) and thus, more substrate would be available for biosynthesis of cell wall polysaccharides for the dividing cells in the ELB. Generally, UDP-Glucose is highly available in the growing sink tissues such as meristems and developing leaves where the majority of cell wall synthesis takes place (Verbančič *et al.*, 2017). Higher availability of sucrose and glucose in the expanding leaves of RAC875 and Kukri could be due to increased expression of these proteins at elevated [CO_2].

In Kukri, glucose-6-phosphate isomerase, which is involved in the respiratory breakdown of carbohydrates, was down-regulated at elevated [CO_2] (Table 1), suggesting the facilitation of carbohydrate anabolism over catabolism in a high CO_2 environment. In addition, beta-amylase, the main enzyme responsible for starch granule breakdown in leaves was down-regulated in Kukri at elevated [CO_2]. Therefore, less degradation of starch to sucrose could be the reason for the difference in total soluble carbohydrate concentration of Kukri observed at elevated [CO_2]. However, UDP-glucose 6-dehydrogenase, a key enzyme involved in the synthesis of UDP-glucuronic acid (Kärkönen *et al.*, 2005; Klinghammer and Tenhaken, 2007), a common precursor in the formation of pectin and hemicellulose in the plant's primary cell wall, was upregulated at high [CO_2] (Table 1). Upregulation of this enzyme at elevated [CO_2] suggests that cell wall biosynthesis is accelerated at elevated [CO_2].

High energy demand at elevated [CO_2] supported by the induction of proteins in the energy metabolism pathway

Enhanced rates of photosynthesis at elevated [CO_2] demands a greater energy input in the form of ATP (Gonzalez-Meler *et al.*, 2004). Exposure to elevated [CO_2] induced the expression of ATP synthesizing enzymes in our study. A similar pattern in the upregulation of ATP synthesizing enzymes was observed by Bokhari *et al.* (2007) under elevated [CO_2]. In Kukri, ATP synthase subunit d (mitochondrial) and pyruvate kinase enzyme involved in ATP synthesis was highly expressed suggesting that ATP synthesis is higher at elevated [CO_2] (Table 1). At elevated [CO_2], expression of ATP synthase subunit d (mitochondrial) and Fructose bi-phosphate aldolase was 6 and 3 folds higher in RAC875 than in Kukri (Table 3). These findings suggest that protein expression related to energy metabolism is largely cultivar dependent. Expression of proteins related to energy generation implies that apart from carbohydrate synthesis, elevated [CO_2] also stimulates the respiratory breakdown of the carbohydrates in order to obtain sufficient energy for leaf expansion and carbon skeletons required for further anabolic purposes (Ainsworth *et al.*, 2006).

Cell wall metabolism and cell division related proteins were upregulated by elevated [CO_2]

Cell cycle functions tend to change as a result of increased sugar supply to the growing sink tissues at elevated [CO_2] (Gamage *et al.*, 2018). It has been demonstrated that cell division, cell expansion and cell wall loosening increased at elevated [CO_2] and results in increased growth (Masle, 2000; Ranasinghe and Taylor, 1996). Consistent with the above findings, upregulation of proteins related to these cell cycle functions was observed at elevated [CO_2]. In RAC875, a translationally controlled tumor protein homolog that stabilizes microtubules during cell division (Toscano-Morales *et al.*, 2015) was prominently upregulated (Table 2). Essential components in cell microtubules such as α and β chains of tubulin, that facilitate chromosome segregation during cell division (Hashimoto, 2015) were upregulated in Kukri at elevated [CO_2] (Table 1). Important nucleosome proteins (GTP-binding nuclear protein and histone H4 protein) involved in chromatin formation and condensation during cell cycle processes (Bischoff *et al.*, 1999; Loginova and Silkova, 2017) were differentially expressed at elevated [CO_2] (Table 1 and 2). Additionally, α type proteasome subunit was found to be highly expressed in highly proliferating cells (Genschik *et al.*, 1994) and was upregulated at elevated [CO_2] in RAC875 (Table 3). This protein performs an important proteolytic activity in cell

cycle-related proteins involved in the progression of the cell cycle and thereby facilitating growth and development of the plant.

Beta-galactosidase, identified in both Kukri and RAC875, is an important enzyme related to cell wall expansion in growing tissues, however, the expression of this protein was found to be downregulated in our study (Table 2). On the other hand, upregulation of cell wall peroxidases at elevated [CO_2] was prominent in both cultivars (Table 1 and 2). Peroxidases favor cell elongation either by generating reactive oxygen species (ROS) and/or by regulating the apoplastic concentrations of H_2O_2 (Passardi *et al.*, 2004). Reactive oxygen species play an important role in cell wall loosening by cleaving cell wall components such as xyloglucans and pectins (Passardi *et al.*, 2004). This allows other cell wall loosening enzymes such as xyloglucan endotransglucosylase/endohydrolases (XTHs) to act on xyloglucans to loosen the plant cell wall permitting cell elongation (Van Sandt *et al.*, 2007). Consistent with this, expression of XTH gene members was found to be upregulated at elevated [CO_2] which may have facilitated increased leaf elongation at high [CO_2] (unpublished data, Table 4). However, the mechanism by which ROS mediate leaf development still remains unclear (Schmidt *et al.*, 2016).

Protein synthesis, processing and transport respond to elevated [CO_2]

Several important proteins involved as structural constituents of ribosomes were upregulated at elevated [CO_2] (Table 1 and 2). Increased photosynthesis rates observed at elevated [CO_2] requires a corresponding increase in other cellular proteins, including structural (i.e. light harvest protein complexes, components in electron transport chain and thylakoids) and metabolic proteins (i.e. carbon assimilating enzymes such as Rubisco). Thus, a sufficient turnover of these proteins is constantly required to provide the building blocks for these structural components and maintain their related enzymatic activities (Lawlor, 2002). Further, significant ribosome biosynthesis is required to support increased cytoplasmic growth and cell proliferation occurring in the expanding leaf blade (Ainsworth *et al.*, 2006; Sugimoto-Shirasu and Roberts, 2003). Increased protein turnover in the ELB could be due to the increased nitrogen partitioning observed at elevated [CO_2]. This is further confirmed by the high transcript abundance of the key nitrogen assimilation genes (*GS2b*, *Fd-GOGAT* and *NADH-GOGAT*) in both cultivars under high [CO_2] conditions (unpublished data, Table 4).

A number of molecular chaperone proteins that facilitate proper folding of the polypeptide chains and process proteins, were identified at elevated [CO_2] in this study. Of them, heat shock protein 90 was a highly expressed protein found in both cultivars (Table 1 and 3). This protein plays an important role in maintaining cellular homeostasis through protein folding, assembly and translocation (Park and Seo, 2015). Along with these, a significant upregulation of clathrin heavy chain proteins (5 fold higher than ambient [CO_2]) was observed at elevated [CO_2] (Table 1), which facilitates intra-cellular protein transport required for proper cell division and functioning (Popova *et al.*, 2013).

Post-translational modifications (PTM) of proteins improves the functional diversity of a proteome increasing protein responsiveness to external factors (Friso and van Wijk, 2015). Proteolysis is one of the PTMs in plants which is critical in maintaining cellular protein concentrations within a cell (Vierstra, 1996). Several proteolytic proteins such as alpha type proteasome subunits and ATP-dependent Clp protease subunit were upregulated in RAC875 at elevated [CO_2]. Expression of Aminopeptidase differed in the two cultivars. Kukri showed an upregulation while RAC875 showed a down-regulation at elevated [CO_2] (Table 1 and 2). These proteolytic enzymes and components are responsible for cleaving polypeptide bonds for different cellular activities, removing unassembled protein subunits or misfolded proteins from the cell and thereby controlling cell metabolism.

Signal transduction proteins were upregulated at elevated [CO_2]

Since plants change their morphologies in response to environmental cues, different signaling pathways interact together to co-ordinate biochemical and physiological responses, such as photosynthetic regulation, with plant growth responses (Mulligan *et al.*, 1997). Our results showed an upregulation of Calmodulin (Table 2), a ubiquitous Ca^{2+} binding protein which mediates control of various ion channels, transcription factors, protein kinases and other metabolic enzymes (Zeng *et al.*, 2015). The cytosolic Ca^{2+} concentration changes in response to environmental stimuli (Zhu *et al.*, 2014) including elevated [CO_2]. For example, one of the main mechanisms driving stomatal conductance at elevated [CO_2] involves the interaction of CO_2 with the guard cell membranes, which operate largely via Ca^{2+} signaling pathway (Brodribb and McAdam, 2013). Up-regulation of S-formylglutathione hydrolase (Table 2), the key enzyme that catalyzes the production of glutathione, indicates its increased production at elevated [CO_2]. Glutathione has been shown to participate in several signaling processes

including protein synthesis, amino acid transport and control of cell division (Hossain *et al.*, 2017). In addition, phospholipases (PL), which are often regulated by external factors such as Ca^{2+} concentration, have also been upregulated at elevated [CO_2] (Table 3, Singh *et al.*, 2015). This enzyme was suggested to be involved in various metabolic and cell signaling networks and subsequent phosphatidic production by PL. It is suggested that such changes are likely to determine plant growth responses under a particular environmental condition (Singh *et al.*, 2015).

Apart from these proteins, there were many uncharacterized proteins that showed a differential expression at elevated [CO_2]. Some of the uncharacterized proteins were present in both cultivars and showed a similar pattern of expression. For example, W5H8Z0 uncharacterized protein showed a significant upregulation in both the cultivars at elevated [CO_2], suggesting that this might play a role in moderating plant metabolism in a high CO_2 environment. Since, the whole proteome of wheat is not completely interpreted, developing a complete understanding of the protein network associated with growth responses to elevated [CO_2] is a great challenge, but it is essential in order to adapt to increasing [CO_2] in the atmosphere.

Conclusion

We investigated the physiological and proteomic-wide responses of the ELB of two winter wheat cultivars to elevated [CO_2] in order to understand the underlying molecular mechanisms of plant growth responses to elevated [CO_2]. Elevated [CO_2] increases the sugar accumulation and affects different regulatory mechanisms, which results in altered protein expression to maintain plant carbon and nitrogen metabolism and cell homeostasis.

We conclude that elevated [CO_2] increases the rate of carbon assimilation and carbohydrate metabolism in a significant manner. At the same time, the respiratory breakdown of carbohydrates is upregulated to meet the high energy demand required for maximum leaf growth and elongation at elevated [CO_2]. Consistent with the increased sugar and nitrogen supply to the ELB, proteins including cell wall loosening, expansion and cell proliferation increase at elevated [CO_2], which contributes to the accelerated growth of plants. There was a notable increase in the molecular chaperones and PTM through proteolytic proteins, implying the plants' ability to improve the functionality of their proteome in response to environmental stimuli such as elevated [CO_2]. This evidence clearly suggests that enhanced plant growth at

elevated [CO_2] is not only due to the direct effect of [CO_2] on photosynthesis and stomatal conductance. Variations in other associated key metabolic processes such as carbon/nitrogen metabolism and cell cycle functions also contribute to plant growth responses to elevated [CO_2]. However, protein expression between the two genotypes significantly varied at elevated [CO_2] suggesting a significant genetic variation for CO_2 responsiveness. Therefore, identification of genotypes that can thrive at elevated [CO_2] is essential to improve the crop productibility under a CO_2 enriched environment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

This study was funded by the Strategic Research Fund of the University of Southern Queensland, Australia. The parental lines of the doubled haploid mapping population were obtained from the Australian Centre for Plant Functional Genomics, University of Adelaide, Australia to carry out the research. The proteomics analysis was conducted by La Trobe Comprehensive Proteomics Platform (La Trobe University, Melbourne, Australia).

List of Figures and Supplementary Figures

Figure 1. (a) rate of photosynthesis, **(b)** stomatal conductance, **(c)** above ground biomass accumulation of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

Figure 2. Total nitrogen and total protein concentration of **(a)** & **(b)** Last fully expanded leaf blades (LFELB), **(c)** & **(d)** expanding leaf blades (ELB), and **(e)** & **(f)** cell elongation and shoot apex regions of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

Figure 3. Ven diagram showing percentages of the identified proteins belonging to different categories in **(a)** Kukri and **(b)** RAC875 in response to elevated (700±10 µmol mol^{-1}) [CO_2]. Data are presented as percentages. **A**-photosynthesis, **B**-carbohydrate metabolism, **C**-cell division and cell wall metabolism, **D**-energy pathway, **E**-protein synthesis and processing, **F**-proteins involved in Post-translational modifications (PTM), **G**-anti oxidant enzymes, **H**-Signal transduction proteins, **I**-nitrogen and amino acid metabolism, **J**-lipid metabolism, **K**-hormonal metabolism, **L**- other intermediate metabolic proteins.

Supplementary Figure S1. (a) Total soluble sugar content, **(b)** sucrose content, **(c)** glucose content and **(d)** fructose content in expanding leaf blades of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

Supplementary Figure S2. Total carbon concentration of **(a)** Last fully expanded leaf blades (LFELB), **(b)** expanding leaf blades (ELB), and **(c)** cell elongation region of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

List of Tables and Supplementary Tables

Table 1. Leaf proteome response of Kukri in response to elevated [CO_2] (e[CO_2]) (Protein expression relative to Kukri proteome at ambient [CO_2], Fold change is given in Log_2 transformation, ** P<0.01, * P<0.05).

Table 2. Leaf proteome response of RAC875 in response to elevated [CO_2] (e[CO_2]) (Protein expression relative to RAC875 proteome at ambinet [CO_2], fold change is given in Log_2 tranformation, **P<0.01, * P<0.05).

Table 3. Comparison of leaf proteome response of RAC875 relative to Kukri at elevated [CO_2] (Protein expression relative to Kukri proteome at elevated [CO_2], fold change is given in Log_2 transformation, **P<0.01, * P<0.05).

Table 4. Heatmap for transcript abundance of key genes in carbon, nitrogen and cell wall metabolism in Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript abundance at elevated [CO_2] are presented in Log_2 scale.

Supplementary Table S1. Concentration of macro and micronutrients in the growing solution provided during wheat growth during early vegetative stage. Half strength nutrient solution was provided. Adapted from (Fernando *et al.*, 2017; Makino *et al.*, 1983).

Supplementary Table S2. Comparison of leaf proteome response of RAC875 relative to Kukri at ambient [CO_2] (Protein expression relative to Kukri proteome at ambient [CO_2], fold change is given in Log_2 transformation, **P<0.01, * P<0.05).

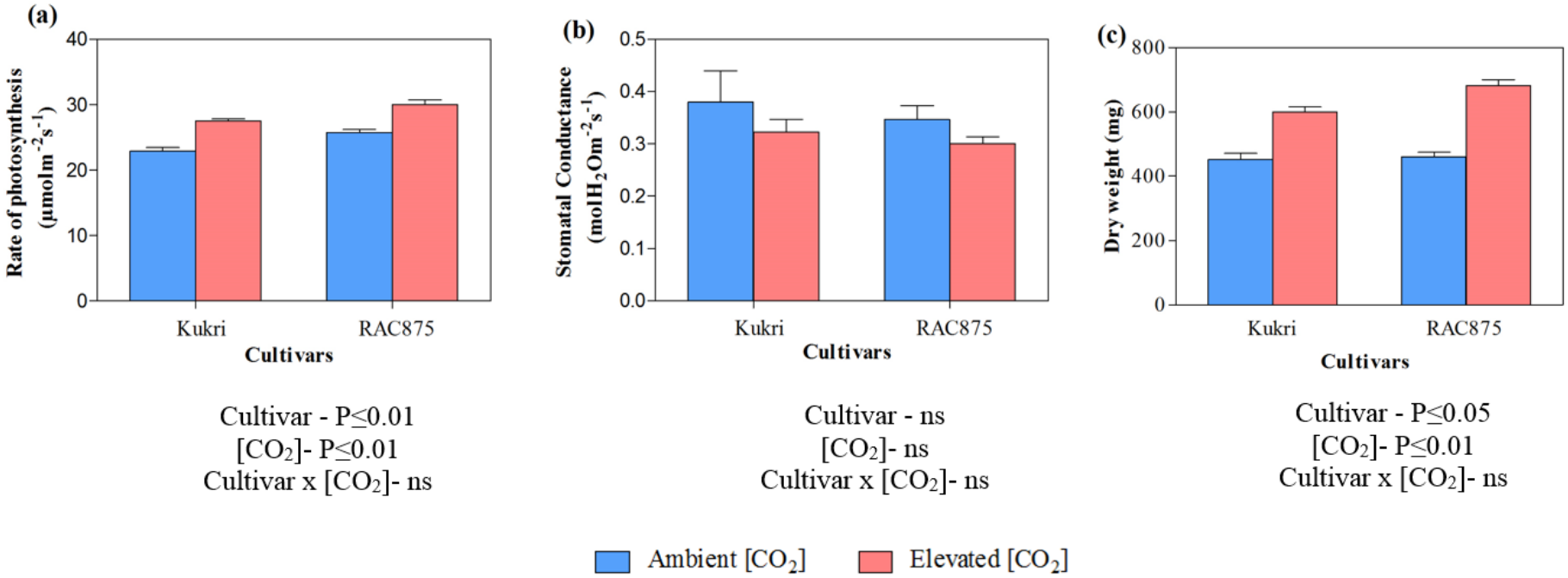
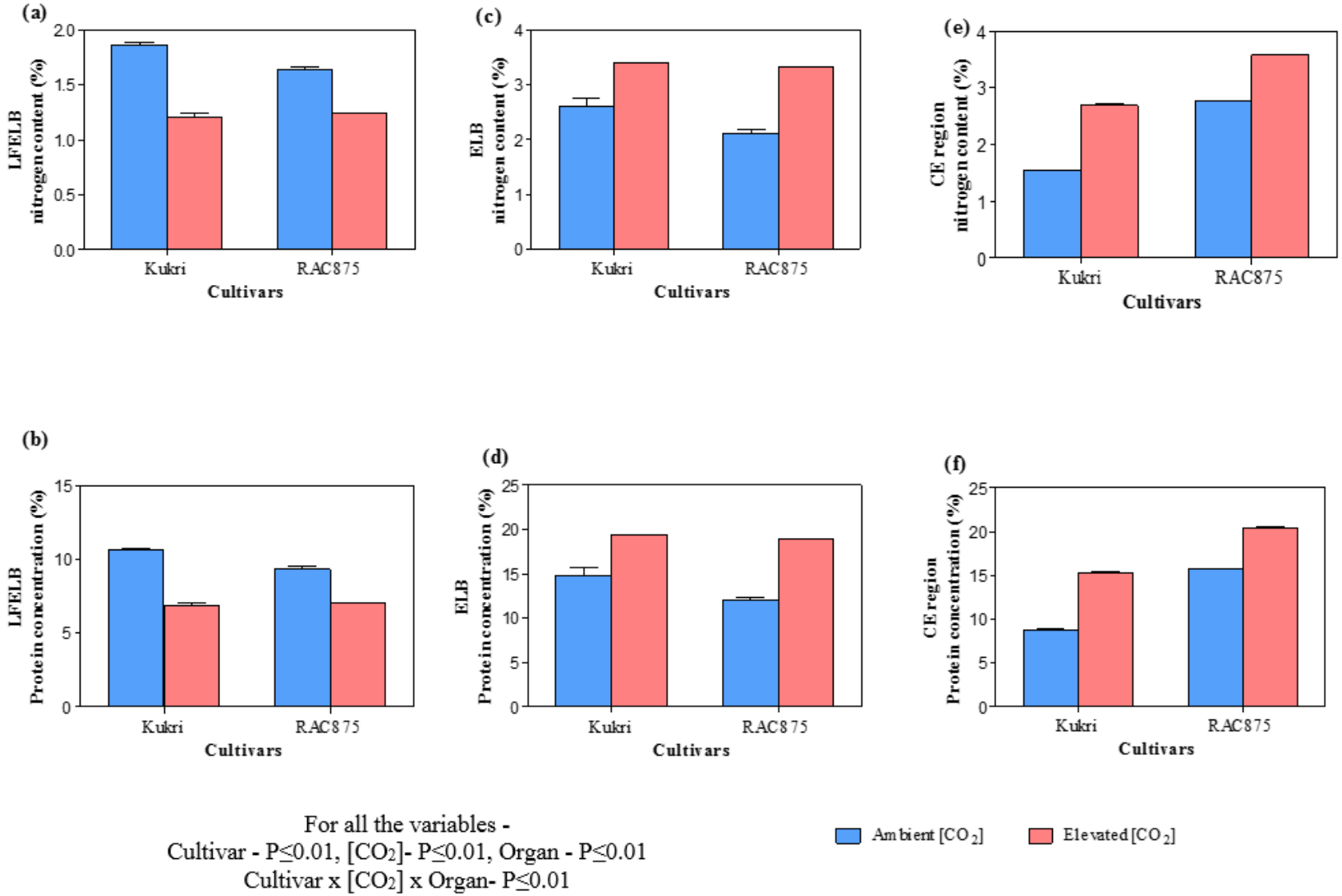

(a)
Rate of photosynthesis (µmolm-2s-1)
40
30
20
10
0
Kukri
RAC875
Cultivars
Cultivar - P≤0.01
[CO2]- P≤0.01
Cultivar x [CO2]- ns
(b)
Stomatal Conductance (molH2Om-2s-1)
0.5
0.4
0.3
0.2
0.1
0.0
Kukri
RAC875
Cultivars
Cultivar - ns
[CO2]- ns
Cultivar x [CO2]- ns
(c)
Dry weight (mg)
800
600
400
200
0
Kukri
RAC875
Cultivars
Cultivar - P≤0.05
[CO2]- P≤0.01
Cultivar x [CO2]- ns
Ambient [CO2]
Elevated [CO2]

Figure 1



For all the variables -
Cultivar - $P \leq 0.01$, $[CO_2]$- $P \leq 0.01$, Organ - $P \leq 0.01$
Cultivar x $[CO_2]$ x Organ- $P \leq 0.01$

Figure 2

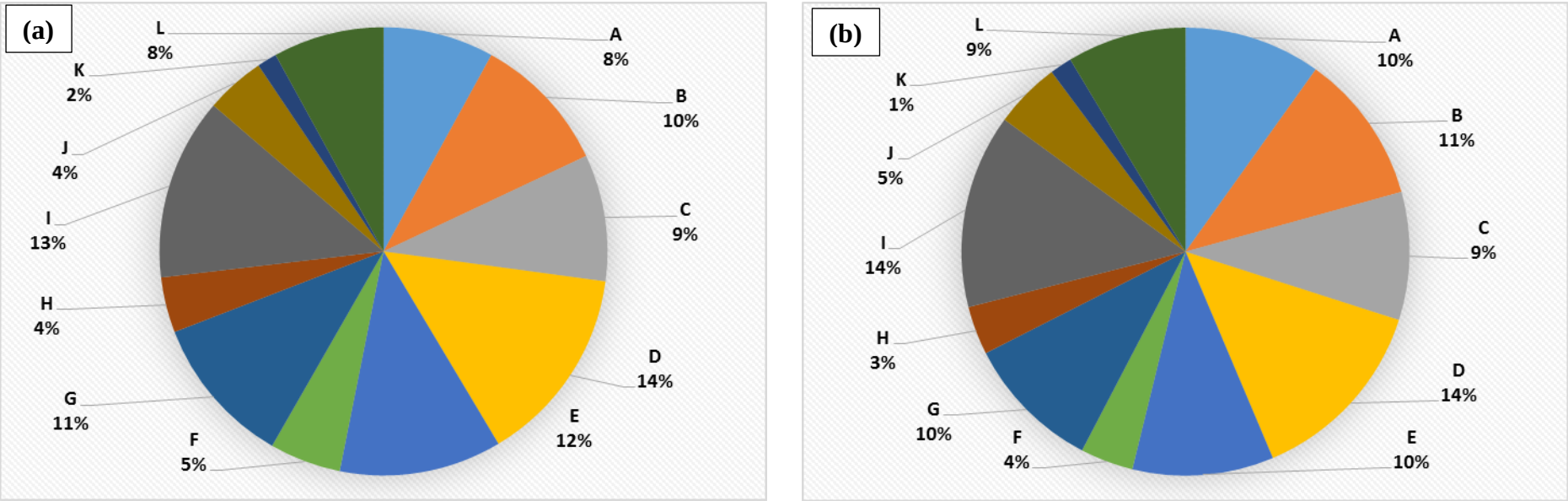

(a)
A
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Figure 3

Table 1. Leaf proteome response of Kukri in response to elevated [CO_2] (e[CO_2]) (Protein expression relative to Kukri proteome at ambient [CO_2], Fold change is given in Log_2 transformation, ** P<0.01, * P<0.05).

Accession No	Protein Name	Fold change at e[CO_2]	Molecular weight (kDa)	P value
	Carbohydrate metabolism			
A0A1D5S2P8	Glucose-6-phosphate isomerase	-1.64	62.308	*
A0A1D6DBP9	Beta-amylase	-2.20	56.102	*
A0A1D5ZJB9	UDP-glucose 6-dehydrogenase	4.68	51.527	**
	Energy pathway			
W5BEP1	ATP synthase subunit D, mitochondrial	6.00	19.548	**
A0A1D5UPJ6	Pyruvate kinase	3.92	35.432	*
C7AE91	Blue copper protein	3.28	17.393	*
	Cell proliferation and differentiation			
A0A1D5SQY8	Tubulin alpha chain	5.97	45.863	*
A0A1D5Z8I6	Histone H4	5.72	16.412	*
A0A1D6BQY9	Tubulin beta chain	4.48	50.211	*
	Protein synthesis			
Q5I7K5	Ribosomal protein P1	-0.89	11.193	*
W5E2W7	40S ribosomal protein SA	4.02	33.271	*
	Protein processing and transport			
Q0Q0I7	Heat shock protein 90	8.43	80.460	**
W5D5R6	Cold-induced protein	-1.33	16.199	*
A0A1D5ZA24	Clathrin heavy chain	5.43	189.889	*
	Post-translational modifications – Proteolytic enzymes			
A0A1D6B0C9	Aminopeptidase	2.10	98.207	*
	Anti-oxidant enzymes – responds to ROS			
A0A1D5SM88	Dihydrolipoyl dehydrogenase	-2.50	52.795	*
W5C5X0	Peroxidase	-1.93	33.077	*
W5ANF5	Peroxidase	2.67	37.449	*

F1DKC1	Catalase	4.47	56.883	*
	Signal transduction			
W5CUZ3	FVE	-1.18	50.298	*
A0A1D6CA52	4-hydroxy-4-methyl-2-oxoglutarate aldolase	1.74	16.581	*
A0A1D6RJH6	Plasma membrane ATPase	8.49	102.253	*

Table 2. Leaf proteome response of RAC875 in response to elevated [CO_2] (e[CO_2]) (Protein expression relative to RAC875 proteome at ambinet [CO_2], fold change is given in Log_2 tranformation, **P<0.01, * P<0.05).

Accession No	Protein Name	Fold change at e[CO_2]	Molecular weight (kDa)	P value
	Photosynthesis			
A0A1D6BXL7	Ferredoxin--NADP reductase, chloroplastic	-2.06	39.843	*
P27665	Oxygen-evolving enhancer protein 1, chloroplastic	2.66	34.740	*
	Porphyrin biochemical pathway – Chlorophyll Synthesis			
W5DYL0	Uroporphyrinogen decarboxylase	0.96	42.951	*
A0A1D5VZ17	Uroporphyrinogen decarboxylase	0.61	45.934	*
	Carbohydrate metabolism			
W5I774	Sucrose synthase	2.42	92.400	**
	Energy pathway			
P20858	ATP synthase subunit beta, chloroplastic	-1.96	53.857	*
	Cell proliferation and differentiation			
Q8LRM8	Translationally-controlled tumor protein homolog	1.01	18.806	*
A0A1D5RY72	GTP-binding nuclear protein	0.77	19.912	*
W5C3H4	Proteasome subunit alpha type	2.05	25.828	*
	Cell wall metabolism			
A0A1D6RX96	Beta-galactosidase	-0.87	87.227	*
A0A1D6CMU1	Beta-galactosidase	-0.93	75.744	*
	Protein synthesis			
E2F3W4	40S ribosomal protein S15a	1.40	14.790	**
A0A1D5UFM0	40S ribosomal protein S12	1.22	15.235	*
A0A1D5T990	Elongation factor G, mitochondrial	1.18	72.841	*
	Post translation modifications – Proteolytic enzymes			
A0A1D6B0C9	Aminopeptidase	-1.50	98.207	**
A0A1D5TF03	ATP-dependent Clp protease proteolytic subunit	1.95	27.092	**

W5F826	Proteasome subunit beta type	2.65	24.333	*
A0A1D6AKZ2	ATP-dependent Clp protease proteolytic subunit	2.02	32.197	*
	Amino acid metabolism			
A0A1D6S822	S-adenosylmethionine synthase	-2.37	42.827	*
	Anti-oxidant enzymes – responds to ROS			
A0A1D5XAW6	Superoxide dismutase [Cu-Zn]	-1.13	16.576	*
H9NAV6	Superoxide dismutase [Cu-Zn]	-1.26	15.092	*
A0A1D6BEM4	Peroxidase	1.06	33.623	*
A0A1D6CM42	Superoxide dismutase [Cu-Zn]	-0.95	19.325	**
W5AS89	Peroxidase	-0.73	36.521	*
W5BAV5	Peroxidase	-0.69	33.724	*
	Signal transduction			
Q7DMG9	Calmodulin TaCaM1-1 (Ca^{2+} signalling)	1.08	16.832	*
P04464	Calmodulin	0.91	16.847	*
W5D6S5	S-formylglutathione hydrolase	1.70	31.599	*

Table 3. Comparison of leaf proteome response of RAC875 relative to Kukri at elevated [CO_2] (Protein expression relative to Kukri proteome at elevated [CO_2], fold change is given in Log_2 transformation, **P<0.01, * P<0.05).

Accession No	Protein Name	Fold change at e [CO_2] relative to Kukri	Molecular weight (kDa)	P value
	Carbohydrate metabolism			
A0A1D6S518	UTP-glucose-1-phosphate uridylyltransferase	4.20	51.664	**
	Energy pathway			
W5BEP1	ATP synthase subunit D, mitochondrial	6.35	19.548	**
W5D5L4	Fructose bi-phosphate aldolase	2.17	38.810	*
A0A1D6AQV8	Aconitate hydratase	-0.42	106.306	*
	Cell proliferation and differentiation			
A0A1D5SQY8	Tubulin alpha chain	5.34	45.863	**
A0A173FEH2	TSK1 protein	1.50	19.059	*
A0A1D5VHL0	Phospholipase	2.07	92.062	*
	Protein synthesis			
A0A1D5YL72	Elongation factor 1-alpha	7.85	47.806	**
W5E2W7	40S ribosomal protein SA	2.11	33.271	*
A0A1D6C4Q5	60S acidic ribosomal protein PO	3.73	33.770	*
	Protein processing			
Q0Q0I7	Heat shock protein 90	7.29	80.460	**
	Post-translational modifications – Proteolytic enzymes			
A0A1D6S4L2	Carboxypeptidase	1.77	51.327	*
A0A1D6B0C9	Aminopeptidase	2.15	98.207	*
D2KZ08	Aminotransferase	0.77	55.546	*
A0A1D5TM94	Carboxypeptidase	1.31	55.914	*
	Amino acid metabolism			
A0A1D5XQ85	Alanine--tRNA ligase	1.94	109.594	*
A0A1D6B308	S-adenosylmethionine synthase	2.12	42.766	*

	Signal transduction			
A0A1D5ZA24	Clathrin heavy chain	4.64	189.889	**
A0A1D5VHL0	Phospholipase	2.07	92.062	*
	Nitrogen assimilation			
A0A1D6AQV8	Aconitate hydratase	-0.42	106.306	*

Table 4. Heatmap for transcript abundance of key genes in carbon, nitrogen and cell wall metabolism in Kukri and RAC875 grown at ambient (a[CO_2], 400 µmol mol^{-1}) or elevated (e[CO_2], 700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean values of gene expression. Fold change of transcript abundance at elevated [CO_2] are presented in Log_2 scale.

Plant metabolism	**Gene Name**	**Gene expression level**				**Log_2 fold change**	
		Kukri		RAC875		Kukri	RAC875
		a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	e[CO_2]/a[CO_2]	
Phtotosyntesis	*rbcL*	2.70	3.69	1.40	3.12	0.45	1.16
	rbcS	1.26	2.65	1.42	2.00	1.07	0.49
Carbon metabolism	*SPP1*	4.47	4.48	3.48	10.32	0.00	1.57
	SPS1	0.95	1.50	1.26	1.27	0.65	0.02
	SUS1	0.43	1.81	0.56	1.50	2.07	1.43
Nitrogen metabolism	*NiR*	0.30	0.56	0.41	1.19	0.89	1.55
	Fd-GOGAT	13.24	29.92	15.61	28.57	1.18	0.87
	GS2a	24.98	46.56	14.65	26.80	0.90	0.87
	GS2b	0.09	0.15	0.11	0.32	0.70	1.49
	GS1a	3.36	4.28	3.15	4.26	0.35	0.44
	GSr1	0.33	0.68	0.25	0.27	1.04	0.12
	NADH-GOGAT	0.29	0.71	0.26	0.26	1.31	0.04
Cell wall metabolism	*TaEXPA3*	1.32	1.23	1.77	5.48	-0.10	1.63
	TaEXPB6	6.11	7.51	7.46	17.14	0.30	1.20
	TAEXPB23	11.55	28.49	13.23	13.55	1.30	0.03
	TaXTH1	39.54	46.23	23.16	66.77	0.23	1.53
	TaXTH2	36.34	65.60	46.83	94.22	0.85	1.01
	TaXTH3	26.76	8.70	4.31	36.13	-1.62	3.07
	TaXTH4	0.61	0.40	0.88	1.92	-0.60	1.12
	TaXTH5	1.94	2.58	0.94	2.44	0.42	1.37

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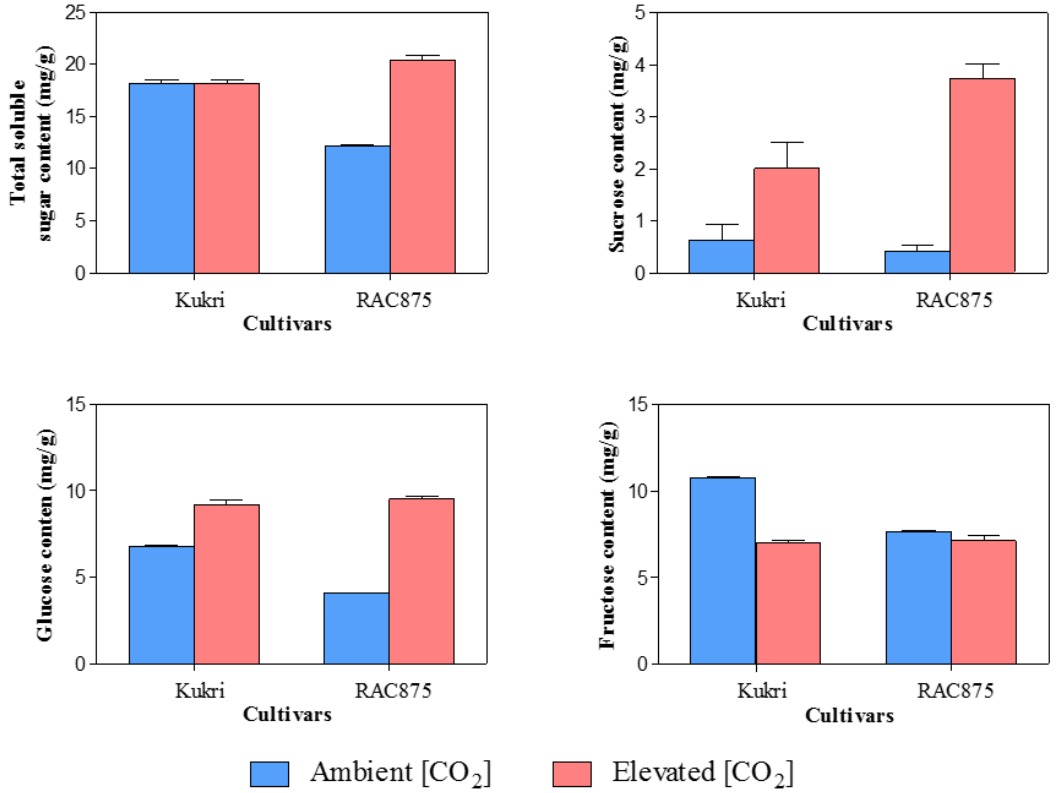
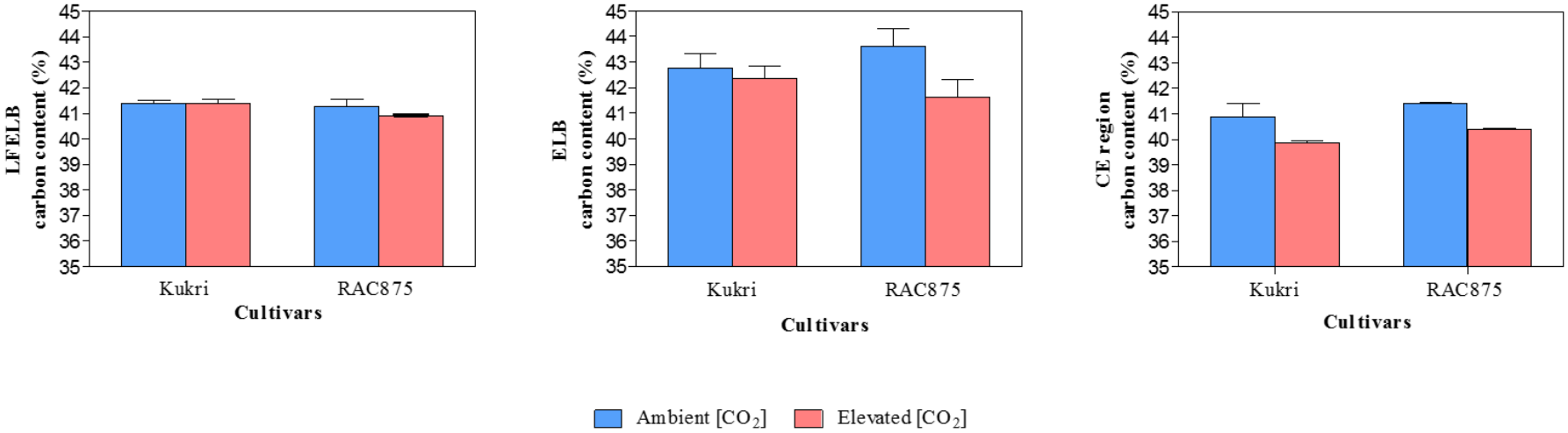
Supplementary Data



For all the variables - Cultivar - P≤0.01, [CO_2]- P≤0.01, Cultivar x [CO_2]- P≤0.01

Supplementary Figure S1. (a) Total soluble sugar content, **(b)** sucrose content, **(c)** glucose content and **(d)** fructose content in expanding leaf blades of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.



Cultivar - ns, [CO_2]- P<0.01, Organ- P<0.01, Cultivar x [CO_2] x Organ- ns

Supplementary Figure S2. Total carbon concentration of **(a)** Last fully expanded leaf blades (LFELB), **(b)** expanding leaf blades (ELB), and **(c)** cell elongation region of Kukri and RAC875 grown at ambient (400 µmol mol^{-1}) or elevated (700±10 µmol mol^{-1}) [CO_2]. Data presented are the mean (±SE) of n=3. P values indicate the significance: *, P<0.05; **, P<0.01; ns, not significant.

Supplementary Table S1. Concentration of macro and micronutrients in the growing solution provided during wheat growth during early vegetative stage. Half strength nutrient solution was provided. Adapted from (Fernando *et al.*, 2017; Makino *et al.*, 1983).

Chemical given in the reference	Available form	Element	Full strength of final solution (mM)	Molecular weight (g/mol)	Nutirent concenteation in final solution (X)	Final concentration in the solution (mM)	Stock solution concentration (mM)	Amount of chemicals to be dissolved in stock solution (g/L)	Volume of stock solution per litre of final solution (ml)	Half strength solution (ml)	Volume of stock solution per litre of final solution (mM)
Macronutrients											
NH_4NO_3	NH_4NO_3	N	1	80.04	1000	0.5	500	40.02	1	0.25	0.5
KH_2PO_4	KH_2PO_4	K	0.8	136.09	1000	0.8	800	108.87	1	0.4	0.5
		P	0.8								
$CaCl_2.2H_2O$	$CaCl_2.2H_2O$	Ca	0.6	147.02	1000	0.6	600	88.21	1	0.3	0.5
		Cl	1.2								
$MgSO_4$	$MgSO_4.7H_2O$	S	0.5	246.48	1000	0.5	500	123.24	1	0.25	0.5
		Mg	0.5								
Micronutrients											
Fe-EDTA	$C_{10}H_{12}FeNaO_8$	Fe	0.05	367.05	1000	0.05	50	18.35	1	0.025	0.5
H_3BO_3	H_3BO_3	B	0.04	61.83	10000	0.04	400	24.73	0.1	0.02	0.05
$MnSO_4$	$MnSO_4.H_2O$	Mn	0.007	169.02	10000	0.007	70	11.83	0.1	0.0035	0.05
$ZnSO_4$	$ZnSO_4.7H_2O$	Zn	0.0005	287.54	10000	0.0005	5	1.44	0.1	0.00025	0.05
$CuSO_4$	$CuSO_4.5H_2O$	Cu	0.0002	249.69	10000	0.0002	2	0.49	0.1	0.0001	0.05
Na_2MoO_4	$Na_2MoO_4.2H_2O$	Mo	0.0001	241.95	10000	0.0001	1	0.24	0.1	0.00005	0.05

Supplementary Table S2. Comparison of leaf proteome response of RAC875 relative to Kukri at ambient [CO_2] (Protein expression relative to Kukri proteome at ambient [CO_2], fold change is given in Log_2 transformation, **P<0.01, * P<0.05).

Accession No	Protein Name	Fold change at a[CO_2] relative to Kukri	Molecular weight (kDa)	P value
	Carbohydrate metabolism			
A0A1D6S518	UTP-glucose-1-phosphate uridylyltransferase	3.28	51.664	**
A0A1D6DCS0	Ribokinase	1.63	36.971	**
Q1XIR9	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside beta-D-glucosidase 1a, chloroplastic	-3.24	64.508	**
W5I774	Sucrose synthase	2.86	92.400	**
A0A1D5XY50	Glyceraldehyde-3-phosphate dehydrogenase	0.32	46.858	*
	Post-translational modifications			
A0A1D6AKZ2	ATP-dependent Clp protease proteolytic subunit	3.00	32.197	**
W5F826	Proteasome subunit beta type	2.20	24.333	**
W5D591	Small ubiquitin-related modifier	0.36	11.101	**
A9EEM6	Triticain beta 2	-0.08	50.417	*
W5ERM8	Proteasome subunit beta type	1.75	23.198	*
A0A1D6B0C9	Aminopeptidase	-1.22	98.207	*
	Amino acid metabolism			
A0A1D5TXK5	Histidinol dehydrogenase, chloroplastic	0.29	46.697	**
A0A1D5VKI1	Cysteine synthase	1.33	40.934	**
P38076	Cysteine synthase	1.50	34.114	**
A0A1D5YM24	Cysteine synthase	0.81	40.452	**
A0A1D6RR58	Glutamate decarboxylase	1.27	53.899	*
W5GBW4	S-adenosylmethionine synthase	-0.75	43.252	*
A0A1D5SPT9	Branched-chain-amino-acid aminotransferase	-0.83	34.435	*
W5GE58	Aspartate aminotransferase	0.23	47.328	*
	Cell proliferation and differentiation			

W5EMA7	Proteasome subunit alpha type	2.39	27.436	**
A0A173FEH2	TSK1 protein	0.42	19.059	*
W5C3H4	Proteasome subunit alpha type	1.82	25.828	*
W5H3N4	Proteasome subunit alpha type	1.53	31.311	*
A0A1D5SZ32	(1,31,4) beta glucanase	1.49	31.492	*
	Anti-oxidant enzymes			
A0A1D6CV93	Peroxidase	2.13	38.416	**
R9W6A6	ER molecular chaperone	1.52	73.186	**
A0A1D5UU04	Peroxidase	-0.78	35.388	**
Q43206	Catalase-1	0.92	56.808	*
A0A1D6BEM4	Peroxidase	1.09	33.623	*
	Energy pathway			
W5C3E3	Pyruvate dehydrogenase E1 component subunit alpha	0.50	45.927	**
W5D0E3	Fructose-bisphosphate aldolase	1.13	38.895	**
A0A1D5SM88	Dihydrolipoyl dehydrogenase	1.15	52.795	*
A3KLL4	Malate dehydrogenase	-0.36	35.486	*
A0A1D5WER2	Isocitrate dehydrogenase [NADP]	0.09	50.575	*
A0A1D6AQ51	Pyruvate dehydrogenase E1 component subunit alpha	0.13	42.569	*
A0A1D6BYZ7	ATP-dependent 6-phosphofructokinase	1.02	48.041	*
	Photosynthesis			
P11383	Ribulose bisphosphate carboxylase large chain	1.89	52.851	**
	Cell wall metabolism			
A0A1D5XZ43	Beta-galactosidase	-0.47	92.126	**
	Signal transduction			
A0A1D5ST71	Gamma-glutamyl hydrolase	-0.04	41.489	**
I3RN54	Inorganic pyrophosphotase	1.90	24.287	**
	Protein synthesis			
A0A1D6C4Q5	60S acidic ribosomal protein P0	2.39	33.770	*
Q5I7K5	Ribosomal protein P1	1.02	11.193	*

W5FWT6	Aldehyde dehydrogenase 7B1	1.55	54.361	*
	Gene expression			
Q2QKB4	Splicing factor U2af large subunit B	0.32	60.586	*
	Hormone biosynthesis			
Q69G22	Pyridoxal kinase	0.82	34.241	*
	Nitrogen assimilation			
A0A1D5WER2	Isocitrate dehydrogenase [NADP]	0.09	24.287	*

Chapter 6

Discussion, Conclusions and Future Directions

Optimization of crop responses to rising atmospheric carbon dioxide concentration ([CO_2]) is considered as one of the best strategies to achieve future food production targets in a changing climate (Tausz *et al.*, 2013). The atmospheric carbon dioxide concentration ([CO_2]) now exceeds 400 µmolmol^{-1} (Tans & Keeling, 2018) and is predicted to reach up to 1020 µmolmol^{-1} by the end of this century (IPCC, 2014). As [CO_2] is the primary substrate for photosynthesis, crops are benefited by this increase resulting in greater biomass accumulation, growth and thereby the final yield (Ainsworth *et al.*, 2008). These improvements in biomass and growth are higher in C_3 crops such as wheat and rice, as C_3 biochemistry is not photosynthetically saturated at the current [CO_2] concentration (Ainsworth & Rogers, 2007). The magnitude of plant responses to elevated [CO_2], varies even within the same species suggesting that there is a significant genetic variation in CO_2 responsiveness (Thilakarathne *et al.*, 2015).

Increase in photosynthesis capacity and reduction in stomatal conductance are the two fundamental factors influencing the enhanced plant growth observed at elevated [CO_2] (Ainsworth & Rogers, 2007). Increases in photosynthesis rate in the exponential growth phase of plants could translate into higher absolute growth enhancements (Kirschbaum, 2010). Therefore, elevated [CO_2] provides a unique opportunity to increase crop productivity in a changing climate. Growth stimulation at elevated [CO_2] cannot be explained only by changes in photosynthesis rates alone. Modifications in other post-photosynthetic processes; carbon, nitrogen metabolism, cell cycle functions and hormonal regulation are also crucial in moderating plant growth responses to elevated [CO_2] (Gamage *et al.*, 2018). Many research studies to date have focused on characterizing photosynthetic responses, whilst paying little attention to the changes in post-photosynthetic processes in response to elevated [CO_2]. Characterizing this post-photosynthetic response will be beneficial in dissecting the physiological and molecular mechanisms of how plants respond to elevated [CO_2]. To that end, this study focussed on investigating the molecular basis of several key areas of post-photosynthetic processes likely to be linked with the control of growth responses at elevated [CO_2] using wheat (*Triticum aestivum* L.). As the CO_2 responsiveness of plants is high during the early vegetative growth stages, this study mainly focused on characterizing the early growth responses of wheat to elevated [CO_2].

In this research project, quantitative trait loci (QTL) associated with plant growth traits at the early vegetative stage were mapped with the purpose of identifying potential genetic components associated with plant growth responses in a CO_2 rich atmosphere. We then narrowed down our study to understand the transcriptome level changes of key genes associated with carbon, nitrogen metabolism and cell cycle functions in response to increased carbohydrate generation at elevated [CO_2] and their relationship with plant growth responses at elevated [CO_2]. Finally, we investigated the changes in leaf proteome to examine the interplay of different regulatory metabolic processes that may have contributed towards growth enhancement at elevated [CO_2]. Each of these objectives contributes to the advancement of our knowledge in the underlying molecular mechanisms of plant growth responses in a high CO_2 world.

6.1 Significant findings of the study

In the first experimental chapter (Chapter 03), QTL for shoot and root biomass accumulation, root to shoot dry weight ratio, total biomass accumulation, seedling height, leaf elongation rate and leaf width were determined in a doubled haploid (DH) population of wheat grown at elevated [CO_2] (700 µmolmlol^{-1}). Parental line characterization results indicated that the two parental cultivars, Kukri and RAC875 showed different responses at elevated [CO_2]. For both cultivars, elevated [CO_2] substantially increased biomass accumulation, the rate of photosynthesis and different growth parameters. Consistent with this, the DH lines of the mapping population also showed a differential response to all the growth traits tested when grown under elevated [CO_2]. These results suggested that QTL mapped from this data would be of value for further detailed analysis. Overall, in this study we mapped 28 putative QTL under elevated [CO_2] and 24 putative QTL CO_2-response QTL for the above mentioned nine growth traits. Amongst the CO_2-response QTL, three QTL identified on chromosome 2A, 1B and 4B showed an increased responsiveness for biomass accumulation at elevated [CO_2]. Interestingly, the QTL on 2A and 4B also contributed to the increase in shoot dry weight under high [CO_2]. This suggests that these three QTL may play a significant role in increasing biomass accumulation at elevated [CO_2] and impact on the genetic component of phenotypic variation of a particular genotype. Overall, the identification of different QTL for growth traits at ambient and elevated [CO_2] further implied that there might be a differential genetic control for plant growth at elevated [CO_2]. To the best of our knowledge, this study is the first report

to identify genomic regions in wheat, which may influence plant growth traits at elevated [CO_2], focusing on early vegetative growth stages.

Chapter 4 of this thesis focussed on examining the changes in elevated CO_2 mediated gene expression related to photosynthetic and several post-photosynthetic processes and their potential association with growth responses during the early vegetative stage. Physiological analysis of different DH lines selected based on the CO_2 responsiveness of biomass accumulation along with Kukri and RAC875 (parental lines) showed that biomass accumulation, leaf area production and carbohydrate generation are genotypically varied at elevated [CO_2]. This intraspecific variation in response to elevated [CO_2] is of great importance as this opens the way for the selection of higher yielding genotypes under future climate conditions (Tausz *et al.*, 2013). Gene expression analysis of key genes related to photosynthesis, carbon/nitrogen metabolism and cell-wall metabolism further indicates that CO_2 responsiveness among different genotypes varied even at the transcript level. In particular, differential expression of genes in carbon (*SPS1* and *SUS1*) and cell-wall metabolism (gene members of β-expansins and Xyloglucan endotransglucosylase/hydrolases) was observed in response to elevated [CO_2]. Of the two parental cultivars, RAC875 showed higher transcript abundance for most of the genes examined in this study. Any change related to the key metabolic processes (carbon/nitrogen, cell cycle functions) at transcript level would affect the subsequent protein synthesis and thereby influence plant growth at elevated [CO_2]. Further, the differential expression of key genes related to different metabolic processes is organ-specific and expression is closely linked with the availability of soluble carbohydrate of a particular organ. This organ-specific gene expression pattern may be the key controlling force of carbon and nitrogen partitioning to different plant organs and thereby maintain carbohydrate production in source organs (e.g. last fully expanded leaf) and utilization demand in sink organs (i.e expanding leaf and shoot apex region). This source and sink integration will then contribute to the growth of an organ and thereby the whole plant.

In the final experimental chapter (Chapter 5), a comparative proteomic approach was employed to investigate the changes in leaf proteome in response to elevated [CO_2]. Since, Chapter 4 results indicated that there are significant transcriptome level responses of key metabolic processes, a proteomic analysis would be able to investigate changes in protein turnover in response to elevated [CO_2]. Proteome changes were investigated using the expanding leaf blades of Kukri and RAC875 (parental cultivars of the DH population) because of the high

nitrogen concentration observed at elevated [CO_2]. Due to the elimination of Rubisco, the most abundant protein in C_3 crops, we were able to examine the changes of other less abundant proteins under high CO_2 conditions. Results of this study showed that most of the differentially expressed proteins at elevated [CO_2] belong to carbon metabolism (sucrose synthase), energy generation, synthesis and processing of proteins (mainly ribosomal proteins), cell cycle proteins (histones) and cell wall loosening enzymes (cell wall peroxidases). This indicates that along with carbohydrate synthesis through increased photosynthetic carbon fixation, elevated [CO_2] also stimulates the breakdown of carbohydrates through respiration to provide sufficient energy for leaf expansion and growth. Ribosomal protein synthesis was significantly higher at elevated [CO_2], which may have facilitated increased cell proliferation and cytoplasmic content, thereby enhancing leaf expansion. Also, differential expression of anti-oxidant enzymes was observed under elevated [CO_2] suggesting a change in reactive oxygen species (ROS) within the plant. It is possible that these ROS together with cell wall peroxidases perform a noteworthy role in cell wall expansion. In addition to these, several signaling proteins involved in the Ca^{2+} signaling pathway, control of cell division/ signaling and protein synthesis were differentially expressed at elevated [CO_2]. This differential expression of proteins in our study was similar to a proteomics analysis conducted by Bokhari *et al.* (2007) using ten day old rice seedlings under high [CO_2]. To our knowledge, this is the first study to investigate the wheat leaf proteome changes in response to elevated [CO_2] focusing on the early growth stage.

6.2 Proposed mechanism for early growth stimulation of wheat at elevated [CO_2]

The proposed mechanism of growth responses of wheat to elevated [CO_2] during early vegetative stage is represented in Figure 1.

In the source tissues, primarily in the last fully expanded leaf blades (LFELB) at the early vegetative stage, elevated [CO_2] increases the CO_2/O_2 ratio at the site of CO_2 fixation. Therefore, the efficiency of the carboxylation efficiency of Rubisco is promoted through the lowering of photorespiration. The genes encoding Rubisco large and small subunits, *rbcL* and *rbcS* substantially upregulated at early vegetative stage (Chapter 4) supporting optimum Rubisco synthesis to utilize the increased [CO_2]. The higher efficiency of PSII, electron transport rate and photochemical quenching correlated with the increased photosynthesis rate at elevated [CO_2] (Chapter 4) and this may have been involved in producing ATP and NADPH, the vital energy components required to maintain photosynthesis at an optimum rate. This increased photosynthesis capacity at elevated [CO_2], then leads to high levels of sucrose

production in the source leaves (Chapter 4). The key genes encoding primary enzymes of sucrose metabolism, sucrose phosphate synthase (*SPS1*) and sucrose phosphate phosphatase (*SPP1*) were markedly upregulated in the LFELB to facilitate sucrose production (Chapter 4). Sucrose and other photosynthetic products provide energy and carbon skeletons for amino acid biosynthesis (Zheng, 2009). Respiratory breakdown of sucrose generates 2-oxoglutarate (2-OG) and this serves as the carbon skeleton for the synthesis of glutamate (Zheng, 2009). In the process of NO_3^- photo assimilation, conversion of NO_2^- to NH_4^+ by *NiR* showed an upregulation at elevated [CO_2]. However, this expression was less when compared with the expression of genes encoding the GS/GOGAT pathway (Chapter 4). NH_4^+ from this primary nitrogen metabolism is then incorporated to glutamate, which is catalysed, by glutamine synthetase (GS) and results in glutamine production (Stitt & Krapp, 1999). The expression of *GS2a* that encode plastidial GS and *GS1a* and *GSr1*, which encode cytosol, GS was significantly higher in LFELB at elevated [CO_2] (Chapter 4). Further genes encoding enzyme Fd-GOGAT and NADH-GOGAT were substantially higher at elevated [CO_2] indicating efficient nitrogen assimilation in the early vegetative stage of the plants. Glutamine and glutamate serve as the NH_4^+ donors to the synthesis of other amino acids required for protein synthesis (Stitt & Krapp, 1999). The sucrose and the amino acids generated in the LFELB would then be translocated to sink organs via the phloem.

In the early vegetative phase, expanding leaf blades (ELB) and the shoot apex region (SAR) can be considered as the major sink organs. In SAR, sucrose translocated from LFELB will be cleaved by sucrose synthase (*SUS1*), which showed a higher upregulation in growing tissues at elevated [CO_2] (Chapter 4). The resulting UDP-Glucose plays an important role as a substrate for the re-synthesis of sucrose from available glucose and fructose (Yong *et al.*, 2000, Koch, 2004). Also, UDP-Glucose is an important component in cell wall biosynthesis of dividing cells (Verbančič *et al.*, 2017). Increased activity of cell wall metabolism was evident from the higher expression of genes encoding β-expansins and Xyloglucan endotransglucosylase/hydrolases (Chapter 4). This implied that cell wall loosening, and expansion has been promoted at elevated [CO_2]. High availability of glucose and fructose of SAR positively correlates with cell division (Koch, 2004). Therefore, in the shoot apex region, active utilization of carbohydrates and increased production and expansion of cells may have facilitated increased growth rates at elevated [CO_2].

A similar pattern for sucrose metabolism was observed in the ELB of wheat at elevated [CO_2]. The results of proteomics analysis showed that SUS enzyme was significantly upregulated at

elevated [CO_2] resulting in the production of UDP-Glucose. Other than this, UTP-glucose-1-phosphate uridylyltransferase, the enzyme that produces UDP-Glucose from Glucose 1 phosphate and UTP had been significantly upregulated (Chapter 5) indicating a higher production of UDP-Glucose in the ELB at elevated [CO_2]. This may have contributed for increased UDP-Glucose production which is an essential substrate for sucrose and cell wall biosynthesis of growing tissues. The increased sucrose supply from LFELB and resynthesizes of sucrose within the tissue increases the sucrose concentration of ELB. This increased sugar supply then promotes expression of proteins related to cell division such as tubulin and protein synthesis such as ribosomal proteins required for cytoplasmic growth (Chapter 5). As a result of these metabolic changes, increased cell proliferation and expansion takes place within the ELB and thus, increases the leaf elongation rate at elevated [CO_2]. The increased photosynthetic capacity at elevated [CO_2] remains high until the sink utilization of carbohydrate supply remains high. In the early vegetative stages, plants' plasticity to develop sink organs is relatively high and thus, higher growth stimulation can be observed at elevated [CO_2].

However, this growth stimulation observed under elevated [CO_2] varied significantly between wheat cultivars indicating a noticeable intraspecific variability. The CO_2 responsiveness of wheat is genetically determined (Chapter 3), and this may be depending on the plants' ability to expand their sink capacity in response to increased sugar supply at elevated [CO_2]. This intraspecific variability in plant responses to elevated [CO_2] was observed even at transcript (Chapter 4) and proteomic level (Chapter 5). These variable responses are of great importance for selecting the best genotypes that can thrive well in a high CO_2 world.

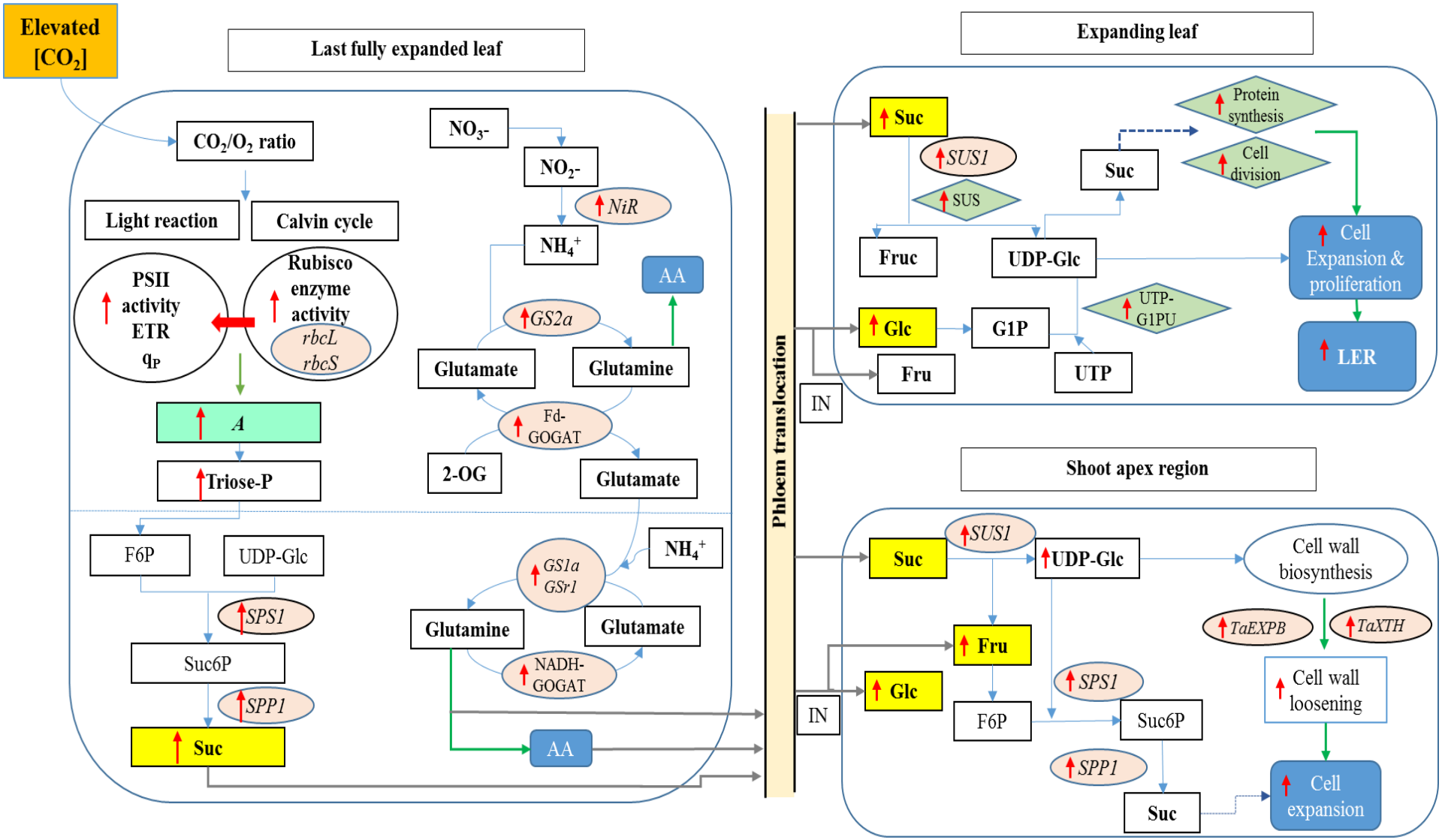

Elevated [CO2]
Last fully expanded leaf
CO2/O2 ratio
Light reaction
Calvin cycle
PSII activity ETR qP
Rubisco enzyme activity
rbcL rbcS
A
Triose-P
F6P
UDP-Glc
SPS1
Suc6P
SPP1
Suc
NO3-
NO2-
NiR
NH4+
AA
GS2a
Glutamate
Glutamine
Fd-GOGAT
2-OG
Glutamate
GS1a GSr1
NH4+
Glutamine
Glutamate
NADH-GOGAT
AA
Phloem translocation
IN
Expanding leaf
Suc
SUS1
SUS
Suc
Protein synthesis
Cell division
Fruc
UDP-Glc
Cell Expansion & proliferation
Glc
G1P
UTP-G1PU
Fru
UTP
LER
IN
Shoot apex region
Suc
SUS1
UDP-Glc
Cell wall biosynthesis
TaEXPB
TaXTH
Fru
Glc
SPS1
F6P
Suc6P
Cell wall loosening
SPP1
Suc
Cell expansion

Figure 1. Schematic diagram of molecular changes of source (last fully expanded leaf) and sink (expanding leaf and shoot apex region) integration of wheat at elevated [CO_2] in early vegetative stage of wheat. Circles with light orange colour represent the transcript level changes of relevant genes at elevated [CO_2]. Green diamonds represent the differential protein expression of relevant metabolic activities at elevated [CO_2]. Blue colour rectangles

A: photosynthesis rate, PSII: photosystem II, ETR: electron transport rate, q_P- photochemical quenching, Glc: glucose, Fru: fructose, Suc: sucrose, F6P- fructose 6 phosphate, UDP-Glc: UDP glucose, Suc6P: sucrose 6 phosphate, G1P: glucose 1 phosohate, UTP: uridine triphosphate, AA- amino acids synthesis & metabolism, UTP-G1PU: UTP-glucose-1-phosphate uridylyltransferase *rbcL* - ribulose 1,5 –bisphosphate carboxylase/oxygenase (large sub unit); *rbcS* - ribulose 1,5 –bisphosphate carboxylase/oxygenase (small sub unit); *SPP*- Sucrose Phosphate Phosphatase; *SPS*- Sucrose Phosphate Synthase; *SUS* - Sucrose Synthase type 1; *NiR* - Ferredoxin Nitrite Reductase; *Fd-GOGAT* - Putative ferredoxin-dependent glutamate synthase; *GS2a* - Glutamine Synthetase (Plastidial), *NADH-GOGAT* - Putative NADH-dependent glutamate synthase; *GS1a* - Glutamine synthetase (cytosolic); *GSr1* - Glutamine synthetase (cytosolic) *TaEXPB* - β – expansins *TaXTH* - Xyloglucan endotransglucosylase/hydrolases

6.3 Future directions

The experiments discussed in this thesis employed a multidisciplinary approach to further elucidate the molecular mechanisms of plant growth responses to elevated atmospheric [CO_2]. The study attempted to dissect the underlying mechanisms of plant growth at the genomic, transcriptomic and proteomic level focusing on the early vegetative growth stage. More research is required both to confirm these initial observations and to establish in much more detail the regulation of growth responses under elevated [CO_2]. This will be beneficial for developing climate-smart wheat genotypes for the future high CO_2 world.

The results of our QTL mapping experiment indicated several putative QTL that might potentially play a significant role in determining biomass accumulation at elevated [CO_2]. However, in our study we could not further validate the QTL to confirm their reproducibility. Before using them in marker-assisted breeding, these QTL regions have to be validated to rule out possible errors associated with QTL mapping. Therefore, re-running the QTL mapping using the same DH mapping population in different locations, especially under field conditions and in different years to test whether the same QTL effect can be detected even under different environmental conditions is necessary. For this purpose, Near-Isogenic lines (NIL) can be selected for a particular trait or molecular marker through a series of backcrosses to a recurrent parent, which does not express the trait of interest. Phenotypic differences between the parental lines and the NIL lines will allow investigation of phenotypic differences due to the QTL of interest. If the identified QTL are good enough to be used in the breeding programmes, these QTL can be combined into the same line through pyramiding and can be used in breeding crops for future CO_2 enriched atmosphere. Further, in-depth analysis of these QTL regions is necessary to identify candidate genes that may be essential for plant growth and adaptation to future CO_2 levels. Our study was limited to the identification of QTL regions during the early vegetative growth stage. Therefore, this study can be further extended to elucidate the CO_2 responsive genomic regions associated with other key developmental stages of wheat.

The results of the second study implied that elevated [CO_2] significantly influences plant transcriptome level changes and organ-specific expression patterns of key genes might play a role in determining source-sink integration under high CO_2 levels. Our study only investigated a few selected key genes related to photosynthesis, carbon/nitrogen metabolism and cell-wall metabolism. To obtain a more complete picture, it is better to conduct an in-depth analysis of

transcript level changes of each metabolic process at elevated [CO_2], which could be possibly achieved through microarray analysis. Therefore, comparative analysis of mature and growing organs using a microarray analysis might provide a more complete overview regarding changes at transcriptome level to elevated [CO_2]. Further in our study, we did not investigate the change of key genes related to hormonal regulation, though this plays a major role in moderating plant growth responses. Hence, studies into the investigation of transcript level changes of genes related to hormonal metabolism would assist in improving our understanding of the underlying molecular mechanisms at elevated [CO_2]. The transcript abundance during the vegetative stage and in the transition to reproductive stage will indicate the plants' transcriptome level changes to elevated [CO_2] and their relation to the source-sink integration of a plant.

Results of the comparative proteomics analysis revealed that key regulatory enzymes, intermediate metabolic proteins/biomolecules and signal transducing proteins were differentially expressed under elevated [CO_2]. The changes in the proteome of expanding leaves allowed us to identify more proteins related to carbohydrate utilization, protein synthesis, cell division and expansion. Similarly, a comparative proteomic analysis of the shoot apex region will give a more complete picture of how cell cycle functions change in response to elevated [CO_2] as Chapter 4 results indicated that cell cycle functions are likely to be more responsive to elevated [CO_2]. Of all the identified proteins, a significant proportion was uncharacterized proteins; the functionality of these proteins is not yet confirmed. There were a few uncharacterized proteins that showed significant differential expression in both cultivars. Studies into annotation and curation of these uncharacterized proteins will be beneficial in unravelling the underlying mechanisms of plant growth responses to elevated [CO_2]. Overall, out of two parental cultivars of the DH mapping population used in this thesis, RAC875 showed a noteworthy CO_2 responsiveness both at the transcriptome and proteome level. Thus, possibilities to use RAC875 as a potential breeding line for crop breeding programmes designed for a future CO_2 enriched atmosphere need to be investigated.

6.4 Conclusion

It is crucial to understand the underlying molecular mechanisms of plant growth responses to elevated [CO_2] in order to develop effective crop breeding strategies for a future CO_2 enriched world. Each of the experimental chapters of this thesis, contributed to elucidating the molecular mechanism of how plants respond to elevated [CO_2] at the genomic, transcriptomic and proteomic level using wheat, focusing on the early vegetative growth stages. In Chapter 3, three main CO_2-response QTL for biomass accumulation were identified on chromosome 2A, 1B and 4B, of which QTL on chromosome 2A and 4B also contributed to increasing shoot dry weight at elevated [CO_2]. These QTL can be validated and used for further in-depth analysis to identify the candidate genes involved in moderating plant adaptations to elevated [CO_2]. The results of Chapter 4 indicated that plants show an organ-specific expression pattern for key genes associated with photosynthesis and post-photosynthetic processes at elevated [CO_2]. Carbon metabolism, nitrogen metabolism and cell-wall metabolism are highly influenced by increased sugar supply under high CO_2 conditions and of them, several genes related to cell-wall metabolism showed strong positive correlations with the soluble carbohydrate content. These changes in the transcriptome may have influenced protein synthesis that governs plant growth and development. Results of Chapter 5 showed that proteins involved in carbohydrate synthesis and respiratory breakdown, protein synthesis and processing, cell division and cell-wall metabolism significantly changed their expression at elevated [CO_2] and are likely to be involved in enhanced leaf elongation observed in the high CO_2 environment. Overall, this project contributes to the lessening of the knowledge gaps in our understanding of molecular mechanisms of plant growth responses to elevated [CO_2] and yet more work is required to develop a more refined mechanism of how elevated [CO_2] regulates plant growth. However, in the real world, elevated [CO_2] closely interacts with other climatic parameters such as increased temperatures and prolonged drought conditions. Therefore, elucidation of the underlying mechanisms of plant growth responses to elevated [CO_2] is very challenging under this triple whammy of challenges (elevated CO_2, high temperature and water scarcity) anticipated in the future.

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