Mechanisms of the decline in grain protein concentration in wheat under elevated atmospheric CO_2 concentration

A Thesis submitted by

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Abstract

Wheat is one of the most produced crops worldwide and is an important source of protein for many people. Climate conditions predicted for the future will have a major impact on the growth and nutritional quality of wheat, due to the increasing atmospheric carbon dioxide concentration ($[CO_2]$). By the end of the century, the $[CO_2]$ is predicted to increase to at least 700 µmol mol^{-1}, rising from the current concentration of approximately 400 µmol mol^{-1}. Many studies show that wheat grown under these conditions assimilates more carbon and, therefore, increases in biomass and yield. On the other hand, studies have also demonstrated that wheat grown under future $[CO_2]$ declines in grain protein concentration (GPC). Currently, there is a lack of information on what causes this decline in wheat under elevated $[CO_2]$. Current explanations for the decline in GPC consist of biomass dilution, whereby an increase in grain biomass dilutes the grain protein, and inhibition of nitrate assimilation, where nitrogen remains in the form of nitrate in leaves and is unable to be assimilated and remobilised to the grain. However, these mechanisms do not completely explain the decline in GPC. Due to the lack of understanding in this area, this thesis aimed to investigate three unexplored aspects of GPC decline: i) Identifying whether difference exists in the GPC of three wheat types (tetraploid, hexaploid and synthetic hexaploid) in response to e$[CO_2]$; ii) Determining the traits with the greatest contribution to wheat GPC under e$[CO_2]$; iii) Investigating potential sugar sensing pathways in roots of wheat which control expression of nitrogen uptake and assimilation related genes under e$[CO_2]$.

Wheat belonging to three types (tetraploid, hexaploid and synthetic hexaploid) were grown under ambient and elevated $[CO_2]$ (e$[CO_2]$) to identify whether the response of GPC was different between the three wheat types. In addition, biomass measurements were taken to explore the extent of biomass dilution in explaining GPC decline. The response of GPC to e$[CO_2]$ was found to be genotype dependent, rather than wheat type dependent and biomass dilution could not completely explain the change in GPC. Understanding the extent that e$[CO_2]$ affects other traits that contribute to the plant's GPC is important in order to identify any specific mechanisms controlling GPC response. Traits associated with nitrogen uptake and remobilisation were measured in addition to plant biomass and rate of photosynthesis. Elevated $[CO_2]$ did not have a consistent effect on each of the traits studied. The decline in GPC appeared to be caused by a combination of traits, rather than a single trait, although GPC typically declined due to both an increase in grain biomass and decline in nitrogen uptake. Studies have shown that photosynthesis is downregulated under e$[CO_2]$ due to sugar sensing. As such, this

study aimed to identify whether a change in sugar was associated with a decline in nitrogen uptake and assimilation in roots of wheat seedlings through gene expression and proteomics analysis. While increased sugar was associated with an increase in expression of an ammonium transporter and glutamine synthetase, there was not sufficient evidence to indicate regulation of nitrogen uptake and assimilation related gene expression by e[CO_2] through sugar sensing pathways. Overall, this thesis further increases the knowledge available on the mechanisms affecting GPC in response to e[CO_2].

Certification of Thesis

This Thesis is the work of Michael Thompson 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.

Principal Supervisor: Professor Saman Seneweera

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Student and supervisors signatures of endorsement are held at the University.

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Statement of Authorship

Chapter 2: Effects of Elevated Carbon Dioxide on Photosynthesis and Carbon Partitioning: A Perspective on Root Sugar Sensing and Hormonal Crosstalk. **Thompson M**, Gamage D, Hirotsu N, Martin A & Seneweera S 2017, Frontiers in Physiology, 8:578, 10.3389/fphys.2017.00578

Michael Thompson (MT) contributed to 70% 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. Anke Martin (AM), Dananjali Gamage (DG) and Naoki Hirotsu (NH) contributed to 10% of the paper, consisting of revision and editorial input.

Chapter 3: Effect of elevated carbon dioxide on plant biomass and grain protein concentration differs across bread, durum and synthetic hexaploid wheat genotypes. **Thompson M**, Gamage D, Ratnasekera D, Perera A, Martin A and Seneweera S. (Under review)

MT contributed 55%, consisting of experimental design, data collection and analysis, and writing and revising the manuscript. SS and AM contributed 20% and 10%, respectively, consisting of experimental design, data analysis and revision of the manuscript. Disna Ratnasekera (DR) and Anton Perera (AP) contributed 10%, consisting of data collection and revision of the manuscript. DG contributed 5%, consisting of the manuscript's revision.

Chapter 4: Grain nitrogen concentration at elevated [CO_2] is mainly determined by genotype dependent variations in nitrogen uptake and nitrogen utilisation efficiency of wheat. **Thompson M**, Okamoto M, Martin A, Seneweera S. (Prepared for submission)

MT contributed 60%, consisting of the experimental design, data collection and analysis, and writing and revising the manuscript. SS and AM contributed 20% and 10%, respectively, consisting of experimental design, data analysis and revision of the manuscript. Mamoru Okamoto (MO) contributed to 10%, supplying the population the genotypes were selected from and revising the manuscript.

Chapter 5: Effect of increased root sugar supply on expression of nitrogen uptake and assimilation genes under elevated carbon dioxide. **Thompson M**, Gamage D, Fukushima A, Hirotsu N, Martin A, Seneweera S. (Prepared for submission)

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Chapter 1

Introduction and Literature Review

1.1 Wheat and its importance

In 2015, world cereal production was estimated to be 2,570 million tonnes, with wheat's global production estimated at 735 million tonnes, almost a third of the total cereal production (FAO, 2016). Wheat is the most cultivated cereal crop in the world, grown over more land area than any other cereal and provides approximately 21% of the world's daily dietary intake of protein, making it the most important source of protein globally (Shiferaw et al., 2013). Wheat can be separated into bread wheat (*Triticum aestivum*) and durum wheat (*Triticum turgidum spp. Durum*). Durum wheat is a tetraploid (2n = 4x = 28; AABB genome) wheat species thought to have arisen due to the hybridisation of *Triticum urartu* (AA genome) and *Aegilops speltoides* (SS genome), while bread wheat is a hexaploid (2n = 6x = 42; AABBDD genome) wheat species which arose after the hybridisation of the durum ancestor *T. turgidum* (AABB genome) and the diploid grass species *Aegilops tauschii* (DD genome) (Petersen et al., 2006; IWGSC, 2014). Bread wheat is the dominant wheat species produced, accounting for 95% of wheat produced in contrast with durum wheat (5%) (Peng et al., 2011). Due to the global importance of wheat it is necessary to maintain, if not improve, its yield and nutritional properties. However, as part of climate change, the atmospheric carbon dioxide concentration ([CO_2]) is increasing and past research has observed a decline in the grain protein concentration (GPC) of wheat under the future predictions of [CO_2]. The focus of this study is therefore primarily centred on the effect e[CO_2] has on wheat in terms of GPC and what mechanisms lead to its commonly observed decline.

1.3 Elevated [CO_2] and wheat grain protein concentration

The concentration of CO_2 ([CO_2]) in the atmosphere has drastically increased since the beginning of the industrial revolution, rising from 280 ppm (~1750) to 381 ppm in 2006 (Canadell et al., 2007), an increase of 36%. Since then it has continued to increase and currently sits around 400 ppm (NOAA, 2018). An increase of carbon emissions into the atmosphere is thought to be the main reason for such increases and predictions indicate that continued emissions will cause further increases (Meehl et al., 2007). The main contributor to the increase in atmospheric [CO_2] is the burning of fossil fuels, with changes to land-use a smaller, but still significant, contributor (IPCC, 2007).

The effects of elevated [CO_2] (e[CO_2]), as predicted for future climates, have been documented over years of research for a variety of crop species. Understanding the impact CO_2 has on crops is of great importance for the future food security of the world. Throughout the literature there is evidence of both benefits and detriments to the nutritional status of crops when exposed to e[CO_2].

It is widely reported that the protein concentration of wheat grain declines when the plant is grown under e[CO_2]. The first report of declines in grain protein came from Conroy et al. (1994), where the protein concentration of flour produced from grain grown under e[CO_2] declined. The decline in GPC was as great as 22% in one study (Erbs et al., 2010) while other studies have shown lesser declines (3.5% decline in the case of Högy et al. (2009a)). Other studies vary in GPC decline, but fall within this range (Piikki et al., 2008; Högy et al., 2009b; Fernando et al., 2015). From these studies, it is evident that there is no clear consistency to the severity of GPC decline, but it supports the hypothesis that GPC decreases under e[CO_2]. Furthermore, reviews and meta-analyses on the subject agree that there is a negative effect of e[CO_2] on protein concentration (Taub et al., 2008; Myers et al., 2014). The decline in GPC in each study commonly coincided with an increase in grain yield and an analysis by Amthor (2001) of fifty studies found that, on average, doubling [CO_2] from 350 to 700 ppm caused an increase in grain yield in wheat of 31%.

In contrast to the previously mentioned studies, results have also found e[CO_2] to have no effect on grain protein concentration. Lam et al. (2012) found that for wheat grown under 550 ± 17 ppm [CO_2] there was no significant effect on grain protein concentration. Elevated [CO_2] instead appeared to promote the translocation of nitrogen from the stem to the grain, maintaining the grain protein concentration with the increase in grain yield. This study, however, seems to be the exception. These findings indicate genetic variation in grain protein response of wheat to e[CO_2].

The decreases seen in GPC under e[CO_2] have been suggested to be caused by biomass dilution. This occurs due to the increased accumulation of non-structural carbohydrates relative to the amount of nitrogen in grains as a consequence of enhanced photosynthesis at e[CO_2] (Gifford et al., 2000, Seneweera et al., 2005). However, it has been demonstrated that the increase of non-structural carbohydrates at e[CO_2] does not fully explain the reduction of GPC at e[CO_2] (Gifford et al., 2000, Taub and Wang, 2008). Dilution may only account for a small part of the decreases of GPC, with increased starch accounting for less than one third of GPC decrease in

one study (Wu et al., 2004, Taub et al., 2008). Other studies have indicated that part of the decline could be explained by an inhibition of NO_3^- in leaves (Bloom et al., 2014; Bahrami et al., 2017), however, the exact physiological mechanisms for GPC decline and the magnitude of the impact each has is not fully understood.

While GPC often declines, the total grain protein (TGP) content can still increase (Kimball et al. 2001; Fernando et al. 2017). The TGP content indicates how many total grams of protein exists within the grain. The GPC, on the other hand, is a measure of TGP as a percentage of the total grain biomass. This means that if the grain biomass increases proportionally more than the TGP, GPC will decline. An increase in TGP does not necessarily improve the nutritional quality. A decline in GPC lowers the quality of wheat-based food products. Fernando et al., (2015) showed that e[CO_2] lowered both bread volume and dough strength by 11% and 7%, respectively. This occurred alongside an increase in the total protein. Therefore it is important for studies to focus on GPC as a marker of nutritional quality rather than TGP.

GPC is also regulated by environmental factors other than [CO_2] (Fernando et al., 2014). The use of irrigation with wheat has been shown to cause a negative effect on protein concentration (Erekul et al., 2012, Fernando et al., 2014). Fernando et al. (2014) also found that the time of sowing has an effect on GPC outcome with a later sowing time causing a decline in GPC. These findings suggest a greater interaction of other environmental factors with [CO_2] on grain protein quality and concentration. GPC and quality response to e[CO_2] also varied between cultivars. Modern wheat cultivars have displayed greater reductions in grain and flour protein concentration than older cultivars (Ziska et al., 2004). Through ^{15}N labelling, Aljazairi et al. (2014) found that a modern durum cultivar was better adapted to assimilate nitrogen at higher CO_2 levels than a traditional durum cultivar. With ^{13}C labelling, Aljazairi et al. (2014) also found modern durum invested more C in spikes than the traditional genotype, which invested more in non-reproductive shoot tissue.

While decreases in the GPC of wheat have been observed, it is not understood exactly how e[CO_2] causes this to happen. The aim of this study is to fill in some of the missing information about GPC decline under e[CO_2]. In order to understand the mechanisms controlling GPC, it is crucial to first understand how nitrogen flows through wheat to be stored in the grain.

1.4 Nitrogen use by plants

Nitrogen is the most important macronutrient required by plants and deficiency in this nutrient often limits plant growth (Crawford and Glass, 1998). Terrestrial plants primarily take up

nitrogen as soil inorganic nitrogen in the forms of nitrate (NO_3^-) and ammonium (NH_4^+) (Bloom et al., 2010), though to a lesser extent there is evidence some plants can also take up organic nitrogen in the form of proteins and peptides (Paungfoo-Lonhienne et al., 2008). Several steps are involved in nitrogen usage by plants, consisting of uptake, assimilation, translocation and remobilisation (Masclaux-Daubresse et al., 2010).

Nitrogen is taken up by two processes, namely the active and passive transport systems. Among them, active transport plays a vital role. During active transport, NO_3^- is taken into roots via NO_3^- transporters of two systems: the high- and low-affinity transport systems (HATS and LATS, respectively) (Crawford and Glass, 1998). There are two types of NO_3^- transporters, which are known as the NRT1/NPF family and the NRT2 family, where NRT1 transporters are high-affinity transporters and most NRT2 transporters are low-affinity transporters (Tsay et al., 2007, Léran et al., 2014). High affinity transporters function in very low external NO_3^- concentrations, while low-affinity transporters function at higher NO_3^- concentrations (>1mM) (Orsel et al., 2002). The other main source of nitrogen, NH_4^+, is taken up into roots by the ammonium transporter (AMT) family of transporters (Ludewig et al., 2007). Transporting NO_3^- out of the roots occurs by loading NO_3^- into the xylem where it can then be transported to the other tissues. A known transporter that functions in this xylem loading belongs to the NRT1 family, and is known as NRT1.5 (Lin et al., 2008). However, mutation of this transporter does not halt NO_3^- transport to aerial tissues, which suggests it is not the only method of NO_3^- xylem loading (Lin et al., 2008). Another NRT transporter, labelled NRT1.8, is utilized in the plasma membrane of xylem parenchyma cells with the purpose of removing NO_3^- from the xylem sap (Li et al., 2010).

Assimilation of nitrogen can happen in both roots and leaves, however, it is most common for NO_3^- to be assimilated in the leaves (Xu et al., 2012). Nitrate assimilation begins with the reduction of NO_3^- to nitrite (NO_2^-) by the enzyme nitrate reductase before being further reduced by nitrite reductase into NH_4^+ (Tischner, 2000; Krapp, 2015). Nitrate assimilation occurs in the cytosol, while NO_2^- reduction to NH_4^+ occurs in the chloroplast (Krapp, 2015). Ammonium is then assimilated into glutamate through the GS/GOGAT cycle (Masclaux-Daubresse et al., 2010; Krapp, 2015). Ammonium assimilation begins with the enzyme glutamine synthetase (GS) creating glutamine by attaching an NH_4^+ ion onto a molecule of glutamate, before glutamate synthase (GOGAT) then converts the molecule of glutamine into two molecules of glutamate (Masclaux-Daubresse et al., 2010).

In wheat, nitrogen taken up by plants prior to anthesis is mostly remobilised to the grain, contributing around 60 – 95% of the total nitrogen in grains (Palta and Fillery, 1995). This means that nitrogen remobilisation efficiency (NRE) is an important trait for determining the GPC of wheat. Specifically, NRE refers to how much of the plant's total nitrogen stores are remobilised to the grain, which may be affected by the duration of senescence (Gaju et al. 2014). Remobilisation of pre-anthesis stored nitrogen is determined by both genetics and amount of nitrogen fertilizer applied (Palta and Fillery, 1995, Kichey et al., 2007). The level of activity of glutamine synthetase has been shown to be a good marker in wheat for nitrogen remobilisation to grains (Kichey et al., 2007). Rubisco makes up about 30% of the total protein invested in leaves and is thought to be the most abundant protein in the world (Parry et al., 2003, Raven, 2013). During senescence, Rubisco is degraded and becomes a major internal source of nitrogen for remobilisation. Nitrogen from Rubisco degradation can then be translocated to grains and young leaves and the remobilised nitrogen utilized for the protein in mature seeds (Feller et al., 2008). Rubisco is a rate limiting enzyme in photosynthesis, catalysing the reaction of either CO_2 or O_2 with ribulose-1,5-bisphosphate and initiating photosynthetic carbon assimilation (C3 reductive pathway) or photorespiration (C2 oxidative pathway) for CO_2 and O_2 respectively (Bloom, 2006). At leaf senescence, the amino acids asparagine and glutamine (glutamine preferred in cereals) increase in concentration in the phloem and can be remobilised to the grain (Masclaux-Daubresse et al., 2010).

1.5 Nitrogen use efficiency

With a continuously growing human population comes a concomitant increase in food demand. Improving crop productivity is a key area of research into securing a food source for sustaining the growing population. Nitrogen fertilizer is important for maintaining crop productivity, however, its production is highly energy consuming and it is becoming increasingly more expensive and less affordable to farmers (Rothstein, 2007). Crops are only able to utilize 30-40% of applied nitrogen resulting in greater than 60% of the nitrogen from the soil being lost due to leaching, surface run-off, denitrification, volatilization, and microbial consumption (Kant et al., 2011). In order to minimize nitrogen loss and reduce costs for farmers, it is important to improve crop nitrogen use efficiency (NUE). NUE is the yield of grain (or harvested product) achieved per unit of nitrogen available to the crop from soil or applied fertilizer (Sylvester-Bradley and Kindred, 2009). NUE can be broken down into two components; nitrogen uptake efficiency (NUpE), defined as crop nitrogen uptake per unit of nitrogen available from the soil and fertilizer, and; nitrogen utilisation efficiency (NUtE),

defined as grain dry matter yield per unit crop nitrogen uptake at harvest (NUtE) (Gaju et al., 2011). There have been few studies which have investigated the effect of e[CO_2] on either NUpE or NUtE, however, contrasting results have been found. In one study by Bahrami et al. (2017), e[CO_2] appeared to have no effect on the NUpE or NUtE of wheat, however, Tausz et al. (2017) observed that NUtE increased under e[CO_2], although NUtE declined when high nitrogen was applied.

1.6 Elevated [CO_2] effect on photosynthesis

One of the main responses of plants to e[CO_2] is an increase in photosynthetic rate (Ainsworth and Rogers, 2007). The photorespiratory pathway decreases the efficiency of photosynthesis by 20-50% by utilising the reducing power generated from the photosynthetic light reaction; however, at high [CO_2], the [CO_2] increase at the site of fixation competitively inhibits the reaction of photorespiration (Drake et al., 1997). Thus, a doubling of atmospheric CO_2 concentration initially inhibits photorespiration, accelerating photosynthesis and growth in C3 plants by around 30% (Bloom, 2006).

However, increased photosynthesis only occurs in the short-term at e[CO_2], with long-term exposure causing a reduction in photosynthesis; a phenomenon known as photosynthetic acclimation (Drake et al., 1997, Seneweera et al., 2002, Liu et al., 2011). Reduced photosynthesis appears to be associated with an accumulation of non-structural carbohydrates causing suppression of photosynthetic related genes (Jang and Sheen 1994). Cheng et al. (1998) found that *Arabidopsis thaliana* grown in e[CO_2] had a 2-fold increase in non-structural carbohydrates and a decline in Rubisco protein levels and Rubisco gene transcript levels. In contrast, Ludewig and Sonnewald (2000) found that in plants exposed to e[CO_2] with subsequently down-regulated photosynthetic gene transcripts, no increased levels of sugars were found; instead they suggested accelerated leaf senescence as the cause.

For the optimal response of wheat crops to e[CO_2], Aranjuelo et al. (2011) suggested that sufficient sink strength is required in order to utilize the increased carbohydrates and prevent the accumulation of carbohydrates in the shoot. Ainsworth et al. (2003) studied *Trifolium repens* grown under e[CO_2] for eight years and found that despite photosynthetic acclimation, the plant still retained a 37% increase in photosynthesis. This finding showed that photosynthetic acclimation may not completely prevent increases in photosynthesis under e[CO_2].

Photosynthetic acclimation can also be affected by genetic and environmental factors not related to accumulation of non-structural carbohydrates, and these factors can also affect photosynthetic acclimation by affecting the plant's carbon sink capacity (Wolfe et al., 1998). Environmental factors affecting photosynthetic capacity include nutrient availability, temperature and water (Wolfe et al., 1998).

More detail on how e[CO_2] affects photosynthesis is given in Chapter 2.

1.7 Sugar sensing in plants and its effect on gene expression

As previously reported, e[CO_2] increases leaf level photosynthesis, which leads to greater production of carbohydrates. Carbohydrates, or sugars, are well known for their roles in carbon and energy metabolism, but they also have the ability to act as signalling molecules, leading to regulation of gene expression. Smeekens (2000) defines sugar sensing as the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. For example, Chiou and Bush (1998) identified sucrose as a signalling molecule responsible for regulating the proton-sucrose symporter.

There is also evidence that sugar sensing plays a role in repression of photosynthetic gene expression. Jang and Sheen (1994) found that sugars repress photosynthetic related gene expression through the hexokinase signalling pathway, at concentrations as low as 1-10 mM. This further supports the idea that acclimation of photosynthesis at e[CO_2] is also associated with suppression of photosynthetic gene expression through sugar sensing pathways. Hexokinase has been established as a central enzyme in glucose sugar signalling pathways through the use of *glucose insensitive2* (*gin2*) mutants (Jang et al., 1997, Moore et al., 2003).

Many sugar responsive genes exist throughout plants. For example, Kunz et al. (2014) found 290 genes in *Arabidopsis thaliana* which responded to low concentrations of glucose, fructose and/or sucrose. Sugars stimulate expression of genes in roots required for nitrogen uptake in studies on *Arabidopsis thaliana* (Lejay et al., 2003). Sugars are also able to regulate various plant processes via 'crosstalk' with hormones. Such processes include embryogenesis, seed germination, early seedling development, tuberization, and the regulation of α-amylase activity; as reviewed by Gibson (2004).

The ability of sugars to affect expression of genes via sensing and signalling mechanisms is currently well established. However, the extent that e[CO_2] regulates the synthesis of different types of sugars, as well as the role this plays in regulating gene expression, has not been well

studied. As described in the previous section, photosynthesis and thus carbohydrate production are increased under e[CO_2] and this project aims to identify whether an increase in carbohydrate supply to the roots is responsible for altered nitrogen uptake and assimilation related gene expression and the decreases in GPC observed in wheat under e[CO_2]. The effect of e[CO_2] on sugar sensing is discussed in more detail in Chapter 2.

1.8 Objectives of the study

Wheat is one of the most cultivated crops in the world, however, the GPC of wheat grown under CO_2 concentrations predicted for the end of the century will decline. In order to prevent a decline in the protein quality of future wheat products, it is important to understand how e[CO_2] affects the control of GPC in wheat. Therefore, this study's overall aim is to investigate various aspects of the response of wheat to e[CO_2] that may play a role in altering the GPC, in order to further the current understanding of why wheat GPC declines under e[CO_2]. Furthermore, this study is broken down into three main objectives which each contribute to the overall aim:

1. Identify whether any significant difference exists in the GPC of each wheat type (tetraploid, hexaploid and synthetic hexaploid) in response to e[CO_2] (Chapter 3)

Almost all of the previous studies investigating the effect of e[CO_2] on GPC have focussed solely on hexaploid bread wheat, while scarce information is available for tetraploid durum wheat. There also does not appear to be any literature on how the GPC of synthetic hexaploids responds to e[CO_2]. Identifying differences between wheat types could lead to identifying the mechanisms involved with GPC decline. Exploring different genotypes of each wheat type in this study also contributes to the knowledge of how individual genotypes within each wheat type respond to e[CO_2].

2. Determine the traits with the greatest contribution to wheat GPC under e[CO_2] (Chapter 4)

While it is established that GPC declines in wheat under e[CO_2], little is known about the mechanisms controlling this decline. This chapter focusses on traits related to NUE and NRE as well as various biomass related traits in order to identify which traits appear to control the response of GPC to e[CO_2]. Identification of the important traits associated with GPC response will allow closer investigations into these traits in future studies to identify the mechanistic responses to e[CO_2].

3. Investigate potential sugar sensing pathways in roots of wheat which control expression of nitrogen uptake and assimilation related genes under e[CO_2] (Chapter 5)

Elevated [CO_2] leads to increased carbohydrate production, which has been shown to alter the expression of photosynthesis related genes via sugar sensing. The effect of e[CO_2] on sugar content or sugar sensing in roots has been scarcely studied and it is uncertain whether it has any effect on nitrogen uptake. Without sufficient nitrogen being taken up, the plant will be unable to maintain the GPC due to the increase in grain biomass, regardless of whether e[CO_2] affects other processes in wheat that determine GPC, such as nitrogen assimilation and remobilisation. This chapter investigates whether more sugar is allocated to roots of young wheat plants under e[CO_2] and whether a change in sugar correlates with changes to expression of nitrogen uptake and assimilation related genes. Additionally, the root proteome will be analysed via mass spectrometry to identify the difference in abundance of nitrogen transporters or assimilatory proteins under e[CO_2]..

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Chapter 2

Literature Review

In addition to the literature review in Chapter 1, this thesis has a published literature review entitled 'Effects of Elevated Carbon Dioxide on Photosynthesis and Carbon Partitioning: A Perspective on Root Sugar Sensing and Hormonal Crosstalk'. This review discusses how the sugar content of plants changes under e[CO_2] and how this affects the sugar sensing pathways in roots. The review begins with an overview of how e[CO_2] affects photosynthesis and the allocation of different types of sugars to each organ. The remainder of the review shifts focus onto sugar sensing, briefly giving some background into sugar sensing before looking specifically at roots. This chapter is linked with Chapter 5, which discusses sugar sensing as a potential mechanism for affecting the nitrogen status of wheat grown under e[CO_2].

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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REVIEW
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Effects of Elevated Carbon Dioxide on Photosynthesis and Carbon Partitioning: A Perspective on Root Sugar Sensing and Hormonal Crosstalk

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Plant responses to atmospheric carbon dioxide will be of great concern in the future, as carbon dioxide concentrations ([CO_2]) are predicted to continue to rise. Elevated [CO_2] causes increased photosynthesis in plants, which leads to greater production of carbohydrates and biomass. Which organ the extra carbohydrates are allocated to varies between species, but also within species. These carbohydrates are a major energy source for plant growth, but they also act as signaling molecules and have a range of uses beyond being a source of carbon and energy. Currently, there is a lack of information on how the sugar sensing and signaling pathways of plants are affected by the higher content of carbohydrates produced under elevated [CO_2]. Particularly, the sugar signaling pathways of roots are not well understood, along with how they are affected by elevated [CO_2]. At elevated [CO_2], some plants allocate greater amounts of sugars to roots where they are likely to act on gene regulation and therefore modify nutrient uptake and transport. Glucose and sucrose also promote root growth, an effect similar to what occurs under elevated [CO_2]. Sugars also crosstalk with hormones to regulate root growth, but also affect hormone biosynthesis. This review provides an update on the role of sugars as signaling molecules in plant roots and thus explores the currently known functions that may be affected by elevated [CO_2].

Keywords: elevated carbon dioxide concentration (e[CO_2]), sugar sensing and signaling, photosynthesis, hormone crosstalk, photosynthetic acclimation, carbon partitioning, hexokinase

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INTRODUCTION

Since the industrial revolution, global atmospheric CO_2 concentrations have rapidly increased, rising from 280 ppm to currently exceed 400 ppm (Canadell et al., 2007; Tans and Keeling, 2016). Predictions warn that the global CO_2 concentration will continue to rise due in part to humanity's continued carbon emissions (Meehl et al., 2007). The resulting increase in CO_2 will lead to a variety of both positive and negative effects on major agricultural crops used to feed the global population, many of which may yet be unknown. Elevated CO_2 concentrations, written henceforth as e[CO_2], cause increased photosynthesis in plants, which subsequently lead to positive effects

such as greater growth, above-ground biomass, and yield (Ainsworth and Long, 2005; van der Kooi et al., 2016). However, e[CO_2] also causes negative effects which could have serious consequences for the quality of the crop species, such as, declines in a variety of nutrients including protein concentrations of food crops (Fernando et al., 2015; Broberg et al., 2017), vitamins and some macro- and micro-elements (Högy and Fangmeier, 2008; Myers et al., 2014). Due to these negative effects, understanding plant responses to e[CO_2] will become increasingly important as CO_2 levels rise.

The increase in photosynthesis caused by e[CO_2] results in an increase in carbohydrate production, which alters the plant's carbon and nitrogen metabolism. Apart from this direct effect on photosynthesis, many physiological processes are regulated indirectly, particularly via sugar sensing and signaling pathways. Sugar sensing and signaling plays an important role in the plant response to e[CO_2], however, this is not well understood in relation to plant nutritional quality. Sugars are well known for their use as a source of energy and organic building blocks, and in plants they also play a role in regulating gene expression (Price et al., 2004), germination (Dekkers et al., 2004), and hormonal crosstalk (Mishra et al., 2009) among other functions.

Plant growth and development requires the uptake of soil nutrients by the roots, however, the concentration of nutrients in soil can vary and plants must adapt to the environment in order to fulfill their nutrient requirements. Sugars produced from photosynthesis are transported into roots where they can assist in regulating nutrient uptake via sugar sensing (Camañes et al., 2007; Lejay et al., 2008), though little research has been done in this area. How e[CO_2] affects root function is not entirely understood, but we do know that it can affect the acquisition of soil nutrients (Taub and Wang, 2008; Pandey et al., 2015; Jayawardena et al., 2017). To what extent sugars may play a role in this is not currently known. This review aims to provide the current knowledge and understanding of sugar sensing in roots as well as the limited information available on how this is affected by e[CO_2] in order to facilitate research into this area and safeguard crops from potential negative effects of future [CO_2].

In order to study the effects of e[CO_2] in the field, free-air CO_2 enrichment (FACE) facilities have been established which allow plants to be grown in large scale open air environments. Utilizing either FACE or chamber experiments can affect the outcome of the experiment. For example, in comparison to FACE experiments, chamber studies using e[CO_2] have been shown to further increase the yield of globally important food crops (Ainsworth et al., 2008). Plant growth differences between FACE and chamber experiments are likely influenced by the root growth, as restricting the available area for root growth reduces plant biomass (Poorter et al., 2012). Most of the studies discussed in this review were conducted with chamber experiments and to our knowledge no experiments have currently been done in FACE facilities for sugar sensing studies. As such, it is uncertain how the results of many of these sugar sensing studies will potentially change in plants grown in field conditions.

Many reviews have focused on various aspects of sugar sensing, however, this review discusses the limited amount of literature published on sugar signaling and sensing as it relates to plant root function, nutrient acquisition, and hormone crosstalk. As such, we have chosen roots as the focus of our review due to the current absence of reviews in this area, but more importantly due to their importance in determining the nutrient profile of plants. This review also discusses the effect of e[CO_2] on the content of sugars in plants, including how photosynthesis and carbohydrate partitioning is affected, and how e[CO_2] may affect sugar sensing in roots. The aim of this review is to provide the information necessary for scientists developing research projects involving sugar sensing in roots or the effect of e[CO_2] on roots and sugar sensing.

ELEVATED [CO_2] AND PHOTOSYNTHESIS

Photosynthesis is a crucial process for controlling variables of crop growth and exposing C3 plants to e[CO_2] generally increases photosynthesis (Drake et al., 1997; Ainsworth and Long, 2005; Wang et al., 2012; **Figure 1**). Increased photosynthesis under e[CO_2] mainly occurs due to an increase in ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) activity. Rubisco catalyzes the carboxylation of RuBP, which is required for CO_2 fixation, but also uses O_2 as a substrate to oxygenate RuBP in a process called photorespiration (Makino and Mae, 1999). The carboxylation reaction of RuBP is not saturated at the current atmospheric [CO_2], therefore, as the availability of CO_2 increases under e[CO_2] conditions so too will the rate of carboxylation (Drake et al., 1997). The other process, photorespiration, is wasteful in terms of energy, as it costs the plant more energy and does not lead to any gains in energy or carbon (Peterhansel et al., 2010). However, increasing the atmospheric CO_2 levels increases the [CO_2] surrounding Rubisco, shifting the ratio of CO_2:O_2 and thereby increasing the rate of carboxylation while decreasing the rate of oxygenation (Makino and Mae, 1999).

Despite the initial stimulation of photosynthesis seen at e[CO_2], under long-term exposure to e[CO_2] the plant incurs a down-regulation of photosynthesis in both FACE studies (Ainsworth and Long, 2005) and chamber experiments (Warren et al., 2014). This occurrence is known as photosynthetic acclimation. Photosynthetic acclimation, however, does not always completely negate the positive effects e[CO_2] has on photosynthesis. For example, in one study white clover was grown under elevated (600 ppm) [CO_2] for 8 years and retained a 37% increase in photosynthesis after acclimation was observed (Ainsworth et al., 2003). These findings suggest that final growth response to e[CO_2] is largely determined by the magnitude of plant acclimation to e[CO_2].

Various explanations as to the cause of photosynthetic acclimation have been made. Decreased leaf nitrogen (N) is one such explanation. In a study on rice, e[CO_2] caused a decline in N allocation into leaf blades, which subsequently reduced Rubisco and other protein synthesis (Seneweera et al., 2011). In support of this, in a 12 year study on *Liquidambar styraciflua* no acclimation response occurred during the time when leaf N was sufficient for photosynthetic requirements (Warren et al., 2014). Without

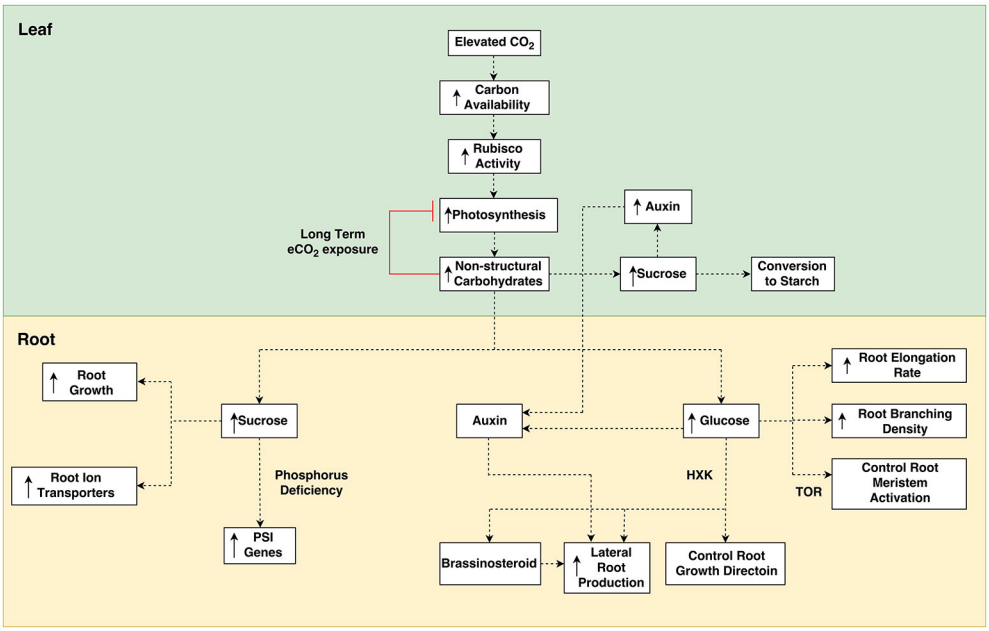

FIGURE 1 | Overview of potential pathways for elevated [CO_2] mediated sugar sensing responses. Elevated [CO_2] increases the availability of carbon in leaves causing greater Rubisco activity and higher rates of photosynthesis. Greater photosynthesis increases the content of non-structural carbohydrates in leaves which can lead to greater starch reserves and increased auxin biosynthesis. Over long term e[CO_2] exposure, photosynthesis is downregulated by increased carbohydrates. Carbohydrates are transported to roots, where they lead to greater root growth and stimulation of gene transcription. Root growth is also altered from the crosstalk of carbohydrates with hormones. HXK, Hexokinase; PSI, Phosphate Starvation Induced; TOR, Target-of-rapamycin.

sufficient N to invest in Rubisco, the photosynthetic capacity of the leaf declines. Low availability of soil nitrate increases the severity of photosynthetic acclimation and seems also to be associated with an inhibition of leaf nitrate assimilation (Vicente et al., 2016). Inhibition of leaf nitrate assimilation also occurs under e[CO_2] (Bloom et al., 2014). It is not known whether the reduction of Rubisco synthesis at e[CO_2] is directly related to lower N assimilation or if Rubisco is just regulated to balance the source and sink activity.

Another explanation for plant acclimation to e[CO_2] is that an increase in sugar production tips the source—sink balance of plants, potentially resulting in more sugars being produced in source tissues than can be utilized in sink tissues. This was the case in a FACE experiment by Ainsworth et al. (2004), who used single gene mutations to test the hypothesis that photosynthetic acclimation is due to inadequate sink capacity. In the study, a soybean cultivar with an indeterminate growth trait (Williams) was compared with a line mutated for determinate growth (Williams-*dt1*). Only the determinate line showed photosynthetic acclimation. On the other hand, mutation of a determinate soybean cultivar (Elf) to an indeterminate form showed no increased photosynthesis. While this may provide evidence for single gene mutations being responsible for photosynthetic acclimation, this could also be explained by the fact that Elf is a cultivar bred to avoid sink limitations (Ainsworth et al., 2004).While sink capacity remains high, plants are able to continue to utilize the greater CO_2 availability. However, with limited carbon sink capacity the plant must decrease photosynthesis in order to maintain source activity. As such, when e[CO_2] causes photosynthesis to surpass what the plant is capable of utilizing or exporting to sinks, an accumulation of non-structural carbohydrates (NSC) occurs (Ainsworth et al., 2004) and leads to feedback inhibition of photosynthesis (**Figure 1**).

These NSCs are then able to affect gene transcription through their role as signaling molecules (Mishra et al., 2009; de Jong et al., 2014). As such, sugars are known to be involved in photosynthetic acclimation, whereby the extra carbohydrates produced under e[CO_2] cause a down-regulation of photosynthetic gene transcripts and suppress protein synthesis, thereby decreasing the rate of photosynthesis (Cheng et al., 1998). In this way, there is a feedback inhibition where the products of photosynthesis cause suppression of photosynthesis, leading to photosynthetic acclimation.

Rubisco, an essential enzyme in the photosynthetic pathway, is known to be decreased in leaves that have an accumulation of carbohydrates (Cheng et al., 1998; Aranjuelo et al., 2008). Despite this evidence, a study by Ludewig and Sonnewald (2000) opposed the hypothesis that accumulation of sugars leads to photosynthetic acclimation when they found that high [CO_2] caused accelerated leaf senescence in *Nicotiana tabacum*, leading to down-regulation of leaf photosynthetic related genes and thus accelerated leaf senescence. Only senescing leaves were found to show down-regulation of photosynthetic genes and increased sugar levels were not observed. They concluded that photosynthetic acclimation was caused by leaf senescence rather

than sugar accumulation. Both Aranjuelo et al. (2008) and Cheng et al. (1998), however, reported that the down-regulation of photosynthesis occurred prior to senescence of the plants. All three studies used different plant species, which suggests that some species acclimate to e[CO_2] differently than others. Therefore, this suggests that photosynthetic acclimation has no single cause, with multiple processes each contributing to a different degree.

EFFECT OF ELEVATED [CO_2] ON CARBOHYDRATE BIOSYNTHESIS AND ALLOCATION BETWEEN ORGANS

As discussed in the previous section, e[CO_2] causes an increase in carbohydrate production via the stimulation of photosynthesis. It has been observed that increased photosynthesis under e[CO_2] results in greater production of certain carbohydrates compared to others. The concentration of sucrose, the main product of photosynthesis, increases in all organs of pea plants exposed to e[CO_2] in growth chambers, however, glucose concentrations are largely unaltered (Aranjuelo et al., 2013). Glucose measurements may be inaccurate as glucose content can fluctuate throughout the day in some plants, increasing and then decreasing as the day progresses (Seneweera et al., 1995; Grimmer et al., 1999). As such, hexose to sucrose ratio will differ depending on what time period the glucose levels are measured. Glucose measurements taken when glucose levels are naturally low, will give a lower hexose to sucrose ratio than if glucose was measured during a period of high glucose levels. Sucrose levels also increased in castor oil plants grown in growth chambers under 700 ppm CO_2 compared to 350 ppm, increasing by an average of one third (Grimmer et al., 1999). Levels of sucrose are higher than that of hexoses under e[CO_2] in both chamber and field studies (Grimmer et al., 1999; Rogers et al., 2004), however, in soybean the leaf hexose-carbon to sucrose-carbon ratio increases with exposure to e[CO_2], where a five-fold greater ratio of hexose-carbon to sucrose-carbon was observed near the end of the growing season (Rogers et al., 2004). Perhaps, such variation in hexose to sucrose ratio during plant development may affect plant source and sink activities. In addition, the preference of a plant to produce one type of carbohydrate over another could potentially be linked to the control of genes by a specific carbohydrate (glucose, sucrose, etc.), though this is not known. For example, if a plant requires the presence of sucrose to initiate the repression of a specific gene, it would be ineffective to produce greater glucose quantities than sucrose. The effect that carbohydrates have on gene expression is a topic discussed further in this review, however, the impact that a change in sugar composition has on plant gene regulations is not well understood.

Starch, a major storage carbohydrate in plants, is also increased in plants growing in e[CO_2] (Aranjuelo et al., 2008). The increase in starch likely contributes to the high levels of sucrose observed with e[CO_2], due to the conversion of starch to sucrose overnight. This conversion is important for normal plant growth under ambient conditions (Smith et al., 2005), however, under e[CO_2] it may contribute to the accumulation of sucrose. In plants grown under ambient [CO_2] the starch content builds up during the day and disappears overnight. The increased production of starch under e[CO_2], however, means that not all of the plant's starch reserves are depleted during the night, leading to a gradual accumulation in leaves over time (Grimmer et al., 1999). Different plant species accumulate different amounts of sucrose compared to starch, for example spinach accumulates more sucrose and cotton more starch (Goldschmidt and Huber, 1992). These responses are likely to affect the sugar sensing pathways in either type of plant. The degree of carbon partitioning between sucrose and starch is influenced by the length of daylight. In shorter periods of light, carbon partitioning shifts toward starch synthesis, while sucrose synthesis and consumption is decreased (Pokhilko et al., 2014). Less starch is accumulated during days with long light periods, while sucrose synthesis is increased (Pokhilko et al., 2014). Sucrose content is greater during the day than night, but the amount of sucrose remaining at the end of the day, as well as the end of the night, decreases as day length decreases (Sulpice et al., 2014). The degradation of starch at night is influenced by the amount of trehalose-6-phosphate (T6P). Increased T6P was found to inhibit starch degradation at night in *Arabidopsis* plants, resulting in much higher starch reserves at the end of the night (Martins et al., 2013). In addition, Martins et al. (2013) found that T6P also slightly increases starch synthesis. As such, increased T6P concentrations result in more starch at both the end of the day and night. Combined with limitations on starch degradation set by the plant's circadian clock, these findings suggested a model for overnight starch metabolism (Martins et al., 2013; Lunn et al., 2014). High sucrose demand causes lower T6P, alleviating the inhibition of starch degradation and increasing sucrose content. Under low sucrose demand, T6P increases and inhibits starch degradation. The plant's circadian clock prevents the total depletion of starch at night by setting limits on starch degradation based on the length of the night period (Martins et al., 2013).

The extra carbohydrates that accumulate in leaves are allocated to the rest of the plant in varying amounts, where some organs receive more of these carbohydrates than others. Little research has been done into the allocation of carbohydrates under e[CO_2], but the following studies have investigated this. Carbohydrate allocation under e[CO_2] varies with species. Some species allocate more carbon to the seeds and others to the shoots, leaves or roots (Salsman et al., 1999; Sasaki et al., 2007; Aljazairi et al., 2014; Butterly et al., 2015). For example, during the grain filling stage of rice e[CO_2] promotes the translocation of carbohydrates stored in vegetative tissues to the panicle, as well as allocating newly fixed carbohydrates to the panicle, where it is stored as starch (Sasaki et al., 2007). A difference in carbon allocation between durum wheat and bread wheat occurs under e[CO_2]. Durum cultivars Blanqueta and Sula allocated more carbon into roots, rather than shoots (Aljazairi et al., 2014), while the bread wheat cultivar Yitpi allocated more carbon into shoots (Butterly et al., 2015). Furthermore, Sula (a modern cultivar) allocated more carbon into spikes compared to Blanqueta (a traditional cultivar), which allocated more carbon into non-reproductive shoot tissue. This indicates

that variation exists within as well as between species and suggests that genetics contributes to these differences. In the case of the two durum cultivars, both differed in yield potential. Sula, which invested more carbon in spikes, is a higher yielding wheat than Blanqueta. Elevated [CO_2] also increased growth of roots and shoots of tepary bean, where the roots saw a ten-fold increase in starch (Salsman et al., 1999). Allocating more carbon into roots under e[CO_2] would contribute to greater root growth, allowing improved nutrient and/or water uptake and thus would help to maintain the balance of nutrients within the plant.

Carbon dioxide concentration is not the sole regulator of carbohydrate partitioning, with many other environmental factors involved in shaping the outcome. Which carbohydrate the increased carbon is partitioned into can be affected by the method plants use to take up nitrogen. An experiment by Aranjuelo et al. (2013) found N_2-fixing and NO_3^--fed plants varied greatly in sucrose content while exposed to e[CO_2]. Sucrose increased by 366% in NO_3^--fed plants but only by 56% in N_2-fixing plants. As e[CO_2] is known to affect the uptake and assimilation of N in plants (Bloom et al., 2014; Vicente et al., 2015a), this could point to a link between N uptake and carbohydrate allocation to roots and thereby facilitating more nutrient uptake. Plant growth method (glasshouse, field, etc.) also affects carbon allocation. Elevated [CO_2] causes increased carbon allocation to roots of perennial rye-grass resulting in increased root dry matter when grown in field conditions, however, no such results occur when grown in controlled environment chambers (Suter et al., 2002). This outcome in rye-grass was attributed to a difference in N availability, plant age and shoot sink strength. Results from Aranjuelo et al. (2013) also indicate that sink strength affects carbon allocation, where increased carbon sink strength of N_2-fixing plant's nodules allows greater storage of carbohydrates which in turn prevents the inhibition of photosynthesis by increased carbohydrates. This could mean that control of carbon allocation could be partially affected by the availability of carbon sinks. Another factor that may affect the allocation of carbohydrates under e[CO_2] is the effect e[CO_2] has on leaf area, as appeared to be the case for N allocation in rice (Makino et al., 1997). Plants which show less variable responses to leaf area under e[CO_2] (e.g., rice; Makino et al., 1997) compared to others, may allocate more carbohydrates to roots, as their leaf sink capacity doesn't change to accommodate the greater carbohydrate production. For some plants, root growth is increased under e[CO_2] (George et al., 2003), which may increase their sink capacity, allowing for greater allocation of carbohydrates to this organ. Carbon allocation under e[CO_2] can also be influenced by pH, as seen in plants grown in a low pH media under e[CO_2], where much of the carbon from photosynthesis accumulates in the shoots (Hachiya et al., 2014).

SUGAR SENSING AND SIGNALING: AN OVERVIEW

There are many reviews already written on the role of sugars as signals in plants including Granot et al. (2013), Rolland et al. (2006), and Sheen (2014) to name a few. However, to the best of our knowledge there are no reviews written specifically for sugar sensing in roots, which is a major focus of this review. As such, before moving on to our discussion of sugar sensing in roots, this section will serve to provide general information on sugar sensing not specific to roots. There is much more information known on sugar sensing than written in this section, however, we direct you to other reviews, such as those mentioned above, for more detailed discussions on sugar sensing not specific to roots.

Glucose has long been known to play a role in photosynthetic gene repression, with the enzyme hexokinase acting as a sensor (Jang and Sheen, 1994). It has since been established that hexokinase is a central enzyme in glucose sugar signaling pathways (Moore et al., 2003). Through sugar sensing, hexokinase appears to be able to promote plant growth by causing greater cell expansion in roots, leaves, and inflorescences when exposed to high light conditions (Moore et al., 2003).

In addition to hexokinase, SnRK1 has been indicated as another sugar sensor which is involved in a sucrose/T6P signaling network and operates as a starvation response (Baena-Gonzalez et al., 2007). It has been observed that SnRK1 may be inhibited by the presence of sucrose. KIN10, a part of the SnRK1 complex, is activated under sugar starvation, leading to up-regulation and down-regulation of various genes (Baena-Gonzalez et al., 2007). SnRK1 also contributes to increasing sugar content in plants by phosphorylating both sucrose phosphate synthase (SPS) and trehalose-phosphate synthase (TPS; Nukarinen et al., 2016), of which the resulting sugars, sucrose and T6P, may lead to inactivation of SnRK1 (Baena-Gonzalez et al., 2007; Zhang et al., 2009). Sucrose concentrations are linked with T6P levels, as increased sucrose leads to stimulation of TPS which in turn increases T6P concentrations (Yadav et al., 2014). High T6P then causes a decline in sucrose content which prevents further increases in T6P (Yadav et al., 2014). The regulation of T6P content is primarily linked with sucrose content, as studies have shown that only sucrose and hexoses able to be converted to sucrose have a significant effect on T6P levels (Lunn et al., 2006; Yadav et al., 2014). Sucrose and T6P may also be involved together with nitrogen assimilation, where increases in T6P signal the plant to synthesize organic and amino acids rather than sucrose (Figueroa et al., 2016). In conjunction with T6P other similar sugar phosphates, glucose 1-phosphate (G1P) and glucose 6-phosphate, are able to inhibit SnRK1, with G1P working together with T6P to significantly increase this inhibition (Nunes et al., 2013). Altogether SnRK1 appears to be involved in the plant's starvation response, inactivating during times of sufficient sucrose/T6P and activating when these signals are low.

Sugar signaling in plants begins as early as seed development and germination. At low levels, sugars are able to delay germination of *Arabidopsis thaliana* seeds. Other sugars have displayed this function as well, with sucrose, glucose, and the non-metabolically active glucose analog 3-O-methyl glucose exhibiting a greater delay on germination than others (Dekkers et al., 2004). The ability of the glucose analog to delay germination indicates a pathway independent of hexokinase.

Sucrose functions as a signaling molecule in a variety of ways. It is capable of inducing gene expression, such as, the *Citrus* ammonium transporter gene *CitAMT1* (Camañes et al., 2007), as

well as affecting the cell cycle. During the G1 phase of the cell cycle, sucrose induces the expression of the two CycD cyclins *Cyc2* and *Cyc3*, which influence cell cycle progression and cell division (Riou-Khamlichi et al., 2000). The role of sucrose in regulating the cell cycle likely correlates with its role in plant growth. As a plant produces more sugars, sucrose stimulates the cell cycle and allows utilization of the produced sugars for growth. As such, e[CO_2] is likely to facilitate this process. The greater sugar production caused by e[CO_2] could stimulate the cell cycle and allow the excess sugars to be used to produce greater plant biomass (Seneweera and Conroy, 2005).

Sugar signaling pathways also interact with hormones. For example, glucose increases the biosynthesis of auxin, therefore affecting processes regulated by this hormone (Sairanen et al., 2012). Evidence also suggests that sugars interact with pathways of both abscisic acid (Cheng et al., 2002) and ethylene (Price et al., 2004). Among other functions, abscisic acid has an enhancing effect on some genes regulated by sugar (Rook et al., 2001), while glucose downregulates the expression of ethylene biosynthetic genes (*VnACO2* and *VnEIL1*) and a transcription factor involved in the ethylene signaling pathway of narbon bean cotyledons (Andriunas et al., 2011). These findings show the various roles of sugars in gene regulation and thus their contribution to plant growth and development by way of sugar sensing.

SUGAR SENSING AND SIGNALING IN ROOTS

Currently there is a lack of understanding about the effect of e[CO_2] on sugar sensing, however, many studies have conducted experiments applying exogenous carbohydrates to plant roots, thus creating conditions of increased root sugar content which may mirror the conditions of greater root sugar content resulting from increased photosynthesis under e[CO_2]. Most of the research into the role carbohydrates play in plant roots has focussed on sucrose exclusively. While some research has brought to light several effects of other carbohydrates, such as, glucose and fructose, there may yet be many more roles that non-sucrose carbohydrates play. Much of this work is limited to *A. thaliana*, but it is likely that sugars play many other diverse roles in root function that may be discovered among other plant species. The following section discusses the potential outcomes for roots of plants grown under e[CO_2], whereby excess carbohydrates in leaves are transported to roots and lead to altered gene expression (**Figure 1**). The effects of sugar sensing in roots has had less attention then in shoots, as is especially the case for sugar sensing under e[CO_2]. As such, there is insufficient data to draw conclusions at this time, however, we provide an insight into how e[CO_2] may affect sugar sensing in roots, as well as sugar crosstalk with hormones.

Sugar Sensing and Gene Expression in Roots

NO_3^- uptake is diurnally regulated in a variety of plants (Lejay et al., 1999; Ono et al., 2000; Feng et al., 2011). In *A. thaliana* the NO_3^- transport genes *Nrt2.1* and *Nrt1*, which are down-regulated at night, are induced by sucrose application at night (Lejay et al., 1999), a result also seen with rice *Nrt2* genes (Feng et al., 2011). This could mean that if sugars accumulate in roots of e[CO_2] grown plants during the night, the diurnal cycle of NO_3^- transport will be affected. In plants that store starch in their roots, this could lead to an accumulation of sucrose in roots throughout the night, leading to altered gene transcription overnight. Sucrose concentration is also responsible for transcriptional regulation of other diurnally-regulated root ion transporters. Sucrose regulates three NH_4^+ transporters (*AtAmt1.1, AtAmt1.2,* and *AtAmt1.3*), an SO_4^{2-} transporter (*AtHst1*), a phosphate transporter (*AtPt2*), a K^+ transporter (*AtKup2*), a metal transporter (*AtIrt1*), and a K^+ channel (*AtSkor*), though each to a different degree (Lejay et al., 2003). Sucrose also contributes to regulation of ammonium uptake in *Citrus* plants, via stimulating expression of *CitAMT1* (Camañes et al., 2007). Though sucrose has the ability to regulate root ion transporters, they are not all regulated by the same mechanism. Lejay et al. (2008) found that three different signaling pathways regulated the expression of 16 sugar-induced root ion transporters. Most genes (ten) appeared to be regulated by a pathway dependent on the catabolic activity of hexokinase, rather than its sensing function, whereby the downstream metabolites of glycolysis act as signals for gene regulation. A second pathway, affecting five genes, involved a sucrose and/or glucose signal prior to hexokinase activity. Hexokinase sensing was proposed as the third pathway, which affected a single gene. All three pathways are briefly reviewed in Rolland et al. (2006) where they are referred to as the glycolysis-dependent pathway, HXK1-independent signaling pathway, and HXK1-dependent pathway, in order of those mentioned above. Among these genes, the majority appeared to also respond to [CO_2] (Lejay et al., 2008). If no sucrose was applied exogenously to the plants, 11 of the 16 genes responded to light exposure, provided there was also CO_2 in the atmosphere. In addition to this, ten of the genes were observed to respond further at higher [CO_2] (600 $\mu L\ L^{-1}$ CO_2) rather than low [CO_2] (300 $\mu L\ L^{-1}$ CO_2). This may suggest that these genes display a varied response depending on the amount of photosynthate produced. As such, these results may support our argument that greater photosynthesis caused by e[CO_2] will change the level of expression of some genes in roots.

Sucrose can also stimulate nitrogen assimilation via the oxidative pentose phosphate pathway (OPPP). An increase in sucrose concentration in roots of *A. thaliana* causes the induction of OPPP genes (*G6PDH2, G6PDH3, 6PGDH2*) and nitrate/nitrite reduction genes (*NIA1, NIA2, NiR;* Bussell et al., 2013). This induction requires plants to have a functional plastidial OPPP, which suggests that sucrose influences the OPPP to produce a signal that leads to transcription of N assimilation genes (Bussell et al., 2013). Not only is the OPPP important for sucrose mediated nitrogen assimilation, but it is also required for glucose mediated *Nrt2.1* expression. Glucose affects the OPPP via HXK1, which ultimately leads to the stimulation of *Nrt2.1* transcription (de Jong et al., 2014). Glucose also appears to post-transcriptionally regulate Nrt2.1 protein levels and transport, however, this appears to be independent of the mechanism used to stimulate *Nrt2.1* transcription via HXK1 (de Jong et al., 2014).

Utilization of the *glucose-insensitive2-1* (*gin2-1*) mutant, which lacks the hexokinase sugar sensing mechanism, showed that glucose regulates *Nrt2.1* transcription independently of nitrate-mediated regulation (de Jong et al., 2014). However, it is not known how these genes function under dynamic changes to sugar composition at e[CO_2]. There is evidence that transcription of OPPP genes in leaf tissue is down-regulated under e[CO_2] (Vicente et al., 2015b), but there was no evidence to suggest sugars as the cause of the down-regulation. Given that sucrose and glucose can affect the OPPP in roots, it is reasonable that a similar system may exist in leaf tissue. The down-regulation seen in Vicente et al. (2015b) may then be attributable to increased sugar production under e[CO_2]. As increased sucrose in roots cause induction of OPPP genes, an increase in sucrose due to increased photosynthesis under e[CO_2] may cause a similar interaction in leaves, but down-regulating the genes instead.

Sugars may also contribute to nutrient uptake by control of genes involved in root formation. Sucrose regulates the gene *CYCD4;1*, a member of the D-type cyclins (De Veylder et al., 1999) which belongs to a family of proteins, called cyclins, that regulate cell cycle progression (Mironov et al., 1999). The cyclin *CYCD4;1* is expressed in pericycle cells of the root apical meristem and is involved in lateral root primordia formation (Nieuwland et al., 2009). This may be, in part, how sugars are able to regulate root growth, as discussed in the next section. In addition, this may explain one way that e[CO_2] is able to increase root growth (Lee-Ho et al., 2007).

Sugars are important regulators in phosphate deficient plants. During phosphate starvation, carbohydrates are used to regulate various phosphate starvation induced (PSI) genes (Karthikeyan et al., 2007). Glucose and fructose can stimulate PSI genes to an extent, however, optimal responses occur with sucrose. During phosphorus deficiency, sucrose is able to increase the expression of a phosphate transporter gene (*LaPT1*) and a phosphoenolpyruvate carboxylase gene (*LaPEPC3*; Zhou et al., 2008). Sucrose also promotes growth of root hairs in phosphate deficient *A. thaliana* (Jain et al., 2007). The increased sugar production under e[CO_2] likely leads to lower inorganic phosphorus in plants due to the use of phosphorus in sugars such as triose phosphate, the synthesis of which will likely increase under e[CO_2]. The lower phosphorus concentration then becomes limiting in ATP synthesis and regeneration of ribulose bisphosphate (Farquhar and Sharkey, 1982). Whether the increased sugar production under e[CO_2] provokes the same expression of PSI genes mentioned above, is not currently known. Research has shown that e[CO_2] increases the expression of the phosphate uptake gene *AtPHR1* in phosphate deficient *Arabidopsis* plants (Niu et al., 2013), however, more research is needed to elucidate the role of e[CO_2] in sugar mediated PSI gene regulation.

There may be many genes in the root that are unrelated to nutrient acquisition which are activated by a sugar signal. For example, almost every aspect of auxin metabolism appears to be affected or regulated by glucose. Out of 604 auxin regulated genes in *A. thaliana*, 376 (62%) are transcriptionally regulated by glucose, which range in function from the biosynthesis of auxin to its transport, perception, and signaling (Mishra et al., 2009). Amino acid synthesis may also be impacted by sugar sensing. Silvente et al. (2008) found that glucose, acting through hexokinase, increased production of asparagine synthetase in roots of common bean. This brings to light more ways that e[CO_2] could affect root processes through sugar sensing. Research needs to be conducted in this area before any conclusions can be drawn, however, given that e[CO_2] has been shown to affect sugar regulation of genes in roots (Lejay et al., 2008), these findings show there is potential to find that auxin metabolism and amino acid synthesis can also be regulated to some extent by e[CO_2] through sugar sensing.

Under e[CO_2] conditions, Jauregui et al. (2015) found that expression of 48 genes of various functions, including genes linked with photosynthesis, hormones, and stress, was affected in *A. thaliana* roots, 95% of which were downregulated. The main finding of this study, however, showed that supplying e[CO_2] treated *A. thaliana* plants with ammonium nitrate improved plant protein content and maintained higher photosynthetic rates. This suggests that altering the nitrogen availability of plants may affect the plant's sugar sensing capabilities, as altering the plant's photosynthetic capacity will ultimately alter the carbohydrate content of plants. The mechanism by which e[CO_2] affected the 48 genes was not explored in the paper and as such, we don't know whether they were affected via sugar sensing pathways. The sugar content of the roots under e[CO_2] did not differ significantly from roots of plants grown under ambient [CO_2], however, there was a slight increase in sucrose content. Whether this small increase is enough to alter gene expression in roots is uncertain. Another possibility is that faster sugar catabolism may promote gene expression, however, the process is totally unknown and more research into the effect of e[CO_2] on gene expression in roots is required. Lower nutrient concentration in grains has been widely reported under e[CO_2] (Taub et al., 2008; Högy et al., 2013; Fernando et al., 2015), but whether these declines are associated with sugar mediated gene expression causing altered nutrient assimilation is unknown.

Elevated [CO_2] and Sugars Affect Root Architecture

Storage of the accumulated carbon under e[CO_2] is not consistent across all plants. In some plants, e[CO_2] causes a shift in the shoot/root carbon ratio toward greater root carbon (Aljazairi et al., 2014). How this extra carbon affects roots is not well understood, however, understanding the extent that sugars affect roots will provide a starting point for research into the effect of e[CO_2] on roots.

Elevated [CO_2] has a similar effect on root growth as increased sucrose concentrations. This may suggest that the way in which e[CO_2] affects root growth is through the increased sugars allocated to roots. Elevated [CO_2] increases both total root number and length in *A. thaliana* as well as root diameter (Lee-Ho et al., 2007). Increasing sucrose concentration in plants grown under ambient [CO_2] also gives results similar to e[CO_2] (Lee-Ho et al., 2007). Elevated [CO_2] may increase root growth in order to

balance nutrient uptake with the rate of sugar production from increased photosynthesis or perhaps a larger root system acts as a sink to store excess sugars.

Gaining a better understanding of how e[CO_2] affects the growth of roots could help explain the changes in nutrient status that occur under e[CO_2], such as the deficiencies of iron and zinc in wheat (Myers et al., 2014). With both e[CO_2] and sugars increasing plant root growth, you would expect greater uptake rates of nutrients, thus relieving nutrient deficiencies. While there are other mechanisms that are affected by e[CO_2] that lead to nutrient deficiencies, their discussion is outside the scope of this review. The role that roots play in causing or alleviating nutrient deficiencies needs to be further elucidated.

The carbohydrate status of plants can strongly influence root architecture. For example, increasing concentrations of the hexoses glucose and fructose in the growing regions of *A. thaliana* roots are positively correlated with both root elongation rate and branching density (Freixes et al., 2002). Not all hexoses work to promote root elongation, however, as mannose inhibits root elongation by a signaling pathway initiated by hexokinase (Baskin et al., 2001). Galactose, another hexose, also inhibited root elongation in the study by Baskin et al. (2001), but to a lower extent. Psicose, an analog of fructose, is a third hexose capable of inhibiting root growth. It was found to inhibit root growth of lettuce seedlings, however, in contrast with mannose, it does not appear to cause the inhibition through a hexokinase-mediated pathway (Kato-Noguchi et al., 2005). Elevated [CO_2] generally increases root growth in FACE and open-top chambers (OTC; Milchunas et al., 2005; De Graaff et al., 2006). In *Sedum alfredii*, e[CO_2] is found to increase both root elongation and branching (Li et al., 2012), while other studies have found a variety of plant species show increased fine root production (Pritchard and Rogers, 2000; Tingey et al., 2000). A meta-analysis of FACE and OTCs found a general increase in root biomass in response to e[CO_2], where root length was increased more than root diameter (Nie et al., 2013). The meta-analysis also found that increased fine root biomass was the main component of the total biomass increase. This may suggest that if e[CO_2] plays a role in the sugar stimulated increase in root growth, more carbon is partitioned into sugars such as glucose, which is capable of increasing root growth, rather than psicose or mannose. Therefore, understanding how diurnal changes in sugar composition is affected under e[CO_2] will provide a greater insight into the role that sugars have on root growth and gene expression in response to e[CO_2].

The role of glucose in *A. thaliana* roots is not limited to root elongation rate and branching density. It has also demonstrated the ability to control root growth direction in *A. thaliana*, and it does this independently of changes in root length (Singh et al., 2014b). The directional change induced by glucose occurs via both hexokinase dependent and independent methods (Singh et al., 2014b). The hexokinase glucose sensing pathway also leads to increased lateral root production (Gupta et al., 2015). Furthermore, root meristem activation is stimulated by glucose via a target-of-rapamycin (TOR) signaling network (Xiong et al., 2013). The control of root meristem activation by the glucose-TOR interaction relies on glycolysis–mitochondrial energy relays. This signal network in turn promotes root growth.

Sucrose has been identified as a necessary signal to stimulate primary root growth in *A. thaliana* seedlings, where the sucrose is transported to the roots from the cotyledons by way of the sucrose transporter SUC2 (Kircher and Schopfer, 2012). In addition, secondary root growth is also promoted by sucrose (Freixes et al., 2002). Sucrose also has the ability to rescue plants from certain factors which inhibit root growth. The inhibition of root growth caused by both psicose and mannose, as previously mentioned, is overcome by the addition of sucrose (Kato-Noguchi et al., 2005). This means that in plants that produce more sucrose under e[CO_2] than hexoses, the inhibition by psicose and mannose is unlikely to occur.

As previously mentioned, sucrose is also involved in promoting lateral root primordia formation, however, Macgregor et al. (2008) argues that this regulation is caused by the metabolism of sucrose, rather than sucrose acting as a signal. They concluded this on the basis that sucrose and its downstream metabolites glucose, fructose, and glucose-6-phosphate, all promoted lateral root primordia formation, but the non-metabolized glucose analog 3-*O*-methyl glucose did not, combined with the observation that exogenous sucrose promoted lateral root primordia formation in the hexokinase mutant *gin2*. It could instead be argued that these sugars operate as signals independently of hexokinase, particularly sucrose which is not sensed by hexokinase. Despite evidence that sugars promote lateral root development, a recent study concluded that sucrose and glucose promote the expression of the *A. thaliana WOX7* gene, which inhibits lateral root growth (Kong et al., 2016). Adding to the complexity surrounding the influence of sugars on regulatory pathways, auxin, a hormone that promotes lateral root development and is upregulated by sugars, represses *WOX7* expression (Kong et al., 2016).

Further aspects of the ability for sugars to control root architecture are discussed in the next section, where crosstalk with various plant hormones is required to bring about changes in root architecture.

Elevated [CO_2] Mediates Sugar and Hormone Crosstalk

Along with the ability for sugars to control gene expression and root growth, they also are known to interact with hormones, extending their potential effect as a signaling molecule. For instance, sucrose-mediated induction of the *Nrt* gene may be due to its capability to crosstalk with auxin, a hormone which, among other functions, regulates the *A. thaliana* nitrate transport gene *AtNrt1.1* (Guo et al., 2002). Exogenously introduced auxin stimulates *AtNrt1.1* transcription at the commencement of lateral root formation (Guo et al., 2002). In addition to crosstalk with auxin, sucrose stimulates both auxin production and transport to roots (Lilley et al., 2012). Glucose and sucrose are able to regulate the biosynthesis of the auxin called indole-3-acetic acid (IAA), though sucrose has a greater effect on IAA biosynthesis

(Sairanen et al., 2012). As such, by regulating the production and transport of auxin, sugars are indirectly influencing the plant processes brought about by auxin. Auxin also works with glucose to promote formation of lateral roots in *A. thaliana*. In the presence of glucose, the formation of auxin-induced lateral roots is bimodal, where the number of lateral roots peaks at both low and high concentrations, but not medium (Booker et al., 2010). Glucose acts to inhibit the heterotrimeric G protein complex, which attenuates this bimodality (Booker et al., 2010). Auxin stimulates the cell cycle to promote lateral root initiation and also affects the frequency and position of lateral roots, depending on the amount of auxin and the direction of its flow in the roots (Himanen et al., 2002). This may contribute in part to glucose's ability to promote lateral root growth, as discussed in the previous section, however, this is unknown. Glucose can also cause root hair initiation and elongation, however, elongation is decreased in the absence of auxin (Mishra et al., 2009).

Glucose interacts with another hormone, brassinosteroid, to stimulate lateral root formation. Brassinosteroid works downstream of the HXK1 glucose sensing pathway (Gupta et al., 2015). This glucose and brassinosteroid mediated pathway also affects auxin transport machinery during lateral root production (Gupta et al., 2015), thus contributing to the auxin-mediated lateral root formation. Brassinosteroid also works with glucose to control root growth direction (Singh et al., 2014b). It appears that polar auxin transport is also involved in glucose induced root growth direction, occurring downstream from glucose and brassinosteroid (Singh et al., 2014b). Working antagonistically to this control of root growth direction, however, are ethylene and cytokinin, which, together with glucose, brassinosteroid and auxin, may make up a system for controlling the growth direction of plant roots (Singh et al., 2014a). Exposure of *A. thaliana* root tips to the hormone cytokinin promotes root growth via cell elongation (Kushwah et al., 2011). This root growth is further promoted by the presence of glucose which operates through hexokinase.

There is limited research focusing on the relationship between e[CO_2] and plant hormones, however, several studies have shown the effect of e[CO_2] on hormone synthesis. Results from Hachiya et al. (2014) suggest that e[CO_2] can cause preferential root growth by increasing root IAA content. Increased sugar production under e[CO_2] appears to cause increased biosynthesis of IAA in shoots, which is subsequently transported to roots. That both sucrose and glucose are known to stimulate IAA biosynthesis in roots under ambient [CO_2] could suggest that this is the mechanism used to cause the increase under e[CO_2]. Auxin and sugars also appear to work together in roots of iron (Fe)-deficient plants. A recent study proposed a model whereby Fe-deficiency increases sucrose content of roots, causing an increase in auxin and a subsequent increase in nitric oxide, ultimately causing FIT-mediated transcriptional regulation of *FRO2* and *IRT1* genes and inducing Fe uptake (Lin et al., 2016). If these genes are regulated by the increase of sucrose, then it stands to reason that an increase in sucrose content in roots brought about by e[CO_2] might bring about the same change. In a hydroponics study, IAA content in roots was increased by e[CO_2] in tomato plants by 26.5% (Wang et al., 2009). IAA was not the only hormone increased by e[CO_2]. They also found ethylene release in roots was increased by 100% in tomato plants when grown under e[CO_2], showing that stimulation of hormone production under e[CO_2] is not limited to auxin. Ethylene was also found to be increased in rice plants grown in growth chambers under e[CO_2] (Seneweera et al., 2003). In addition to auxin and ethylene, jasmonic acid has also been reported to be regulated under e[CO_2]. However, as opposed to the stimulation of auxin and ethylene seen in other studies, the synthesis of jasmonic acid was repressed by e[CO_2] in Guo et al. (2012). This was, however, found to occur in leaves. Whether e[CO_2] affects jasmonic acid in roots is unknown. That both e[CO_2] and sugars have been demonstrated to interact with plant hormones may suggest that in future climates, the sugars produced under e[CO_2] may act as intermediates for hormonal crosstalk.

CONCLUDING REMARKS

Much is still unknown about how plants will react to e[CO_2] and with nutrient deficiencies observed in agricultural crops, this will become increasingly more important to understand. The production of carbohydrates is increased in plants grown under e[CO_2] due to an increase in photosynthesis. Some carbohydrates are produced in higher quantities than others depending on the plant, though production of sucrose is reportedly higher compared to hexoses. The studies discussed provide an insight into how these sugars can be used to regulate many functions in roots. Most of the information on sugar signaling discusses the glucose and sucrose pathways. The amount of carbon partitioned into either of those carbohydrates may be in part determined by which carbohydrate the plant requires to regulate specific genes, though this is unknown. Nutrient acquisition appears to be regulated by sugars, as evidenced by the regulation of expression of various ion transporters as well as the ability for sugars to affect root growth. Finally, both e[CO_2] and sugars are able to affect the biosynthesis of certain plant hormones, which may suggest that sugars function as an intermediate in e[CO_2] control of hormones. From these studies we can begin to think about what changes might occur in roots of plants grown in future carbon dioxide concentrations.

AUTHOR CONTRIBUTIONS

MT wrote the manuscript. SS, AM, NH, and DG each contributed to the design of the review as well as revision of drafts and the final manuscript.

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Chapter 3

Effect of elevated carbon dioxide on plant biomass and grain protein concentration differs across bread, durum and synthetic hexaploid wheat genotypes

In this study, wheat genotypes from three different types of wheat (tetraploid, hexaploid and synthetic hexaploid) were grown under both e[CO_2] and a[CO_2] to identify whether wheat type contributed to the response of wheat GPC to e[CO_2]. In addition, grain and shoot biomass measurements were taken to investigate whether the reduced GPC was due to biomass dilution of the grain nitrogen. The ratio of grain biomass to total biomass, known as the harvest index, was also analysed for differences between wheat types. This chapter has been submitted as a research article to the Journal of Cereal Science and is currently under review.

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Effect of elevated carbon dioxide on plant biomass and grain protein concentration differs across bread, durum and synthetic hexaploid wheat genotypes

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Keywords: future climate; total grain protein; climate stress; shoot biomass.

Abbreviations: e[CO_2], elevated carbon dioxide concentration; a[CO_2], ambient carbon dioxide concentration; HI, harvest index; GPC, grain protein concentration

Abstract

Atmospheric carbon dioxide conditions predicted for future climates cause increases in wheat biomass, but also decreases in wheat grain protein concentration. We investigated the response of grain protein concentration of wheat to elevated carbon dioxide in nineteen wheat genotypes, including five tetraploid, eleven hexaploid and three synthetic hexaploid genotypes to test whether decreased grain protein concentration is genotype dependent and whether it is caused by biomass dilution. These were grown in ambient and elevated carbon dioxide conditions simultaneously. Shoot biomass and grain samples were taken at maturity. The grain protein concentration, grain biomass, shoot biomass and harvest index were analysed for each genotype. Despite most genotypes increasing in total grain protein (g), the majority of genotypes decreased in grain protein concentration (%) under elevated carbon dioxide. Elevated carbon dioxide caused an increase in grain biomass for all genotypes and total shoot biomass for most genotypes, which led to an increased harvest index for all genotypes except the two synthetic hexaploids CPI133814 and CPI133811. Most of the differences between wheat types were not statistically significant, suggesting that the individual genotype of wheat plants determines the response to elevated carbon dioxide rather than the wheat type.

1. Introduction

One of the main components of global climate change is the increasing concentration of carbon dioxide (CO_2) in the atmosphere. Under future climates, the increased atmospheric CO_2 concentration ([CO_2]) will directly affect the yield, growth and development of crop plants (Ainsworth and Long 2005; Leakey *et al.* 2009). For wheat (*Triticum aestivum*), although elevated [CO_2] (e[CO_2]) usually improves plant biomass and grain yield (Thilakarathne *et al.* 2013), the nutritional aspects of the grain suffer the opposite effect, where the concentration of protein and many macro and micronutrients declines (Fernando *et al.* 2012). With the global human population expected to increase, there will be a greater demand on food production. As such, the effect of climate change on food crops is of great concern.

Wheat is one of the most important food crops in the world, accounting for nearly a third of the global cereal production in the 2015/2016 season (FAO 2017). Wheat species typically belong to three different ploidy levels, consisting of diploids (2n = 2x = 14), tetraploids (2n = 4x = 28) and hexaploids (2n = 6x = 42). The hexaploid wheat genome is comprised of seven pairs of chromosomes each in three genomes, called the A, B and D genomes. Hexaploid wheat was created from the hybridisation of the tetraploid *T. turgidum* (containing the A and B genomes) with the D donor *Aegilops tauschii* (Matsuoka, 2011). Synthetic hexaploid wheat is created by hybridising these two species, followed by amphidiploidisation (Yang *et al.* 2009). With this method, breeders are able to develop synthetic hexaploid wheat genotypes which incorporate genes from *T. turgidum* and *Ae. tauschii* that were not maintained during hexaploid wheat evolution, including traits such as drought tolerance (Reynolds *et al.* 2007), increased nutrient uptake (Calderini and Ortiz-Monasterio 2003) and pathogen resistance (Wang *et al.* 2016). These synthetic hexaploids can then be crossed with bread wheat cultivars to transfer across the elite genes and improve upon the bread wheat cultivar (Li *et al.* 2014).

Growth under e[CO_2] causes increased yields in wheat (Amthor 2001; Högy *et al.* 2009), but many studies have shown that it also causes a decline in nitrogen stored in the grain at maturity (Taub *et al*. 2008; Högy *et al.* 2013; Fernando *et al*. 2015). Protein composition of wheat grain grown under e[CO_2] is also affected, resulting in lower bread making quality in some cultivars (Fernando *et al.* 2015). Of the proteins in the grain, storage proteins (gluten-forming), rather than structural or metabolic proteins, appear to be the most affected by e[CO_2] (Arachchige *et al.* 2017).

Previous studies have looked at the effect of e[CO_2] across diploid, tetraploid and hexaploid wheat species (Sinha *et al*. 2009; Uprety *et al*. 2009). Uprety *et al.* (2009) observed that the responses of each species to e[CO_2] was different depending on the physiological variable measured. For example, variables such as photosynthesis, leaf area, dry weight, grain yield and harvest index (HI) had a greater response to e[CO_2] in hexaploids and tetraploids than diploids. Sinha *et al.* (2009) also found differing responses of each ploidy level for their variables studied. Protein concentration in grains decreased for all ploidy levels, though the decrease was lowest in tetraploids and highest in hexaploids. How synthetic wheat responds to e[CO_2] has not previously been determined.

Past research has also demonstrated differences across ploidy levels regarding responses to abiotic stresses. For example, hexaploid wheat was observed to have better survivability under salt stress than both tetraploid and diploid wheat (Yang *et al.* 2014). Wheat development is also affected by drought in various regions and much work has gone into developing wheat with drought tolerance. A study by Becker *et al.* (2015) demonstrated the value of synthetic hexaploid wheat genotypes as potential sources of genes conferring drought tolerance. In addition, the response of wheat to high temperature stress, coupled with weak radiation, differs across diploid, tetraploid and hexaploid wheats (Li *et al.* 2018). Li *et al*. (2018) observed that of the genotypes studied, the diploid and tetraploid genotypes adopted an avoidance strategy to cope with high temperatures, while hexaploids possessed tolerance mechanisms. Both the diploids and tetraploids appeared to avoid the higher temperature and radiation stress by reducing the leaf area and ultimately developed a lower biomass and yield in contrast to hexaploids, which were better able to maintain their leaf area. As such, it appears worthwhile to investigate how different ploidy level wheat genotypes respond to e[CO_2].

A major goal for wheat breeders has been to develop cultivars with improved HI. As such, identifying wheat with a high HI is important for the continual improvement of commercial wheat cultivars. Elevated [CO_2] increases both the grain yield (Amthor 2001) and shoot biomass (Kimball 2016) of wheat, with the ratio of these two components determining the plant's HI. The stimulation of both biomass and yield at the same magnitude can lead to no change in HI, which has been seen in both hexaploid bread wheat and tetraploid durum wheat (Wang *et al*. 2013; Aranjuelo *et al.* 2015; Fitzgerald *et al.* 2016). Furthermore, some studies have shown HI to both increase and decrease in some wheat cultivars (Uddling *et al.* 2008; Wang *et al.* 2013). Thilakarathne *et al.* (2013) found that increases in grain yield are associated with increases to leaf mass area due to e[CO_2]. As such, the degree that e[CO_2] increases grain

yield, and in turn HI, may rely partly on how leaf mass area is affected. Increased HI, however, may lead to decreased grain protein concentration (GPC) in wheat due to dilution of N with increased carbohydrates (Taub *et al*. 2008).

In this study, we aimed to identify whether the effect of e[CO_2] on wheat GPC is dependent on wheat type and whether GPC decline is affected by HI and/or biomass dilution. We also investigated how e[CO_2] affects the GPC of synthetic hexaploid wheats. We used nineteen wheat cultivars, consisting of five tetraploid, eleven hexaploid and three synthetic hexaploid genotypes.

2. Materials and Methods

2.1 Plant material and growth conditions

Nineteen wheat genotypes were grown in environmentally controlled glasshouses at the University of Southern Queensland, Toowoomba, Queensland, Australia, in 2014. The genotypes consisted of five tetraploid durum wheats (Tjilkuri, WID802, Hyperno, Jandaroi and Caparoi), eleven hexaploid bread wheats (Hartog, Sunbri, Longreach Dart, Sunvale, Longreach Crusader, Aus29259, LRC2010-157, Longreach Scout, Longreach Lincoln, Sunguard and Longreach Spitfire) and three synthetic hexaploid wheats (CPI133814, CPI133811 and CPI133898) (Supplementary Table 1).

The average day/night temperatures of the glasshouse chambers were maintained at 20 ± 2°C and 17 ± 2°C, respectively, with 60-70% relative humidity. A compartmented glasshouse was used, where ambient [CO_2] (a[CO_2]) (~389 µmol mol^{-1}) and e[CO_2] (~700± 20 µmol mol^{-1}) were maintained in their respective treatments. All nineteen genotypes were grown in both CO_2 conditions at the same time (a[CO_2] & e[CO_2]) and consisted of four replicates. Each replicate consisted of one pot containing four plants. Seeds were pre-germinated and planted into pots containing 2.5kg top soil. All pots were randomized and rearranged weekly to eliminate chamber effects.

2.2 Biomass analysis

Plants were sampled at physiological maturity and separated into leaf blades, stems (including sheaths) and heads, and dried at 60°C for 48 hours. Heads were hand threshed to obtain the grain. The grains were weighed to obtain total seed weight. Plant tissues were weighed to obtain total shoot biomass. Grain number per plant was counted.

2.3 Nitrogen analysis

Grain from each genotype was ground using a Millser IFM-800DG grinder (Iwatani, Japan). A 100mg sample of the ground grain was analysed for its nitrogen concentration using a CN analyser (LECO CN628 analyser, Michigan, USA). The total GPC was calculated by multiplying the total N concentration of grain by the conversion factor of 5.7. Protein content was analysed by multiplying the GPC by total grain biomass.

2.4 Harvest Index

Harvest index was calculated by dividing the total grain biomass by the total plant biomass (grain biomass + shoot biomass):

HI = Total grain biomass/Total plant biomass

2.5 Statistical analysis

Statistical analysis to determine significant difference between means of the dependent variables (Grain biomass, total shoot biomass, GPC and HI) was performed using Compare Means in IBM SPSS Statistics ver. 23 (IBM Corp., 2015, Armonk, NY). Statistical significance was determined using a One-Way ANOVA with the wheat type (tetraploid, hexaploid and synthetic hexaploid) as the independent factor and CO_2 response (GPC, grain biomass, total shoot biomass and HI) as the dependent variable. CO_2 response was calculated as the difference between the e[CO_2] data and the a[CO_2] data. Results were regarded as significant at $P \leq 0.05$.

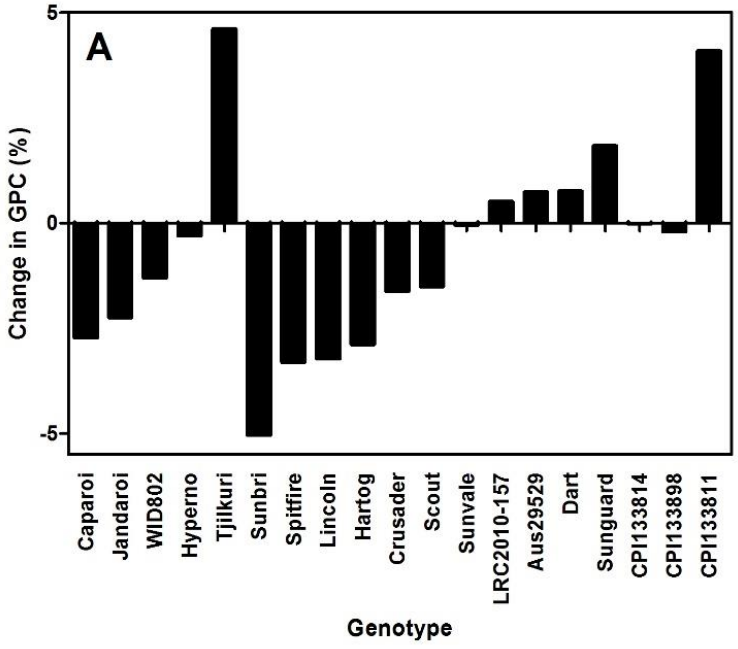
IBM SPSS Statistics ver. 23 was also used to perform a Pearson product-moment correlation test using Correlate to analyse any correlation between grain biomass and total grain protein. Statistical significance was determined with Bivariate Correlations using grain biomass and total grain protein as the variables. Results were regarded as significant at $P \leq 0.01$.

3. Results

3.1 Grain protein concentration and total grain protein

Grain samples from wheat genotypes of three types (tetraploid, hexaploid and synthetic hexaploid) grown under e[CO_2] or a[CO_2] were analysed for their nitrogen concentration. Nitrogen concentration was then converted to GPC by multiplication with a conversion factor of 5.7 and the data analysed for any significant difference between the wheat types. Of the 19 genotypes tested thirteen had a lower GPC under e[CO_2] compared to a[CO_2] (Fig. 1, Table 1). Among the tetraploid wheat genotypes, Caparoi, Jandaroi, WID802 and Hyperno had a lower

GPC in plants grown under e[CO_2]. Similarly, of the hexaploid genotypes, GPC declined in Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout and Sunvale, while it also declined in the synthetic hexaploid genotypes CPI133814 and CPI133898 under e[CO_2] (Fig. 1). Despite the majority of genotypes decreasing in GPC under e[CO_2], however, a paired-samples *t* test found no significant difference between e[CO_2] and a[CO_2] grown wheats for any of the wheat types.



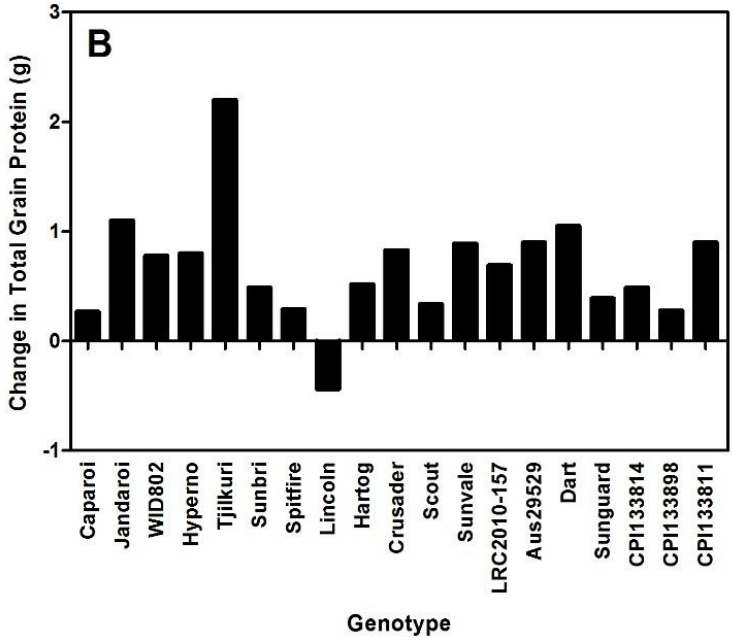


Figure 1. Effect of e[CO2] on A) GPC and B) total grain protein. Data represents the difference between a[CO2] and e[CO2] values. Positive values indicate greater values for e[CO2] than a[CO2]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802, Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout, Sunvale, LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814, CPI133898, CPI133811).

Tetraploids showed the largest variation of GPC between the least and most responsive genotypes in response to e[CO_2] between the three wheat types, with Caparoi incurring the greatest reduction of GPC among tetraploids. The largest decrease in GPC occurred in the hexaploid genotype Sunbri where the GPC at e[CO_2] was 5.04% lower than at a[CO_2]. On average, synthetic hexaploids had the least decline in GPC under e[CO_2], while hexaploids incurred the greatest reduction of GPC. On the other hand, some genotypes in each wheat type increased in GPC. For both tetraploids and synthetic hexaploids only one genotype was found to increase in GPC under e[CO_2], while there were four hexaploid genotypes. Statistical analysis by One-Way ANOVA showed that the effect of e[CO_2] on GPC was not significantly different between tetraploid, hexaploid and synthetic hexaploid wheat genotypes.

Despite the majority of genotypes decreasing in GPC in response to e[CO_2], the total grain protein content increased in 18 out of 19 genotypes when grown under e[CO_2] (Fig. 1, Table 1). Only Lincoln (a hexaploid genotype) decreased in total grain protein content. Similarly to GPC response, Tjilkuri increased the most in total grain protein content when grown in e[CO_2] (Fig. 1). For those genotypes which increased in protein in response to e[CO_2], total grain protein content of tetraploids increased on average by 1.03g and by 0.64g and 0.55g for hexaploids and synthetic hexaploids, respectively. There was no significant difference between wheat types for total grain protein, however, a pairwise t test showed that [CO_2] significantly affected tetraploids ($P = 0.033$) and hexaploids ($P = 0.001$).

		Grain Protein Concentration (%)						Total Grain Protein (g)					
					ANOVA						ANOVA		
Wheat type	**Genotype**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**
Tetraploid	Caparoi	11.08	13.80	-2.72	-	ns	ns	1.67	1.40	0.27	-	ns	ns
	Jandaroi	17.42	19.66	-2.24				3.22	2.12	1.10			
	WID802	11.86	13.16	-1.30				2.29	1.51	0.78			
	Hyperno	11.83	12.13	-0.30				2.33	1.53	0.80			
	Tjilkuri	18.49	13.89	4.61				3.64	1.44	2.20			
Hexaploid	Sunbri	9.68	14.72	-5.04	ns	-	ns	2.56	2.07	0.49	ns	-	ns
	Spitfire	11.81	15.11	-3.30				2.08	1.78	0.29			
	Lincoln	12.11	15.33	-3.22				2.13	2.57	-0.44			
	Hartong	11.13	14.01	-2.89				2.30	1.78	0.52			
	Crusader	13.86	15.47	-1.61				2.98	2.15	0.83			
	Scout	11.90	13.40	-1.51				2.47	2.12	0.34			
	Sunvale	13.65	13.70	-0.05				2.43	1.53	0.89			
	LRC / 2010 / 157	13.29	12.78	0.51				2.69	2.00	0.69			
	Aus 29259	13.66	12.92	0.74				2.80	1.90	0.90			
	Dart	16.17	15.42	0.76				2.83	1.78	1.05			
	Sunguard	14.37	12.54	1.83				1.65	1.26	0.39			
Synthetic Hexaploid	CPI 133814	14.53	14.55	-0.02	ns	ns	-	1.78	1.29	0.49	ns	ns	-
	CPI 133898	12.09	12.29	-0.20				1.02	0.74	0.28			
	CPI 133811	19.62	15.54	4.08				2.46	1.56	0.90			

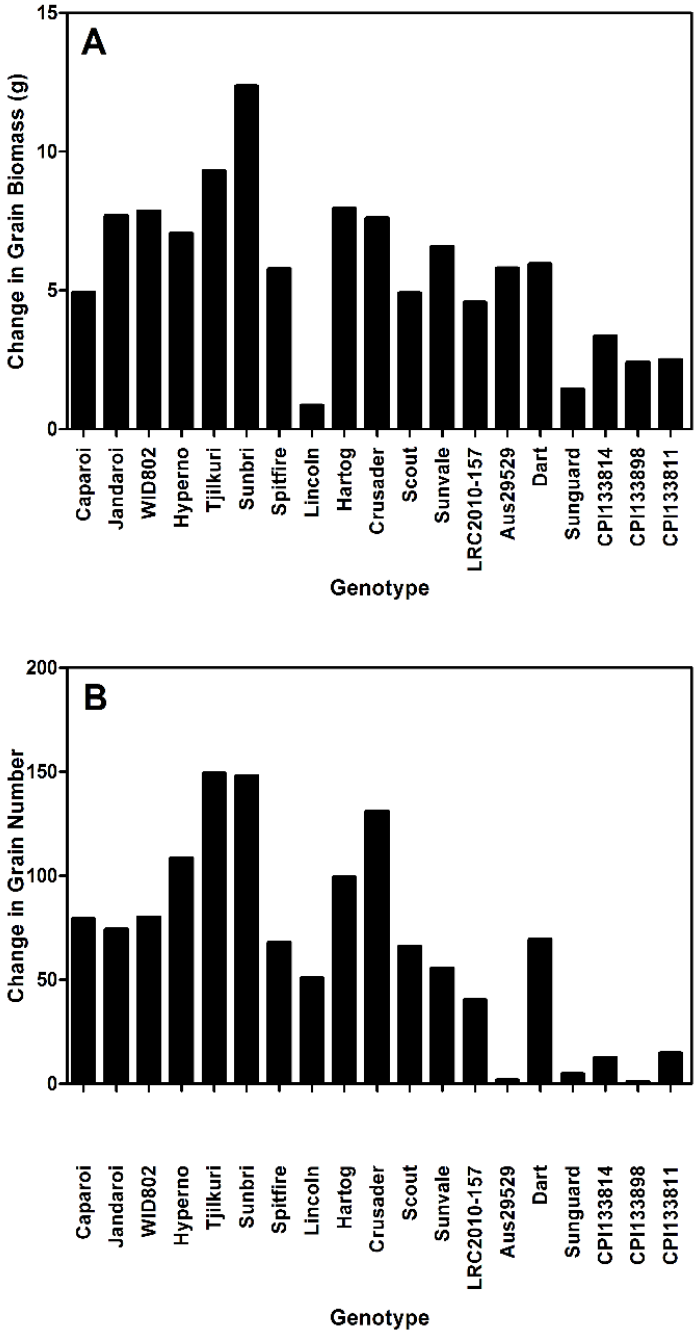
Table 1. Grain protein concentration (%) and total grain protein (g) of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO_2] and elevated [CO_2]. ANOVA results show differences between each wheat type. Abbreviations: P_T, AVOVA results against tetraploids; P_H, ANOVA results against hexaploids; P_S, ANOVA results against synthetic hexaploids; ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$.

3.2 Grain biomass and number

Total grain biomass increased in all genotypes grown under e[CO_2] compared to a[CO_2] (Fig. 2, Table 2). In addition, the grain number per plant also increased in all genotypes (Table 2). On average, e[CO_2] stimulated the greatest increase in grain biomass for tetraploid genotypes compared to both hexaploid and synthetic hexaploid genotypes, with the increase in tetraploids averaging about 67% compared to 44% for hexaploids and 34% for synthetic hexaploids. Tetraploids also had, on average, a greater response to grain number. Both of the genotypes which displayed the greatest and least increase in grain biomass, respectively, were Sunbri and Lincoln, both of which are hexaploids (Fig. 2). Despite the differences in average grain biomass response to e[CO_2], there was no significant difference between the three groups. On the other hand, synthetic hexaploids were significantly different in grain number compared to both tetraploids and hexaploids. A paired-samples t test revealed that the effect of e[CO_2] on grain biomass was significant for all three wheat types (tetraploids, $P < 0.001$; hexaploids, $P < 0.001$; synthetic hexaploids, $P = 0.012$). This was also the case for grain number (tetraploids, $P = 0.043$; hexaploids, $P < 0.001$; synthetic hexaploids, $P = 0.002$). Pearson product-moment correlation analysis revealed that there was a moderately positive correlation between grain biomass and total grain protein ($r = 0.584$, $n = 19$, $P = 0.009$). Grain number had a strong positive correlation with both grain biomass ($r = 0.820$, $n = 19$, $P < 0.001$) and HI ($r = 0.698$, $n = 19$, $P = 0.001$), while there was a moderately negative correlation with shoot biomass ($r = -0.494$, $n = 19$, $P = 0.032$).

3.3 Total shoot biomass

Total shoot biomass increased in all genotypes grown under e[CO_2] compared to a[CO_2] except for the tetraploid wheat Jandaroi (Fig. 3, Table 3). Synthetic hexaploid genotypes had the greatest increase in total shoot biomass under e[CO_2], with an average increase of 71.1%, compared to tetraploids and hexaploids, which each increased on average by 17.4 (excluding Jandaroi) and 14.9%, respectively. Among the synthetic hexaploids CPI133811 increased the most under e[CO_2]. Statistical analysis by One-Way ANOVA found that synthetic hexaploids were significantly different from hexaploids ($P = 0.001$) and tetraploids ($P = 0.002$), however, no significant difference was found between tetraploids and hexaploids. A paired-samples t test found that the effect of e[CO_2] on total shoot biomass was only significant for hexaploids ($P < 0.001$).



232

233 **Figure 2.** Effect of e[CO_2] on A) grain biomass and B) grain number. Data represents the
234 difference between a[CO2] and e[CO_2] values. Positive values indicate greater values for
235 e[CO_2] than a[CO_2]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802,
236 Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout, Sunvale,
237 LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814,
238 CPI133898, CPI133811).

		Grain Biomass (g)						Grain Number					
					ANOVA						ANOVA		
Wheat type	**Genotype**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**
Tetraploid	Caparoi	15.07	10.15	4.92	-	ns	ns	262.00	182.75	79.25	-	ns	ns
	Jandaroi	18.48	10.78	7.70				296.00	221.75	74.25			
	WID802	19.35	11.50	7.85				397.50	317.50	80.00			
	Hyperno	19.70	12.65	7.05				417.25	308.75	108.50			
	Tjilkuri	19.70	10.40	9.30				388.75	239.50	149.25			
Hexaploid	Sunbri	26.43	14.07	12.36	ns	-	ns	639.50	491.67	147.83	ns	-	ns
	Spitfire	17.58	11.80	5.78				356.00	288.00	68.00			
	Lincoln	17.60	16.75	0.85				396.75	346.00	50.75			
	Hartong	20.68	12.73	7.95				391.75	292.50	99.25			
	Crusader	21.50	13.90	7.60				456.50	325.50	131.00			
	Scout	20.73	15.83	4.90				360.75	294.75	66.00			
	Sunvale	17.78	11.20	6.58				357.25	301.75	55.50			
	LRC / 2010 / 157	20.23	15.67	4.56				422.00	381.75	40.25			
	Aus 29259	20.50	14.70	5.80				303.50	301.75	1.75			
	Dart	17.50	11.55	5.95				455.75	386.25	69.50			
	Sunguard	11.50	10.08	1.43				238.25	233.50	4.75			
Synthetic Hexaploid	CPI 133814	12.23	8.88	3.35	ns	ns	-	198.75	186.25	12.50	ns	ns	-
	CPI 133898	12.52	10.03	2.50				308.00	307.00	1.00			
	CPI 133811	8.40	6.00	2.40				172.50	157.75	14.75			

Table 2. Grain biomass (g) and grain number of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO2] and elevated [CO2]. ANOVA results show differences between each wheat type. Abbreviations: PT, AVOVA results against tetraploids; PH, ANOVA results against hexaploids; PS, ANOVA results against synthetic hexaploids; ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$.

		Total Shoot Biomass (g)						Harvest Index					
					ANOVA						ANOVA		
Wheat type	**Genotype**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**	**e[CO_2]**	**a[CO_2]**	**e[CO_2] – a[CO_2]**	**P_T**	**P_H**	**P_S**
Tetraploid	Caparoi	6.9	5.1	1.80	-	ns	**	0.69	0.67	0.02	-	ns	**
	Jandaroi	6.0	5.1	0.90				0.75	0.68	0.08			
	WID802	6.1	7.6	-1.50				0.76	0.60	0.16			
	Hyperno	6.3	5.5	0.80				0.76	0.70	0.06			
	Tjilkuri	4.2	4.1	0.10				0.82	0.72	0.11			
Hexaploid	Sunbri	6.6	6.1	0.50	ns	-	**	0.80	0.70	0.10	ns	-	**
	Spitfire	6.4	5.3	1.10				0.73	0.69	0.04			
	Lincoln	7.1	6.8	0.30				0.71	0.71	0.00			
	Hartong	7.0	6.1	0.90				0.75	0.68	0.07			
	Crusader	6.7	5.8	0.90				0.76	0.71	0.06			
	Scout	7.2	6.7	0.50				0.74	0.70	0.04			
	Sunvale	7.0	6.3	0.70				0.72	0.64	0.08			
	LRC / 2010 / 157	6.1	5.8	0.30				0.77	0.73	0.04			
	Aus 29259	7.3	5.3	2.00				0.74	0.74	0.00			
	Dart	5.5	4.2	1.30				0.76	0.73	0.03			
	Sunguard	5.3	4.9	0.40				0.68	0.67	0.01			
Synthetic Hexaploid	CPI 133814	8.5	4.4	4.10	**	**	-	0.59	0.67	-0.08	**	**	-
	CPI 133898	9.6	7.9	1.70				0.57	0.56	0.01			
	CPI 133811	15.7	7.9	7.80				0.35	0.43	-0.08			

Table 3. Total shoot biomass (g) and harvest index of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO_2] and elevated [CO_2]. ANOVA results show differences between each wheat type. Abbreviations: P_T, AVOVA results against tetraploids; P_H, ANOVA results against hexaploids; P_S, ANOVA results against synthetic hexaploids; ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$.

3.4 Harvest Index

Harvest index was calculated using grain biomass and total shoot biomass (above ground biomass). Harvest index increased in all plants when grown under e[CO_2] compared to a[CO_2], except for the two synthetic hexaploid genotypes CPI133814 and CPI133811 (Fig. 3, Table 3). The genotype with the greatest increase in HI under e[CO_2] was the tetraploid genotype WID802, with an increase of 26.28%. Of the two genotypes which declined in response to e[CO_2], CPI133814 declined the most, decreasing in HI by 19.25%. The tetraploids had an average increase in HI of 12.84%, while hexaploids increased on average by 6.23%. On the other hand the synthetic hexaploids had an average decrease of 15.51%, excluding CPI133898, which increased in HI in response to e[CO_2] by 1.20%. Like total shoot biomass, statistical analysis by One-Way ANOVA found that synthetic hexaploids were significantly different than hexaploids ($P = 0.007$) and tetraploids ($P = 0.001$), however, no significant difference was found between tetraploids and hexaploids. Paired-samples t tests were carried out for each wheat type, which revealed the effect of e[CO_2] to be significant for both tetraploids ($P = 0.022$) and hexaploids ($P = 0.001$), but not for synthetic hexaploids ($P = 0.219$). In addition, we also analysed the correlation between HI and GPC. Statistical analysis by Pearson product-moment correlation found there was no significant correlation between these two variables.

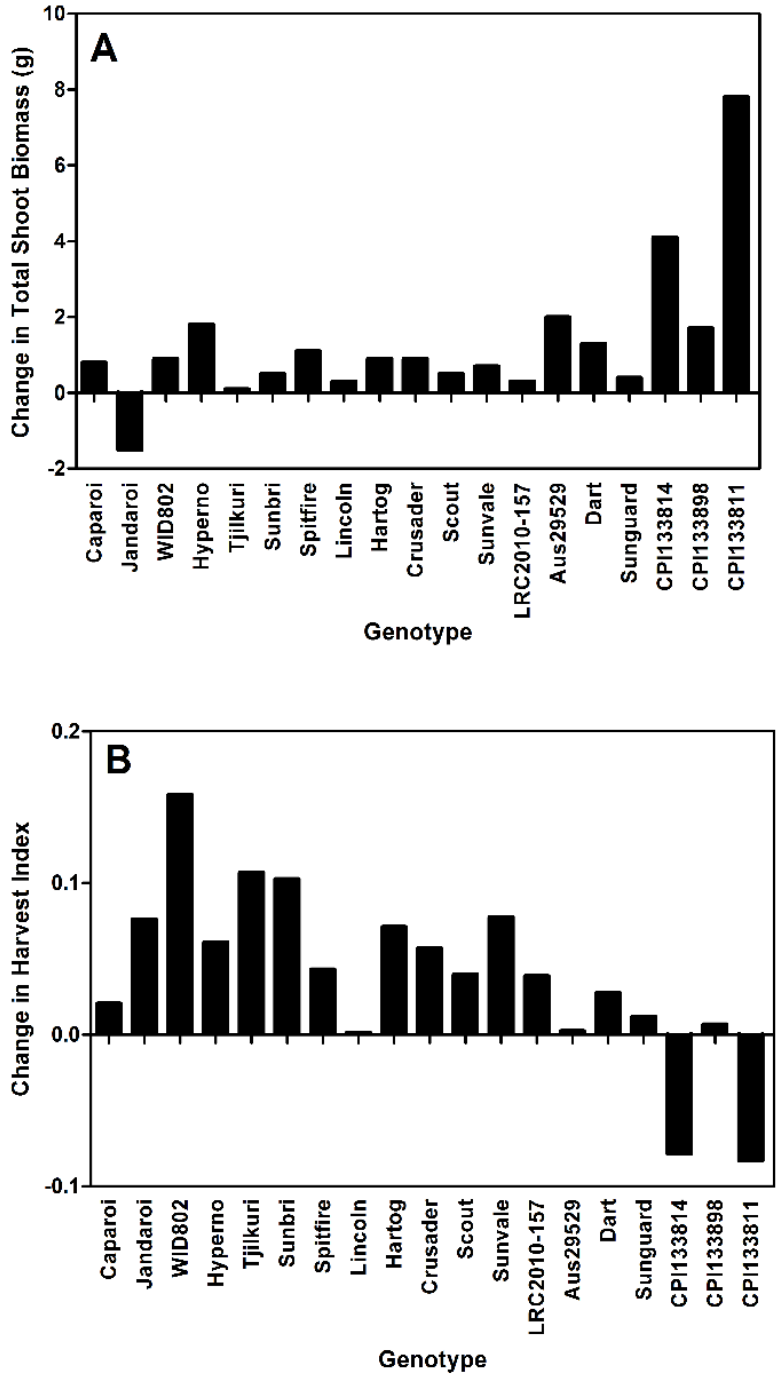


Figure 3. Effect of e[CO_2] on A) total shoot biomass and B) HI. Data represents the difference between a[CO_2] and e[CO_2] values. Positive values indicate greater values for e[CO_2] than a[CO_2]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802, Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout, Sunvale, LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814, CPI133898, CPI133811).

4. Discussion

It is currently unclear the extent to which e[CO_2] affects different types of wheat, and thus our research aimed to investigate how the GPC of wheat is affected by e[CO_2] across three different wheat types: tetraploid, hexaploid and synthetic hexaploid. In addition, we aimed to investigate the relationship between GPC, HI and biomass of each wheat type, in order to elucidate the mechanism behind GPC decline under e[CO_2]. In the present study, we found that rather than the wheat type determining GPC, it is specific genotypes within and between wheat types that determine GPC. On the other hand, we found that the HI of the studied wheat genotypes was significantly different between tetraploids and synthetic hexaploids, showing that while GPC may not be affected by type specific differences, wheat type may affect HI. Our results did not appear to show any significant link between GPC and HI.

In this study we examined the response of GPC, grain biomass, total shoot biomass and HI to e[CO_2] for 19 wheat genotypes, consisting of five tetraploids, eleven hexaploids and three synthetic hexaploids. Overall, the majority of genotypes decreased in GPC. This is a typical response of bread wheat to e[CO_2] (Taub *et al.* 2008), but GPC response to e[CO_2] is scarcely studied in tetraploid wheats, and to our knowledge, this is the first study to observe the effect of e[CO_2] on the GPC of synthetic hexaploids. The effect of e[CO_2] on GPC was not consistent within each wheat type. While most genotypes decreased in GPC, at least one genotype of each type increased in GPC. When looking solely at the genotypes which decreased in GPC, we found that the decrease in GPC for hexaploids on average was greater than tetraploids, supporting the results of Sinha *et al.* (2009), which found that tetraploid wheat had the lowest decline in GPC compared to hexaploid and diploid wheats. More genotypes need to be studied.

Despite the amount of genotypes with lower GPC under e[CO_2], nearly all genotypes (16 of 19) increased in total grain protein (g) per plant. We found that there was a significant correlation between grain biomass and total grain protein. This suggests that as the grain biomass increases due to the greater carbon availability from e[CO_2], the plant remobilizes greater amounts of nitrogen to the grain. However, even though most genotypes have greater grain protein and biomass under e[CO_2], there is a wide variation in GPC. Most genotypes have a lower GPC under e[CO_2] which means that while more protein is being stored in grains of plants grown under e[CO_2] than a[CO_2], the stimulation of grain biomass is too great for nitrogen uptake, transport or assimilation to keep up with. However, we acknowledge the

variation in number of genotypes per wheat type in this experiment and as such, a larger number of cultivars is needed to further support this conclusion.

Synthetic hexaploids differ from other wheats in that they are derived from crosses between *Ae. tauschii* and *T. turgidum*. The resulting GPC of each synthetic hexaploid then, is likely dependant on the responsiveness of both parent genotypes to e[CO_2]. For instance, the increased GPC of CPI133811 is likely a trait inherited from either its *Ae. tauschii* parent or the *T. turgidum* parent. As such, identifying the GPC response to e[CO_2] for the parents of each synthetic hexaploid examined in this study could explain the differences in CO_2 responsiveness of the synthetic hexaploids. In order to develop more synthetic hexaploid cultivars with improved responsiveness to e[CO_2], it may be crucial to screen genotypes of *Ae. tauschii* and *T. turgidum* to identify genotypes with high GPC responses to e[CO_2] that could allow breeders to develop synthetic hexaploid lines with a similar responsiveness. These elite synthetic hexaploid cultivars could then be crossed with bread wheat cultivars to transfer the improved traits, as has been done for other types of traits (Li *et al.* 2014). Synthetic hexaploids could be a solution for improving the bread-making quality of bread wheat grown under e[CO_2]. *Ae. tauschii* has displayed a greater variety in high molecular weight and low molecular weight glutenin subunits, encoded by the *Glu-D^t1* and *Glu-D^t3* loci respectively, compared to the glutenin subunits of bread wheat (Pflüger *et al.* 2001). Within this variety of alleles in *Ae. tauschii* we may find alleles capable of overcoming the poorer bread-making quality of wheat grown under e[CO_2].

One of the main hypotheses explaining the decline in grain protein is the dilution hypothesis, whereby e[CO_2] causes greater biomass stimulation in wheat compared with that observed under a[CO_2], and this increase is too great for the uptake and assimilation of N to keep up with (Taub *et al*. 2008). We investigated whether HI, a measurement of biomass allocation, was linked with GPC across tetraploid, hexaploid and synthetic hexaploid wheats. Harvest index, like total grain protein, increased in the majority of genotypes (17 of 19). Unlike total grain protein, however, where a tetraploid, hexaploid and synthetic hexaploid decreased under e[CO_2] (Jandaroi, Lincoln and CPI133811, respectively), the two genotypes which declined in HI were both synthetic hexaploids (CPI133814 and CPI133811). We found that there was a significant difference between tetraploids and synthetic hexaploids, however, hexaploids were not found to be significantly different to either of the other wheat types. Harvest index is determined by the total shoot biomass and total grain biomass of the plant. These components are each affected by e[CO_2], which means that HI will be determined by the extent that either

component is affected. For example, a genotype which is greatly affected by e[CO_2] in both grain biomass and total shoot biomass will have a much different HI than a genotype which is mostly affected in grain biomass. Amthor (2001) found the grain yield of wheat increased by 31% on average. This would lead to greater harvest indices for plants with low total shoot biomass responses to e[CO_2], however, as seen with Wang *et al.* (2013), HI remains the same for plants whose shoot biomass increases proportionally to the increase in grain biomass. In our experiment, total shoot biomass and total grain biomass both increased for all genotypes in response to e[CO_2], except Jandaroi, which declined in total shoot biomass. Synthetic hexaploids were significantly different than both tetraploids and hexaploids in total shoot biomass response to e[CO_2]. While the differences between means of tetraploids, hexaploids and synthetic hexaploids were not significant for total grain biomass, the synthetic hexaploids showed the lowest response to e[CO_2]. It appears that the main factor contributing to the lower HI of the synthetic hexaploids is the response of total shoot biomass to e[CO_2] rather than grain biomass. This indicates that the extra carbon being assimilated under e[CO_2] is being stored largely in the shoot, compared to grain, of synthetic hexaploids.

The increase in biomass, both shoot and grain, can be explained by the effect of e[CO_2] on photosynthesis. While the rate of photosynthesis was not measured for the plants in this experiment, it is accepted that e[CO_2] generally increases the photosynthetic rate of C3 plants (Ainsworth and Long 2005). An increase in photosynthesis means there is greater carbon fixation, resulting in increased biomass in the plant. Increasing the HI of a plant involves increasing how much carbon is stored in the grain as opposed to the shoot, as can be seen in this study's results when comparing the HI of each genotype with the two components of grain biomass and total shoot biomass. This increase in carbon could partly explain the decline in GPC for plants grown under e[CO_2]. As previously mentioned, one of the main hypotheses for the decline in protein under e[CO_2] is dilution by carbohydrates, where the increase in biomass is greater than the increase in nitrogen (Taub and Wang 2008). However, as with Taub and Wang (2008), we argue that biomass dilution cannot be the only explanation for the decline in GPC. Our results showed that the change in GPC did not correlate with the change in grain biomass or HI. While some genotypes, such as Sunbri and Hartog had a clear decline in GPC and increase in both grain biomass and HI, others were not as consistent. Some genotypes increased in GPC despite the stimulation of grain biomass, most notably Tjilkuri, which had the greatest increase in GPC and the second greatest increase in grain biomass. This means that there are factors that are affecting the GPC of each genotype other than carbon dilution itself.

In addition, Lincoln declined in GPC despite a small change to grain biomass and HI. As such, while dilution might explain part of the decline in GPC of some genotypes, there are very likely other factors controlling the protein response of wheat to e[CO_2]. Other explanations have been proposed, such as altered nitrogen assimilation (Bloom *et al.* 2014). Our results also found some correlation between grain biomass and total grain protein, suggesting that as e[CO_2] stimulates grain biomass, it also causes the plant to transport more nitrogen to the grain. This was not always the case, however, as Jandaroi in particular put less nitrogen into grain under e[CO_2] despite its increase in grain biomass. This supports the idea that there are other mechanisms being affected by e[CO_2] which control the transport of protein. Lincoln decreased in total grain protein despite the low stimulation of grain biomass. This further suggests that lower total grain protein is not controlled by how great e[CO_2] stimulates grain biomass.

In addition to a decline in protein concentration, the composition of protein is altered and ultimately the baking quality of grain harvested from plants grown under e[CO_2] is affected (Fernando *et al.* 2014; Panozzo *et al.* 2014). Therefore, it is important to not only identify hexaploid genotypes with greater responses to e[CO_2] with regards to GPC, but also those which will not have decreased baking quality. As previously mentioned it will be important to screen a wider range of wheat genotypes for those which are highly responsive to e[CO_2] for their GPCs, but to ensure the end product quality it will also be necessary to screen the highly responsive genotypes for baking quality.

Our results have identified a number of wheat genotypes that increased in both HI and GPC. Repeated confirmation of these results could provide breeders with genotypes that would benefit breeding programs for developing wheat cultivars capable of maintaining or improving upon current GPCs and HIs for future climates. These genotypes could also be used in further research to investigate the mechanisms of GPC decline by providing wheat with contrasting CO_2 responsiveness. The tetraploid genotype Tjilkuri, which increased in GPC in response to e[CO_2], may be a potential parent for generating synthetic hexaploid genotypes. However, in addition to the generation of synthetic hexaploids, there exists another possibility for developing wheat genotypes with improved GPC and HI under e[CO_2]. While there are many barriers to success, crossing tetraploid genotypes with hexaploids can result in pentaploid wheats (Padmanaban *et al*. 2017). Pentaploid wheat can be a source of great genetic variability and has shown promise for improving resistance to both biotic and abiotic stress (Padmanaban *et al*. 2017). Crossing highly [CO_2] responsive tetraploid and hexaploid genotypes together could lead to pentaploid genotypes with improved GPC and HI under e[CO_2]. These pentaploid

genotypes could then be crossed into either tetraploid or hexaploid genotypes, thus allowing the transferral of durum genes into bread wheat and vice versa.

In summary, we conclude that wheat type is not a major factor for determining GPC or HI response to e[CO_2]. GPC and HI both varied among the cultivars within each wheat type and no significant difference could be found between wheat types, except for the difference between the HI of tetraploids and synthetic hexaploids. The difference in HI for the synthetic hexaploids was due to the high response of total biomass to e[CO_2], which itself was significantly different than both tetraploids and hexaploids. There also does not appear to be a strong connection between GPC and HI regardless of wheat type. Our results suggest that biomass dilution is not the sole cause of the decline in GPC seen in this study. Ultimately, our results suggest that the individual genotype is more important than wheat type in determining the response of wheat GPC and HI to e[CO_2], however, more genotypes need to be studied to arrive at a definitive conclusion.

Conflict of interest

The authors declare no conflicts of interest.

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Chapter 4

Grain nitrogen concentration at elevated [CO_2] is mainly determined by genotype dependent variations in nitrogen uptake and nitrogen utilisation efficiency of wheat

The second study investigated the response of nitrogen and biomass related traits to e[CO_2]. Wheat was grown in glasshouse conditions to assess traits including nitrogen use efficiency and remobilisation efficiency, grain and shoot biomass, and rate of photosynthesis. This study aimed to identify which of the traits studied are associated with GPC variation in response to e[CO_2]. As such, this chapter addresses the current knowledge gaps for the mechanisms which lead to a decline in GPC under e[CO_2]. This chapter has been prepared as a research article to be submitted to Functional Plant Biology.

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Grain nitrogen concentration at elevated [CO_2] is mainly determined by genotype dependent variations in nitrogen uptake and nitrogen utilisation efficiency of wheat

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Summary text for the table of contents

Atmospheric carbon dioxide concentrations predicted for future climates cause declines in the grain protein concentration of wheat, but the mechanisms which cause this are not completely understood. Using 20 wheat genotypes, we show that grain protein concentration decline is due to a combination of traits, with the response of each trait differing across each genotype. This indicates multiple mechanisms in controlling grain protein concentration that are genotype dependent.

Abstract

Predictions for wheat grown under future climate conditions indicate a decline in grain protein concentration accompanied with an increase in yield due to increasing carbon dioxide concentrations. Currently, there is a lack of understanding as to the complete mechanism that governs the response of grain protein concentration (GPC) to elevated carbon dioxide (e[CO_2]). We investigated the grain protein concentration of 18 wheat genotypes from a doubled haploid wheat population and the two parental genotypes, Kukri and RAC0875. In addition, other nitrogen and biomass related traits were analysed to further elucidate which traits are connected with the decline in grain protein concentration. Wheat was grown under ambient and elevated [CO_2] in an environmentally controlled glasshouse. Plant nitrogen and biomass accumulation was measured at anthesis and maturity. We found that most genotypes declined in GPC. Response of GPC to e[CO_2] was negatively correlated with nitrogen utilisation efficiency and harvest index, yet correlated positively with nitrogen uptake efficiency and plant total nitrogen. The extent that each trait impacted GPC in response to e[CO_2] varied across each genotype, suggesting that multiple mechanisms are responsible for GPC decline at e[CO_2] and that these mechanisms are effected differentially across genotypes.

Keywords: post-anthesis nitrogen uptake, future climate, total grain protein

Introduction

One of the major challenges in agriculture is the improvement of crop production, which is further impacted by the growing population and changing climate. Global atmospheric carbon dioxide concentrations ([CO_2]) are predicted to increase to at least 700 μmol mol^{-1} by the end of the century (IPCC 2007). This increase in [CO_2] typically decreases the grain protein concentration (GPC) of wheat crops, which poses a serious health risk for a large portion of the population who rely on wheat as their main source of protein (Myers *et al.* 2014).

Most observations of the effect of elevated [CO_2] (e[CO_2]) on wheat reveal an increase in yield (Thilakarathne *et al.* 2013). Biomass stimulation forms the basis of the biomass dilution hypothesis, which explains the decline in nitrogen (N) concentration in plant tissues as the result of an increase in biomass that outpaces N uptake (Taub and Wang 2008). This appears to be the foundation for why GPC declines under e[CO_2], however, alterations in the flow of N throughout the plant likely contribute as well. For example, under e[CO_2], plants exhibit reduced stomatal conductance, which is thought to lower transpiration-driven mass flow of nutrients, including N, and thus lead to decreased nutrient concentrations in the plant

(Houshmandfar *et al.* 2018). In addition, e[CO_2] also decreases the amount of N available for translocation into grains by inhibiting nitrate (NO_3^-) assimilation in leaves (Bloom *et al.* 2014). Currently, the extent that each of these mechanisms affect the GPC of wheat under e[CO_2] is not completely understood.

Increasing wheat yields has been a long term goal of wheat breeders. Additionally, the ability of plants to produce greater yields without requiring increased N fertilizer application is also highly desirable. This trait is known as nitrogen use efficiency (NUE) and refers to the total grain yield divided by the amount of N available in soil (Moll *et al.* 1982). Previously it was shown that NUE increases in response to e[CO_2] (Li *et al.* 2003). Furthermore, NUE can be divided into two categories: nitrogen uptake efficiency (NUpE) and nitrogen utilisation efficiency (NUtE). NUpE is a measure of total plant N uptake divided by the total N available in the soil, whereas NUtE is defined as the total grain produced per total plant N. Bahrami *et al.* (2017) found that neither NUpE or NUtE was effected by e[CO_2], however, Tausz *et al.* (2017) observed an increase in the N content of wheat along with an increase in NUtE in plants grown under e[CO_2]. Tausz *et al.* (2017) also found that NUtE declined when plants were supplied with high N, due to an increase in N uptake without a significant impact on biomass, however, this did not affect the GPC enough to mitigate the effects of e[CO_2].

Most of the N stored in grain proteins is remobilised from N assimilated in the vegetative parts of the plant prior to anthesis (Kichey *et al.* 2007). As such, an important trait involved in maintaining GPC under e[CO_2] is nitrogen remobilisation efficiency (NRE). NRE refers to how much of the plant's total N stores are remobilised to the grain and appears to be affected by the duration of senescence (Gaju *et al.* 2014). The quantitative trait locus (QTL) *Gpc-B1* was identified in both tetraploid and hexaploid wheat genotypes, which, under ambient atmospheric conditions, accelerates the timing of senescence in flag leaves, thus stimulating N remobilisation and ultimately GPC (Uauy *et al.* 2006). A similar QTL was found on chromosome 6 in barley by Jukanti and Fischer (2008), where high GPC was also found to be linked with earlier leaf senescence. Both reports suggested that the increase in GPC could be explained by an increase in N remobilisation to the grain, but also because of reduced grain production due to lower carbon acquisition post-anthesis. Increased grain protein has also been linked to early senescence in rice (Seneweera *et al*. 2002).

The timing of N uptake may also be a significant factor in controlling wheat GPC in e[CO_2] grown plants. While pre-anthesis N uptake typically contributes the most N to grains, some

studies suggest that post-anthesis N uptake (PANU) may be crucial as well. For example, in bread wheat grown under ambient conditions, there is usually a negative correlation between GPC and grain yield, however, evidence suggests that increased PANU can attribute to a higher GPC without sacrificing the grain yield (Bogard *et al.* 2010; Taulemesse *et al.* 2016). Fernando *et al.* (2017) found that N fertilizer supplied at 50% ammonium (NH_4^+) + 50% NO_3^- mitigated the decline in GPC seen at e[CO_2]. This even application of NH_4^+ and NO_3^- increased PANU and led to a higher GPC than in wheat grown at 100% NO_3^- or 25% NO_3^- + 75% NH_4^+ (Fernando *et al.* 2017). Whether plants assimilate most of its grain nitrogen from nitrogen taken up before or after anthesis may be dependent on genotype.

In this study we investigated the effect of e[CO_2] on traits associated with GPC, NUE, NRE and biomass of two wheat cultivars with contrasting NUE (personal communication – Mamoru Okamoto) (RAC0875 and Kukri) and 18 RAC0875/Kukri derived lines. The 18 genotypes were selected from an RAC0875/Kukri doubled haploid population based on previously obtained GPC data under e[CO_2] exposure (unpublished data). The doubled haploid population is a mapping population that has been used in NUE studies. The two parent genotypes, RAC0875 and Kukri, are two lines adapted to the Australian Mediterranean-type environment and have shown differences in growth under drought stress (Bennett *et al.* 2012). We aimed to identify the traits conferring the greatest contribution to wheat GPC under e[CO_2] for both cultivars and whether the difference in these traits was consistent amongst the genotypes selected from the doubled haploid population.

Materials and Methods

Plant materials and growth conditions

Eighteen wheat lines from a doubled haploid population, created by crossing cultivars Kukri and RAC0875, were grown alongside their two parental cultivars in glasshouses at the University of Southern Queensland, Toowoomba, Queensland, Australia, from July to November 2016. The eighteen lines were selected from a larger population based on their variation in GPC response from a prior experiment (unpublished data). Two adjacent glasshouse chambers were used. One chamber was maintained at ambient [CO_2] (a[CO_2]) (~ 400 µmol mol^{-1}) and the other at e[CO_2] (~ 700 µmol mol^{-1}). The two parent genotypes and each of the eighteen lines were grown as three replicates in both glasshouse chambers. Each replicate consisted of a single pot which contained five plants to allow sample collection at multiple growth stages. The seeds were pre-germinated on flyscreen trays suspended over

water and transferred into pots containing a brown topsoil with a N percentage of 0.4%. Throughout the experiment, all pots were randomized on a weekly basis to reduce chamber effects.

Sample collection and harvest

Plant material was collected at two different growth points: anthesis (DC 65) and maturity (DC 90). At anthesis the aboveground portion of the plant was separated from the roots, stored in sample packets and dried in an oven at 70°C for 48 hours. Samples were weighed to obtain the biomass at anthesis. At maturity, the aboveground portion of the remaining plants was also separated from roots and stored in sample packets. Wheat heads of samples taken at maturity were hand threshed to obtain the grain. Shoot and grain samples were weighed to obtain the biomass.

Measurements

Gas exchange measurements were taken for the flag leaf using a LI-6400XT portable photosynthesis system (LI-COR, USA). Measurements were taken at two different physiological stages: anthesis and post anthesis (DC 75). Above ground biomass was obtained for the plants sampled at anthesis and maturity. Above ground biomass of the samples taken at maturity was then further separated into grain yield and straw biomass. Grains were ground into a powder using a Millser IFM-800DG grinder (Iwatani, Japan). Straw was also ground into a powder using a CT 193 Cyclotec grinder (Foss, USA). Nitrogen concentration of all samples taken at anthesis and maturity was analysed using a CN628 CN analyser (LECO, USA). Total N content (g) was calculated by multiplying the N% of each sample by the biomass of either the grain or straw. The GPC was determined by multiplying the N% of grains by a conversion factor of 5.7 (Mosse 1990).

Harvest index

The grain yield and total biomass of mature plants were used to calculate the harvest index using the following equation:

(1) HI = Total grain weight/Total above ground weight

Nitrogen uptake efficiency and nitrogen utilisation efficiency

NUpE was determined for plants sampled at anthesis and maturity. It was calculated as the amount of N taken up by the plant (g) per amount of N in the soil (g):

(2) NUpE = Nt/Ns, where Nt is the total N in the plant (g) and Ns is N supply (g).

NUtE was determined for plants sampled at maturity. It was calculated as the grain produced (g) per total N in the plant (g):

(3) NUtE = Gw/Nt, where Gw is grain weight (g) and Nt is total N in plants (g).

Total nitrogen remobilisation and nitrogen remobilisation efficiency

Nitrogen remobilisation was calculated as the difference between the total N of plants at anthesis and the total N of straw samples at maturity. NRE was calculated as the amount of N remobilised to the grain divided by the total plant N (TPN) at anthesis.

Post-anthesis N uptake

The amount of N taken into the plant during post-anthesis was calculated as the difference between TPN at DC90 and TPN at DC65.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics ver. 23 (IBM Corp., 2015, Armonk, NY). Differences in the response of each trait to e[CO_2] observed between the two parental cultivars (RAC0875 and Kukri) were checked for significance by performing a One-Way ANOVA. The entire data were then analysed by Two-Way ANOVA through general linear model to test for genotype and [CO_2] affect, along with genotype×[CO_2] interaction. For all ANOVA analyses, results were regarded as significant at $P \leq 0.05$. A Pearson product-moment correlation test using Bivariate Correlations in IBM SPSS Statistics ver. 23 was performed to test for correlations between GPC and the other traits studied. Results were regarded as significant at $P \leq 0.05$. All graphs were created in the GraphPad Prism 5 software.

Results

Effect of [CO_2] on grain protein concentration, total grain protein and total plant nitrogen

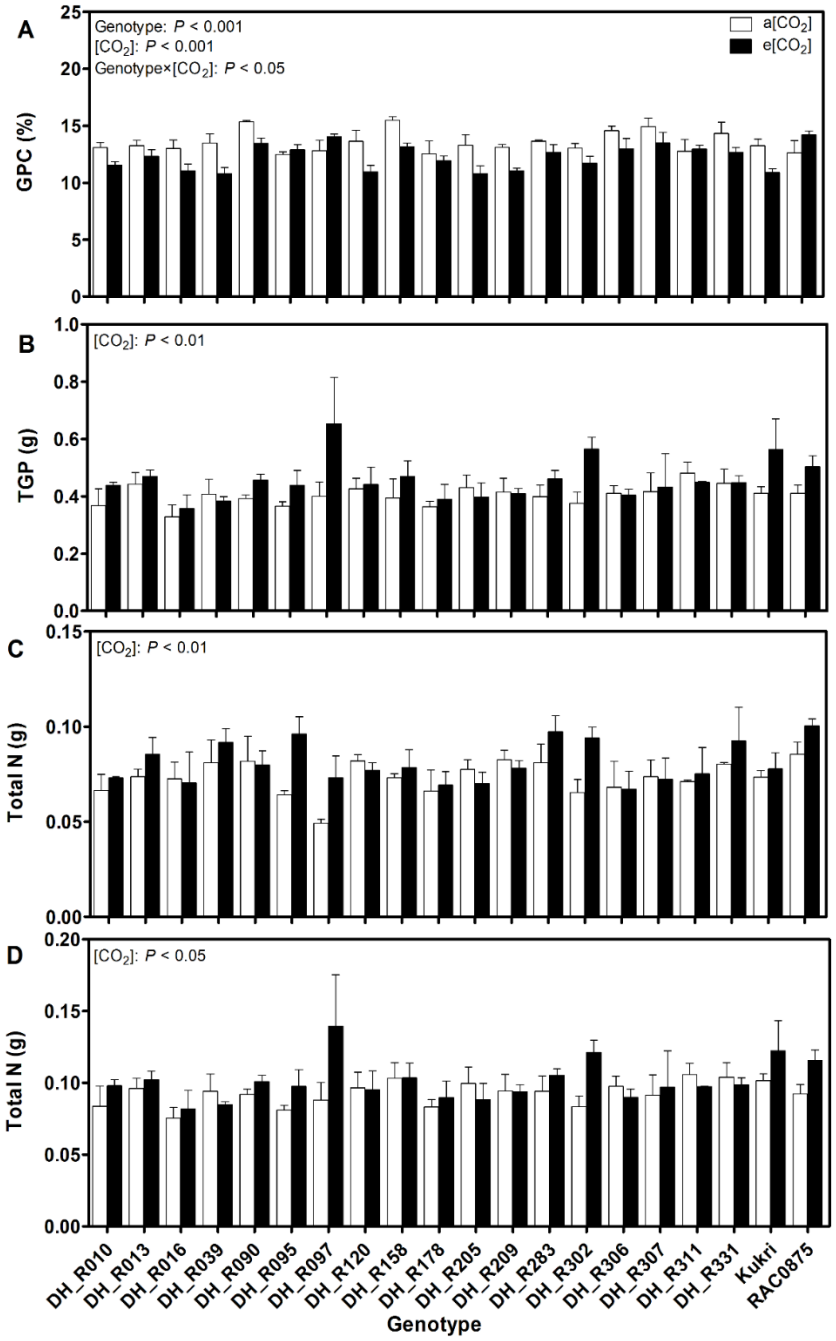
The N percentages of the shoot and grain were analysed for each line, allowing the calculation of GPC, total grain protein (TGP) and TPN. The parental genotypes, RAC0875 and Kukri, differed significantly ($P \leq 0.01$) in their GPC response to e[CO_2], where Kukri decreased in GPC in response to e[CO_2] and RAC0875 increased. (Fig. 1). Under ambient conditions, the GPC of Kukri was on average 0.65% greater than RAC0875, however, the GPC of RAC0875 was on average 3.34% greater under e[CO_2]. Overall, elevated [CO_2] had a significant negative effect on GPC, with the majority of genotypes declining in GPC. However, in addition to

RAC0875, there were three genotypes, DH_R095, DH_R097 and DH_R311, which each saw an increase when compared with growth under a[CO_2]. We found that GPC was significantly affected by both genotype and [CO_2] (Fig. 1). There was also a significant genotype×[CO_2] interaction ($P \leq 0.05$).

Total grain protein increased under e[CO_2] for both RAC0875 and Kukri, however, there was no significant difference between the two genotypes. Overall, total grain protein increased significantly on average in response to e[CO_2], however, a quarter of the genotypes (DH_R039, DH_R205, DH_R209, DH_R306 and DH_R311) incurred a small decrease (Fig. 1). Response of TGP to e[CO_2] was not significantly different between genotypes. Most genotypes which declined in GPC had an observable increase in TGP. On the other hand, DH_R311, which had a non-significant increase in GPC under e[CO_2], declined in TGP.

Total plant N was analysed at anthesis and maturity. At both times of sampling, both Kukri and RAC0875 increased in TPN in response to e[CO_2]. RAC0875 had great TPN at anthesis, while Kukri showed greater TPN at maturity. Most genotypes increased in N content under e[CO_2] at both anthesis and maturity. Seven genotypes were found to have less N under e[CO_2] than a[CO_2] at anthesis, along with seven genotypes which had less N under e[CO_2] at maturity, however, only genotypes DH_R120, DH_R205, DH_R209 and DH_R306 had consistently lower TPN under e[CO_2] at both time points (Fig. 1). Genotype did not appear to significantly affect the variation in TPN response to e[CO_2], however, the effect of [CO_2] was significantly positive at both anthesis ($P \leq 0.01$) and maturity ($P \leq 0.05$).

Figure 1. GPC (A), TGP (B) and Total plant N at anthesis (DC 65) (C) and maturity (DC 90) (D) of 20 wheat genotypes grown under ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) [CO_2]. All data are the means of n = 3 with SE. P values are shown on each graph where significance was found for genotype and [CO_2] effect, along with genotype×[CO_2] interaction. GPC, grain protein concentration; TGP, total grain protein.



196 *Effect of [CO₂] on*

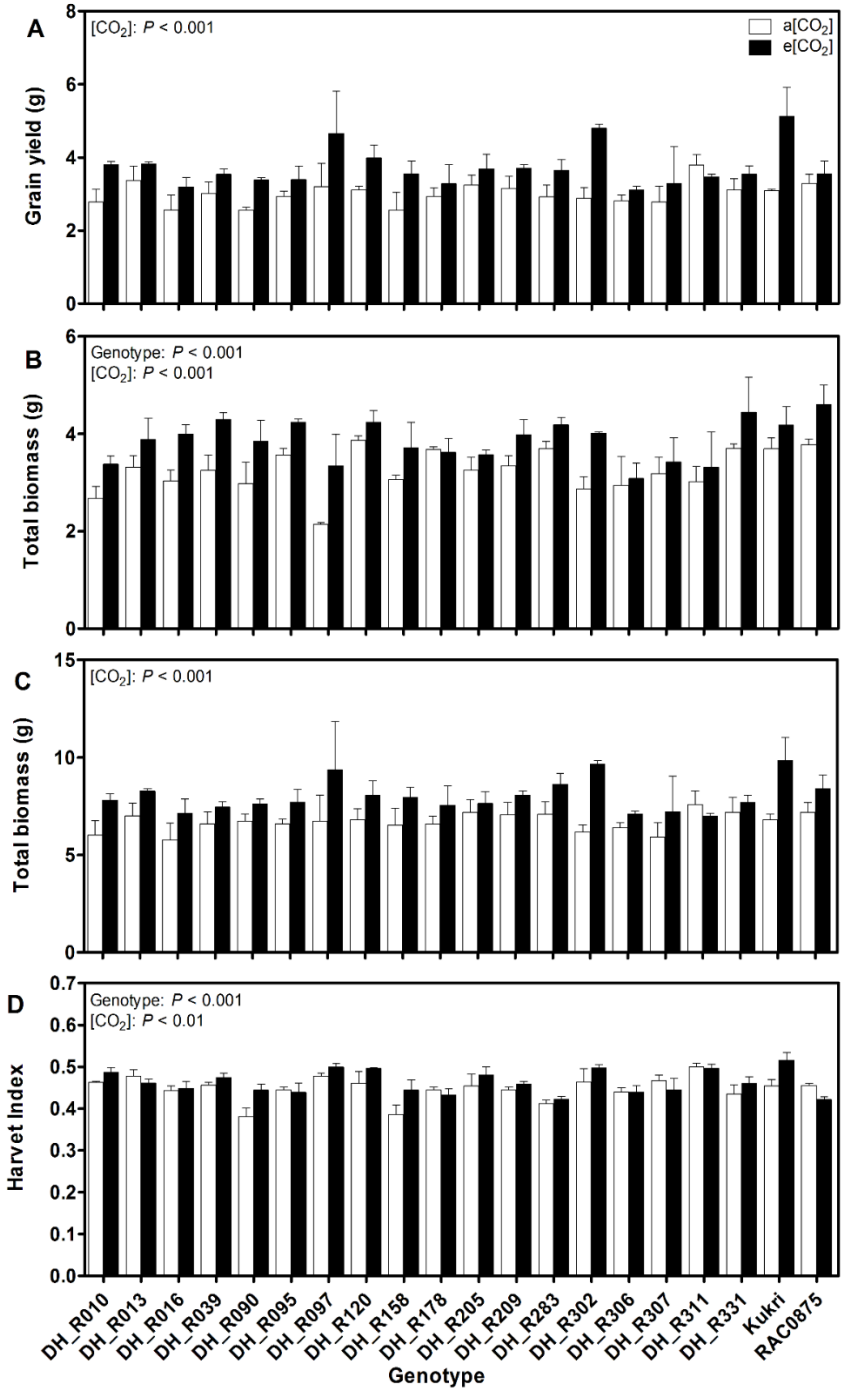
197 *grain yield, total plant biomass, harvest index and photosynthesis*

198 Grain yield increased for both parental genotypes, but this was not significant. While the
199 difference in grain yield between the two genotypes was only 0.192g under a[CO_2], e[CO_2]
200 stimulated the grain yield of Kukri to 1.565g greater than RAC0875. Overall, grain yield was
201 stimulated by e[CO_2] for all genotypes studied except for DH_R311 (Fig. 2). The effect of
202 [CO_2] on grain yield was highly significant ($P \leq 0.001$), although there was no significant
203 difference between genotypes.

The total plant biomass was recorded for each genotype at both anthesis and maturity. Total plant biomass was weighed as the above ground portion of the plant, without roots. Kukri and RAC0875 both increased in total plant biomass and, similar to grain yield, e[CO_2] caused an increase in the total plant biomass of all other genotypes except for one (Fig. 2). Genotype had no significant effect on total plant biomass, regardless of time point, however, there was a highly significant effect of [CO_2] for both anthesis ($P \leq 0.001$) and maturity ($P \leq 0.001$).

From the grain yield and total plant biomass data, HI was calculated. We found that one of the parents, RAC0875, declined in HI in response to e[CO_2], while Kukri on the other hand increased. The HI differed between the two parental genotypes under a[CO_2] by only 0.001, while they differed under e[CO_2] by 0.095. Harvest index increased in 13 out of the total 20 genotypes in this study (Fig. 2). Genotype was found to have a highly significant effect on HI ($P \leq 0.001$). In addition, [CO_2] significantly affected HI ($P \leq 0.01$), though there was no significant interaction between genotype and [CO_2].

Figure 2. Grain biomass (A), Total biomass at anthesis (DC 65) (B) and maturity (DC 90) (C), and Harvest index (D) of 20 wheat genotypes grown under ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) [CO_2]. All data are the means of n = 3 with SE. P values are shown on each graph where significance was found for genotype and [CO_2] effect, along with genotype×[CO_2] interaction.

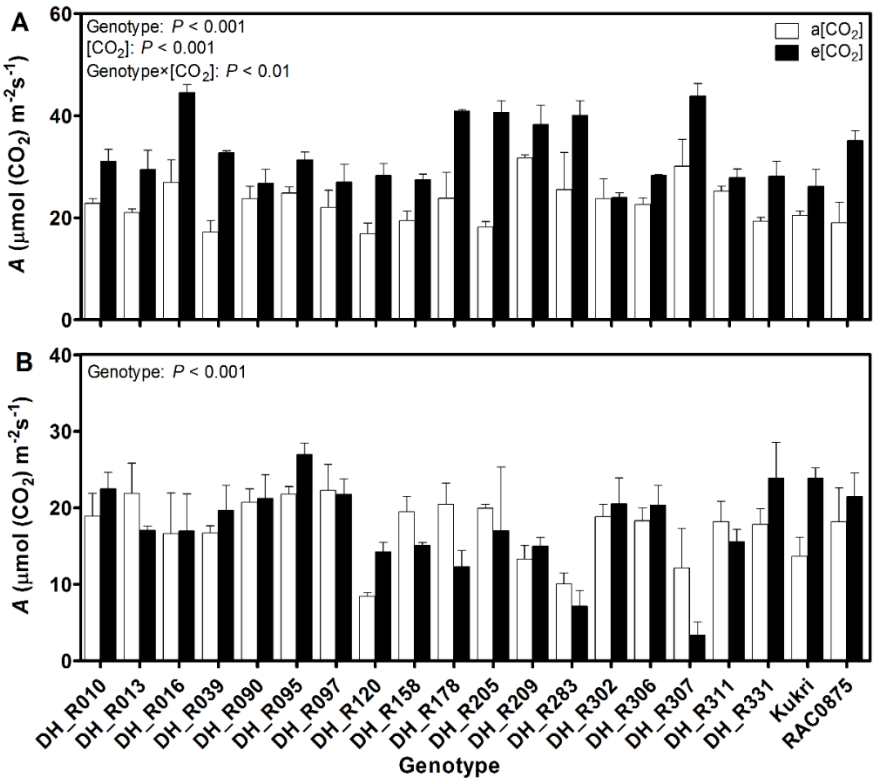


222

223 Rate of photosynthesis was measured at anthesis and post-anthesis (DC 75). Rate of
224 photosynthesis at anthesis was the only trait studied which was improved by e[CO_2] in all
225 genotypes (Fig. 3), including Kukri and RAC0875, with a mean increase of 9.87 mmol (CO_2)
226 $m^{-2}s^{-1}$. The increase in rate of photosynthesis ranged from 0.17 mmol (CO_2) $m^{-2}s^{-1}$ in DH_R302
227 to 22.5 mmol (CO_2) $m^{-2}s^{-1}$ in DH_R205. As such, [CO_2] had a highly significant effect on
228 photosynthesis at anthesis ($P \leq 0.001$). This was not the case during post-anthesis, where only

12 out of 20 genotypes increased in photosynthesis (Fig. 3). The parent genotype Kukri improved the most in photosynthesis under e[CO_2], increasing on average by 10.2 mmol (CO_2) $m^{-2}s^{-1}$, while DH_R307 incurred the greatest decrease, with a change in photosynthesis of -8.83 mmol (CO_2) $m^{-2}s^{-1}$ under e[CO_2]. Genotype significantly affected photosynthesis at both stages.

Figure 3. A at anthesis (DC 65) (A) and A at post-anthesis (DC75) (B) of 20 wheat genotypes grown under ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) [CO_2]. All data are the means of n = 3 with SE. P values are shown on each graph where significance was found for genotype and [CO_2] effect, along with genotype×[CO_2] interaction. A, rate of photosynthesis.

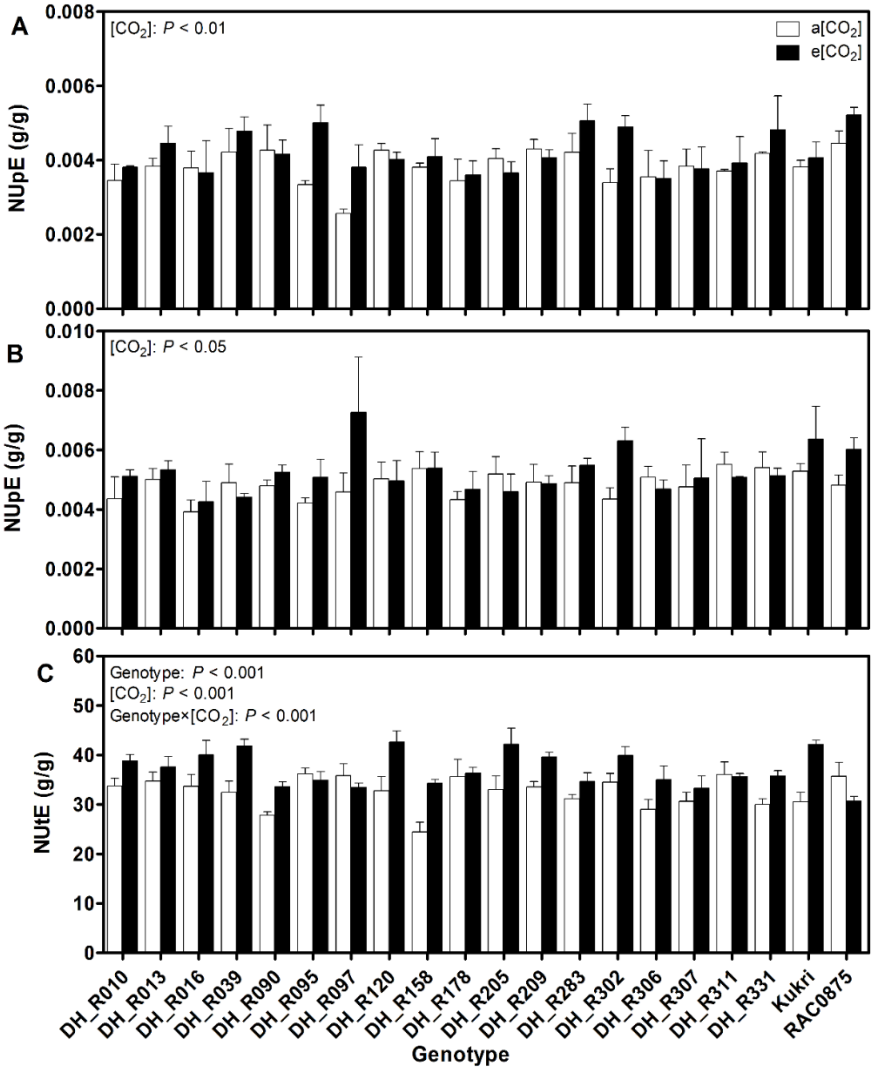


Effect of [CO_2] on nitrogen uptake, utilisation and remobilisation efficiency, and post-anthesis nitrogen uptake

Nitrogen use efficiency was calculated as two components: NUpE and NUtE. The NUpE of each genotype was calculated at both anthesis and maturity, while NUtE was calculated only at maturity. Kukri and RAC0875 both increased in NUpE. Similar to other traits, NUpE increased in 13 out of the 20 genotypes under e[CO_2] for both anthesis and maturity. Only DH_R120, DH_R205, DH_R209 and DH_R306 were consistently negative across both time

points (Fig. 4). On the other hand, NUtE increased in one parental genotype, Kukri, but did not increase in the other, RAC0875. Under ambient conditions, RAC0875 had a greater NUtE than Kukri, while e[CO_2] caused the NUtE of RAC0875 to decline to a similar level as the NUtE of Kukri grown under a[CO_2] (Fig. 4). There was no significant difference between the parents for NUpE, regardless of time point, but the difference in NUtE was significant ($P \leq 0.01$). Regarding the other genotypes, NUtE increased in all but three, DH_R095, DH_R097 and DH_R311 (Fig. 4). Both genotype and [CO_2] had a significant effect on NUtE, while only [CO_2] significantly affected NUpE (both anthesis and maturity). The genotype×[CO_2] interaction was also significant for NUtE ($P \leq 0.001$).

Figure 4. NUpE at anthesis (DC 65) (A), NUpE at maturity (DC 90) (B) and NUtE (C) of 20 wheat genotypes grown under ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) [CO_2]. All data are the means of n = 3 with SE. P values are shown on each graph where significance was found for genotype and [CO_2] effect, along with genotype×[CO_2] interaction. NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilisation efficiency.

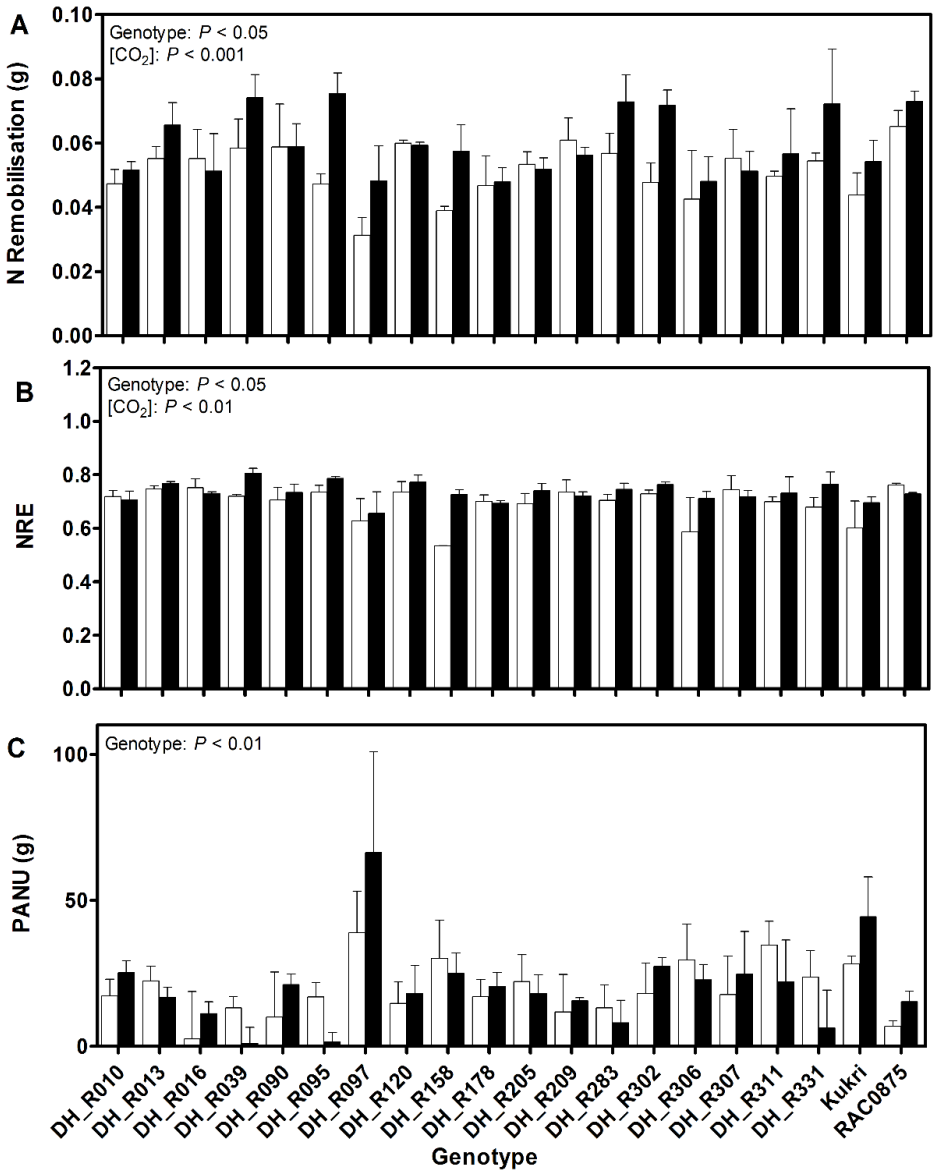


Nitrogen remobilisation increased under e[CO_2] for both Kukri and RAC0875, however, only Kukri increased in NRE under e[CO_2]. Despite this, the difference in NRE between Kukri and RAC0875 was not significant. Both the NRE and total N remobilisation increased for the majority of the other genotypes (Fig. 5). Of those which declined in total N remobilisation, only DH_R016, DH_R209 and DH_R307 also declined in NRE. Genotype and [CO_2] both significantly affected N remobilisation as well as NRE.

Kukri had greater PANU than RAC0875 under both CO_2 conditions (21.2 mg greater under a[CO_2] and 29.1 mg greater under e[CO_2]), but this was not significant. Post anthesis N uptake only increased in about half (11 out of 20) of the total genotypes studied (Fig. 5). As such, there was no significant effect of [CO_2] on PANU. On the other hand, PANU appeared to vary with genotype. DH_R097 had the greatest stimulation in PANU by e[CO_2] (27.5 mg), but also had

the greatest PANU under both e[CO_2] (66.4 mg) and a[CO_2] (38.9 mg) (Fig. 5). On the other hand, DH_R039 had the largest decrease in PANU of 20.1 mg.

Figure 5. N remobilisation (A), NRE (B) and PANU (C) of 20 wheat genotypes grown under ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) [CO_2]. All data are the means of n = 3 with SE. P values are shown on each graph where significance was found for genotype and [CO_2] effect, along with genotype×[CO_2] interaction. GPC, grain protein concentration; NRE, nitrogen remobilisation efficiency; PANU, post-anthesis N uptake.



Correlations between traits

The Pearson correlation method was used to calculate the correlation between GPC response to e[CO_2] and the response of other nitrogen and biomass related traits (Table 1). There was a strong negative correlation between GPC and NUtE (r = -0.963, $P < 0.001$) and a moderate positive correlation between GPC and NUpE at both anthesis and maturity (Anthesis: r = 0.596, $P = 0.006$; Maturity: r = 0.550, $P = 0.012$). GPC was typically lower in plants which were more efficient at producing grains per gram of N under e[CO_2], while plants which were more efficient at taking up the available soil N appeared to maintain a higher GPC. Despite the positive correlation between GPC and NUpE, however, there seemed to be no significant relationship between GPC and PANU. Grain yield also had no significant correlation to GPC, but HI on the other hand, had a moderate negative correlation (r = -0.588, $P = 0.006$). As such, it was often the case that when the biomass partitioning within the plant shifted further into grain production, GPC tended to decline.

In addition to analysing the correlation of each trait's response to e[CO_2] with that of the GPC response, all traits were further analysed against one another (Table 1). All traits, except for the two photosynthesis related traits, displayed some degree of significant correlation with at least one other trait. The only negative correlation observed, other than the correlations calculated with GPC, was between the response of NUpE to e[CO_2] at anthesis and NUtE. Plants which became more efficient in taking up the available soil N under e[CO_2] were more likely to either decline in the amount of grain yield produced per gram of N or incur a lower increase under e[CO_2] than other plants. All of the correlations between the traits are listed in Table 1.

Table 1. Coefficients of correlation (r) between the response of nitrogen and biomass related traits to e[CO_2]. Data used were the difference between measurements from e[CO_2] grown plants and measurements from a[CO_2] grown plants. Measurements were collected from 20 wheat genotypes. Significant data are indicated in bold; *, $P \leq 0.05$; **, $P \leq 0.01$. GPC, grain protein concentration; TGP, total grain protein; NUtE, nitrogen utilisation efficiency; NUpE, nitrogen uptake efficiency; HI, harvest index; A, rate of photosynthesis; TNR, total N remobilised; PANU, post-anthesis nitrogen uptake; NRE, nitrogen remobilisation efficiency; TPN, total plant nitrogen.

	GPC	TGP	NUtE	NUpE (Anthesis)	NUpE (Maturity)	Grain Biomass	HI	*A* (Anthesis)	*A* (Post-anthesis)	TNR	PANU	NRE	TPN (Anthesis)	TPN (Maturity)	Total Biomass (Anthesis)	Total Biomass (Maturity)
GPC																
TGP	0.403															
NUtE	**-.963****	-0.245														
NUpE (Anthesis)	**.596****	**.619****	**-.500***													
NUpE (Maturity)	**.550***	**.958****	-0.443	**.662****												
Grain Yield	-0.218	**.791****	0.362	0.291	**.666****											
HI	**-.588****	0.286	**.710****	-0.139	0.059	**.644****										
A (Anthesis)	-0.137	-0.429	0.063	-0.347	-0.355	-0.357	-0.352									
A (Post-anthesis)	-0.136	0.209	0.265	0.184	0.137	0.369	0.390	-0.297								
TNR	0.319	**.477***	-0.174	**.889****	0.401	0.302	0.128	-0.389	0.267							
PANU	0.179	**.701****	-0.126	-0.048	**.717****	**.616****	0.208	-0.151	0.011	-0.292						
NRE	-0.369	-0.003	**.523***	0.053	-0.261	0.183	**.540***	-0.243	0.196	**.485***	-0.397					
TPN (Anthesis)	**.596****	**.619****	**-.500***	**1.000****	**.662****	0.291	-0.139	-0.347	0.184	**.889****	-0.048	0.053				
TPN (Maturity)	**.550***	**.958****	-0.443	**.662****	**1.000****	**.666****	0.059	-0.355	0.137	0.401	**.717****	-0.261	**.662****			
Total Biomass (Anthesis)	0.133	**.549***	-0.056	**.521***	**.546***	**.451***	0.292	-0.270	0.366	0.437	0.243	-0.029	**.521***	**.546***		
Total Biomass (Maturity)	0.007	**.858****	0.114	**.446***	**.805****	**.936****	0.347	-0.282	0.256	0.341	**.657****	-0.003	**.446***	**.805****	**.466***	

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Discussion

Gran protein concentration, total grain protein and total plant nitrogen

Grain protein concentration declined in response to e[CO_2] in the majority of plants studied in this experiment. This response is typical in wheat grown under e[CO_2] as found throughout the literature (Taub *et al.* 2008; Myers *et al.* 2014). Contrasting this, in the current study we found that four genotypes increased in GPC in response to e[CO_2], including one of the parent genotypes, RAC0875, along with the genotypes DH_R095, DH_R097 and DH_R311, suggesting that GPC response is genotype dependent. Various hypotheses have been proposed for what causes the decline in GPC, however, the exact mechanisms are not completely understood. In this study, we investigated the response of a variety of N related traits to e[CO_2] in order to further elucidate the mechanisms involved in GPC decline. The two parental genotypes in this study, Kukri and RACO0875, declined and increased in GPC, respectively, in response to e[CO_2], which allows us to investigate how other N related traits are linked to GPC in response to e[CO_2] and in doing so, gain a further understanding of the mechanisms behind GPC decline. The diverse responses of each trait studied across the 18 other genotypes can further allow insights into which of the traits that contribute to GPC are most affected by e[CO_2].

The GPC of plants in this study was negatively correlated with NUtE and positively correlated with NUpE (anthesis and maturity), HI and total N content (anthesis and maturity). High NUtE values exist when plants produce either a greater number of grains or more grain biomass for lower amounts of N, which leaves the plant with insufficient N content for remobilization to grains and thus leads to a lower GPC. Increasing NUtE is detrimental to GPC unless the N harvest index (NHI; ratio of grain N to TPN) can also be increased (Barraclough *et al.* 2010). Similarly, increasing the HI of plants will lead to the same issue, unless there is a concomitant increase in the amount of N taken up by the plant and remobilised to the grain. As seen in this study, neither NUpE or NUtE appeared to be consistently regulated by e[CO_2] across genotypes. In support of this, a study by Tausz *et al*. (2017) found that both N uptake and NUtE were increased under e[CO_2], while Bahrami *et al*. (2017) observed no change in either NUpE or NUtE. This suggests there are genetic differences between genotypes which confer different responses to e[CO_2]. When looking at the two parental lines in this study, it seems evident that NUtE played an important role in determining the different responses of GPC to e[CO_2]. The increase and decrease in NUtE for Kukri and RAC0875, respectively, reflects the difference in

grain yield of each genotype based on the total N taken up. While both genotypes increased in TGP and TPN under e[CO_2], Kukri incurred a much greater increase in grain yield than RAC0875. While breeding genotypes for reduced grain yield stimulation under e[CO_2] may help improve the GPC, this is unlikely to be a viable option in the future, due to the rapidly growing population and increase in food demand. Identifying plants with both an increase in grain yield and GPC, like DH_R097 in the present study, might allow breeding programs to breed new genotypes that are able to avoid the negative effect of future [CO_2].

Total grain protein increased in 15 genotypes in response to e[CO_2], including both Kukri and RAC0875. As such, a decline in GPC did not usually indicate a decline in the total amount of N in the grain. Only genotypes DH_R039, DH_R205, DH_R209 and DH_R306 declined in both GPC and TGP, while one genotype, DH_R311 decreased in TGP despite an increase in GPC. Increases in TGP despite a decrease in GPC have been seen in other studies as well (Kimball *et al.* 2001; Fernando *et al.* 2017). Total grain protein did not correlate with GPC in this study (Table 1), which can be explained by the fact that GPC depends on the amount of protein in grains as well as the biomass of the grains. For example, genotypes DH_R090 and DH_R095 both had similar increases in TGP at e[CO_2], however, due to the difference in grain yield, genotype DH_R090 decreased in GPC while line DH_R095 increased. Elevated [CO_2] appeared to have a generally positive effect on TGP, but this did not vary significantly between genotypes. The degree to which TGP is affected by e[CO_2] appears to be affected by many of the other traits that we studied (Table 1). We found that genotypes which had a greater response to NUpE also had greater increases to TGP. An increase in NUpE results from more N taken up from the soil and in turn increases the plants total N. The greater amount of N stored in the plant means that there is likely more N available for remobilisation to the grains that would increase the TGP. Just as there were significant correlations between NUpE and TGP, NUpE also correlated positively with total N at both anthesis and maturity. The response of TGP to e[CO_2] positively correlated with the response of total N and total biomass of the plant at both anthesis and maturity. Furthermore, post-anthesis N uptake may have played an important role in determining the response of TGP. Both PANU and N remobilisation correlated with TGP. Decreased N uptake rates by e[CO_2] is one of the main hypotheses for why plants exhibit lower [N] under such conditions, whether due to lower stomatal conductance or altered root architecture (Taub and Wang 2008). In addition to the correlation between TGP and N uptake, TGP also increased alongside grain yield, however, this was clearly not a strong enough association to prevent the decline in GPC.

We found that [CO_2] had a significant effect on the total N status at both anthesis and maturity. On average, e[CO_2] tended to increase the total N of the plant, however, there was no difference between Kukri and RAC0875, which both differed in GPC. Kukri, which declined in GPC under e[CO_2], had greater total N content under both a[CO_2] and e[CO_2] than RAC0875, which increased in GPC. As such, an increase in the total amount of N taken up into the plant is not in itself sufficient to maintain GPC under e[CO_2].

Biomass stimulation

Elevated [CO_2] stimulated the grain yield of all but one of the 20 genotypes. Between the two parent genotypes, e[CO_2] mostly affected the grain yield of Kukri rather than RAC0875. Grain yield stimulation is widely reported in studies on e[CO_2] (Wang *et al.* 2013) and forms the basis of the dilution hypothesis, which explains the GPC decline under e[CO_2] as being due to the increase in biomass being too large for the N uptake and assimilation to maintain the plant's N concentration (Taub *et al.* 2008). Any increase in grain yield means that there needs to be a proportional increase in total grain protein in order to maintain the GPC. In this study, grain yield alone could not explain the decrease in GPC. For example, although line DH_R097 displayed the third greatest positive change in grain yield in response to e[CO_2], it also increased in GPC. In addition, DH_R302 had the second highest increase in grain yield, but had a lesser decline in GPC than other genotypes which had lower responses of grain yield to e[CO_2]. This demonstrates not only that there are other factors responsible for the GPC, but that it is possible to generate wheat capable of achieving greater yield and greater GPC at the same time.

In addition to the grain yield increase, growth stimulation was also observed in shoot tissue. Of the 20 lines, only line DH_R311 declined in total plant biomass in response to e[CO_2] at maturity (Fig. 2), which was the same line that declined in grain yield. A core driver of growth in plants is the process of photosynthesis, which is known to increase under e[CO_2] (Ainsworth and Long 2005). This leads to an increase in carbon assimilation and consequently plant biomass. The results of the present study found that photosynthesis increased at anthesis for all plants in response to e[CO_2], however, there was no correlation found between photosynthesis and total biomass at anthesis. The change in photosynthesis observed post-anthesis (DC 75) was generally lower under e[CO_2] than at anthesis. The decline in photosynthesis under e[CO_2] is a phenomenon known as photosynthetic acclimation, where total Rubisco decreases and genes involved in photosynthesis are down-regulated (Drake *et al.* 1997). The reduced

photosynthesis in our study observed post-anthesis (Fig. 3) may have been due to acclimation. On the other hand, as these measurements were taken closer to senescence of the flag leaf, the reduced photosynthesis could also have been due to the breakdown of Rubisco or other photosynthesis related proteins.

Elevated [CO_2] stimulates growth through a variety of other means, such as changes in expression of genes linked with the cell cycle and regulating plant hormone metabolism (Gamage *et al.* 2018). Additionally, N supply is important for growth. Nitrogen is one of the most limiting nutrients for plant growth and therefor any change in N status of the plant due to e[CO_2] can alter the plant's growth and biomass. In this study, the total N of most plants increased (13 out of 20 at both anthesis and maturity). We found that TPN at anthesis was moderately correlated with total plant biomass at anthesis and TPN at maturity was strongly correlated with total plant biomass at maturity (Table 1).

Nitrogen uptake and remobilisation

NUpE varied across each genotype in response to e[CO_2] regardless of the timing of measurement. While an increase in NUpE is not sufficient enough in itself to prevent GPC decline, a higher NUpE leads to greater total N being available. High N content is important for maintaining GPC, but as evidenced in this study, some genotypes still decline in GPC despite an increase in total N uptake. This means that either the uptake of N was insufficient for the amount of grain yield produced, or there was an inhibition somewhere along the assimilation and remobilisation pathway to the grain. Elevated [CO_2] is known to inhibit NO_3^- assimilation in leaves (Bloom *et al.* 2014). Bloom *et al.* (2014) observed that in the leaves of field-grown wheat, there is a higher proportion of unassimilated NO_3^- in relation to the total N under e[CO_2] than a[CO_2]. Lower N assimilation therefore affects the NRE of plants as N is typically remobilised into grains in the form of amino acids sourced from the degradation of proteins during senescence (Distelfeld *et al.* 2014). While the total N remobilised and NRE was increased in most lines, some plants incurred a negative response under e[CO_2]. While RAC0875 remobilised more total N at e[CO_2], this is likely due to the greater uptake of N until anthesis. NRE, on the other hand, declined under e[CO_2], suggesting that while more N was available for remobilisation, the percentage of the plant's total N available for remobilisation had declined. The effect that e[CO_2] has on the availability of soil N and the ability of wheat roots to take up N from soil may also play a role in determining how much N is taken up by the plant and transported throughout. A recent review by Uddling *et al*. (2018) discussed some

of the potential causes for crop N and protein decline under e[CO_2]. Uddling *et al*. (2018) firstly points to decreases in soil N under e[CO_2] as a potential cause, but also mentions lower transpiration-driven soil to root transport of N as another cause. While the review concludes that neither can significantly explain the declines in N and protein concentrations under e[CO_2], it is likely that any decline in N uptake would have an effect on the crop N content even if it does not account for the majority of the decline. The review also discusses other potential mechanisms, including lower plant N demand and inhibition of shoot nitrate assimilation, as discussed above, but concludes that all of these mechanisms may only contribute partly to the decline in GPC. From our results we agree that GPC decline cannot be explained by one mechanism.

PANU is often overlooked when it comes to the significant factors affecting GPC. It's thought that the majority (60-95%) of N in grain protein is sourced from N stored in vegetative tissues at anthesis (Palta and Fillery 1995; Kichey *et al*. 2007), and as such, PANU uptake may typically attribute only a lower amount of N to grains. In this study, line DH_R097 had a greater PANU than pre-anthesis N uptake and although line DH_R097 had the third greatest stimulation of grain yield under e[CO_2], it still increased in GPC. The N remobilisation and NRE of this line was among the lowest of all the lines, while the total grain protein at e[CO_2] was the highest, suggesting that most of the N in the grain came from N taken up post-anthesis. This uptake strategy may be a method wheat crops can use to overcome the negative effect of e[CO_2] on GPC.

Conclusion

Currently, there is a lack of knowledge about the mechanisms which control the response of GPC to e[CO_2]. Previous research has identified various mechanisms that each contribute to the decline in GPC, however there is no solid understanding about how each of the various elements of N and carbon metabolism coordinate to control GPC. We found that not all the genotypes we studied declined in GPC. Grain protein concentration correlated negatively with NUtE and HI, but correlated positively with NUpE (anthesis and maturity). It is clear that the general cause of GPC decline under e[CO_2] is due to increases in grain yield without a proportional increase in grain protein, however, it appears that other mechanisms related to N uptake and remobilisation contribute as well. In this study, in regard to the four genotypes which increased in GPC, there appeared to be no single trait that allowed all of them to avoid the decline seen in the other genotypes. The effect e[CO_2] has on GPC starts with how NUpE

is affected. Less efficient N uptake reduces the overall N in the plant. Elevated [CO_2] may then affect the N remobilisation to grains by preventing N assimilation, however, an increase in PANU may mitigate this inhibition by being stored directly in the grain. In order to maintain the current GPC under future climate conditions, it seems beneficial to select for traits such as higher NUpE, NRE and PANU.

Conflicts of Interest

The authors declare no conflicts of interest.

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Chapter 5

Effect of increased root sugar supply on expression of nitrogen uptake and assimilation genes under elevated carbon dioxide

In this study, transcriptomic and proteomic approaches were used to investigate the effect of e[CO_2] on nitrogen uptake and assimilation related genes and proteins in the roots of young wheat plants. Furthermore, sugar content was analysed to identify any potential sugar sensing pathways involved in regulating nitrogen uptake and assimilation in response to e[CO_2]. This chapter furthers the overall aim of the thesis, which is to further understand the mechanisms reducing GPC under e[CO_2]. While not directly assessing the link between altered sugar sensing pathways and GPC decline, this study hypothesises that increased sugar content in roots of wheat grown under e[CO_2] leads to a change in expression of nitrogen uptake and assimilation genes through sugar sensing pathways. Altered nitrogen uptake and assimilation would affect the total plant nitrogen, which is likely to have an effect on GPC. This chapter has been prepared as a research article to be submitted to "Journal of Experimental Botany".

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Effect of increased root sugar supply on expression of nitrogen uptake and assimilation genes under elevated carbon dioxide

Running title: Elevated [CO_2] affects root sugar, N uptake and assimilation

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Highlight

Elevated [CO_2] increases sugar supply to roots of wheat seedlings, which is associated with the expression of an ammonium transporter (*AMT2.1*) and glutamine synthetase (*GS1a*).

Abstract

Elevated carbon dioxide (e[CO_2]) decreases the grain protein concentration of wheat, although the mechanisms behind this are not completely understood. To elucidate how e[CO_2] affects wheat nitrogen status, we tested the hypothesis that e[CO_2] affects expression of nitrogen uptake and assimilation genes through increased sugar supply to roots using wheat seedlings. We analysed the gene expression (nitrogen uptake and assimilation), sugar dynamics and proteome of roots from the wheat genotypes Kukri and RAC0875, along with eight of their progeny. Gene expression response to e[CO_2] varied with genotype and did not show any consistent up- or down-regulation across the genes. Sugar content increased in roots of almost all genotypes for sucrose, glucose and fructose. An increase in sucrose correlated positively with *AMT2.1* expression while an increase in glucose and fructose correlated negatively with *GS1a* expression under e[CO_2]. Root proteomics analysis of both parents indicated that the abundance of glutamine synthetase was increased in RAC0875, but no other proteins associated with nitrogen uptake or assimilation were significantly affected by e[CO_2]. This study reveals that e[CO_2] differentially affects gene expression of nitrogen uptake and assimilation genes in wheat seedling roots based on genotype, but this is mostly independent of the increased sugar supply to roots.

Keywords: ammonium, elevated carbon dioxide, future climate, nitrate, nitrogen assimilation, nitrogen uptake, sugar sensing, sugar signalling

Abbreviations: a[CO_2], ambient carbon dioxide concentration; e[CO_2], elevated carbon dioxide concentration; GOGAT, glutamate synthase; GS, glutamine synthetase; GPC, grain protein concentration;

Introduction

Future food production is of great concern as the global population continues to grow and new challenges arise due to climate change. As part of climate change, the carbon dioxide concentration ([CO_2]) is increasing and is predicted to reach at least 700 µmol mol^{-1} by the end of the century (IPCC 2007). Elevated [CO_2] (e[CO_2]) causes a decline in the nitrogen concentration of crop plants (Taub *et al*., 2008) and results in a lower grain protein concentration (GPC) (Taub *et al*., 2008; Högy *et al*., 2013; Fernando *et al*., 2015). An increase in [CO_2] provides more substrate for ribulose bisphosphate carboxylase oxygenase (Rubisco), which increases the rate of photosynthesis (Ainsworth and Long 2005), and as such, it is generally reported that e[CO_2] stimulates greater biomass of plants (Thilakarathne *et al*., 2013).

This may improve yields for wheat, but the nutritional value of the grain is diminished due to the effect on GPC.

The mechanisms which cause the GPC decline in wheat under e[CO_2] are not completely understood. Most of the protein that constitutes grain protein in wheat comes from remobilisation of nitrogen originating from proteins degradation in leaves during senescence (Barneix, 2007). One of the current hypotheses for the cause of GPC decline is the inhibition of nitrate assimilation in leaves (Bloom *et al*., 2014). Therefore, one potential method for overcoming GPC decline could be the use of ammonium fertiliser over nitrate as the source of nitrogen. Post-anthesis ammonium uptake has been shown to improve wheat GPC under e[CO_2] (Fernando *et al*., 2017). In addition, nitrogen transport may be lessened under e[CO_2] as stomatal conductance is reduced and may lower transpiration-driven mass flow of nitrogen (Houshmandfar *et al*., 2018). Therefore, both uptake and assimilation of nitrogen in wheat are crucial areas to investigate in order to elucidate the mechanisms leading to lower GPC in response to e[CO_2].

Nitrogen transport into roots is the first step in the flow of nitrogen throughout the plant and ultimately results in the storage of protein in grains. Plants mainly take up nitrogen from the soil in the form of either nitrate or ammonium, with each form using a different set of transporter proteins. Nitrate transport relies on two transport systems, the low-affinity transport system (LATS), which is more important under higher soil nitrate content, and the high-affinity transport system (HATS), which operates under lower soil nitrate content (Forde, 2000). HATS transporters are divided into those that are induced by the presence of nitrate (iHATS) and those that are constitutively expressed (cHATS) (Forde, 2000). Nitrate transporters have been labelled depending on which system they use, with LATS transporters labelled as NRT1 and HATS transporters labelled as NRT2 (Daniel-Vedele *et al*., 1998). Similar to nitrate, ammonium transport also uses systems known as HATS and LATS, where HATS and LATS are each important under low and high ammonium soil concentrations, respectively (Cerezo *et al*., 2001). The effect that e[CO_2] has on the expression of these transporters is not well understood.

After nitrogen is taken up into the roots, it is assimilated into amino acids. This can happen in both roots and leaves, although nitrate assimilation predominantly takes place in leaves (Xu *et al*., 2012). Nitrate assimilation begins with reduction of nitrate to nitrite by the enzyme nitrate reductase, which is located in the cytosol and requires NADH or NADPH (Tischner, 2000).

Nitrite is further reduced to ammonium via nitrite reductase, which feeds into the ammonium assimilatory pathway (Krapp, 2015). Ammonium assimilation occurs via the GS/GOGAT cycle, which involves the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) (Masclaux-Daubresse *et al*., 2010). In this process an ammonium ion is fixed to a molecule of glutamate by GS to make glutamine, which is subsequently converted by GOGAT into two molecules of glutamate (Masclaux-Daubresse *et al*., 2010).

As mentioned above, e[CO_2] results in a stimulation of photosynthesis. However, although e[CO_2] initially stimulates photosynthesis, long-term exposure leads to photosynthetic down-regulation (Ainsworth and Long, 2005). Sugar sensing is one of the mechanisms that appears to cause photosynthetic acclimation, where the build-up of excess sugars from e[CO_2] leads to the down-regulation of genes involved in photosynthesis (Cheng *et al*., 1998). In plants, sugars can affect the expression of genes through sugar sensing pathways, where sensory enzymes detect the presence of different sugars and initiate a signal that regulates specific genes (Thompson *et al*., 2017). Different enzymes function as sensing enzymes for different sugars, such as hexokinase for glucose (Moore *et al*., 2003) and SnRK1 for sucrose and trehalose-6-phosphate (Baena-González *et al*., 2007), but the sugar sensing function may not be limited to these enzymes. Under ambient conditions, sucrose was found to affect the expression of various nutrient transport genes (Lejay *et al*., 1999; Lejay *et al*., 2003). Currently, knowledge of the effect of altered sugar content in response to e[CO_2] on plant roots and nutrient uptake is lacking (Thompson *et al*., 2017).

We tested the hypothesis that e[CO_2] increases the sugar supply to roots, which down regulates the genes and proteins associated with nitrogen uptake and assimilation using two wheat genotypes and eight of their selected progeny. Furthermore, this study aimed to improve the understanding of how e[CO_2] affects the nitrogen dynamics of wheat and the mechanisms behind the protein decline in the grain.

Materials and Methods

Plant materials and growth conditions

An experiment was conducted with 10 bread wheat (*Triticum aestivum*) genotypes grown in environmentally controlled growth chambers (Bioline Global) at the University of Southern Queensland, Toowoomba, Australia. The 10 genotypes consisted of two parental genotypes (Kukri and RAC0875) and eight selected lines derived from a Kukri/RAC0875 doubled haploid population. The eight selected lines were chosen based on GPC values from a previous

experiment, consisting of a range of GPC responses to e[CO_2] (data not published). All 10 genotypes were grown in one growth chamber at a[CO_2] (~ 400 µmol mol^{-1}) and another at e[CO_2] (~ 700 µmol mol^{-1}). All other settings were identical across both growth chambers, consisting of 70% relative humidity, 40% light intensity (partial) and a temperature gradient based on the time of day (15°C at 06:00, 20°C at 08:00, 23°C at 11:00, 20°C at 15:00, 15°C at 18:00 and 13°C at 20:00). Seeds were pre-germinated and transferred into pots containing brown topsoil (0.4% nitrogen). Plants were grown in three replicates with three plants per replicate (nine total plants per genotype in each treatment) and the position of each pot was randomized on a weekly basis.

Gas exchange measurements

Gas exchange measurements were conducted for both parental genotypes one month after transferring using a LI-6400XT portable photosynthesis system (LI-COR, USA). Measurements were taken at 3-4 hour intervals over the course of one day per genotype, from 4am to 8pm, to observe the diurnal change in photosynthesis and how it was influenced by e[CO_2]. Measurements for each genotype were taken on separate, adjacent days.

Sample collection

After one month growth, root samples were collected for each plant following the gas exchange measurements. Each plant was removed from the pot and the soil was washed from the roots. Roots were blotted dry with a paper towel and frozen in liquid nitrogen before being stored at -80°C.

Gene expression analysis

RNA extraction and cDNA synthesis

Frozen root samples were weighed to 100 mg for RNA extraction. Roots were ground in liquid nitrogen using a mortar and pestle and RNA was extracted using TRIsure (Bioline, London, UK) according to the manufacturer's instructions. The quantity of RNA in each extraction was measured using a Qubit 3 Fluorometer (Invitrogen, California, USA). DNA was eliminated from the sample by DNase treatment with DNase I, amplification grade (Invitrogen, California, USA) according to the manufacturer's instructions. Then, cDNA was synthesised by reverse transcription using a SensiFAST cDNA Synthesis Kit (Bioline, London, UK) in a total volume of 20 µl according to the manufacturer's instructions.

Quantitative real-time PCR and gene expression analysis

Quantitative real-time PCR (qPCR) was performed to analyse the gene expression of nitrogen uptake and assimilation genes in the roots of one month old wheat plants. Table 1 lists the genes analysed and the primer details. The qPCR reactions were performed in 96 well plates using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, California, USA) with a reaction volume of 20 µl consisting of 4 µl of diluted cDNA (1:10), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 10 µl of 2X PowerUp SYBR Green Master Mix reagent (Applied Biosystems, California, USA). PCR cycling conditions were as follows: 95°C for 10 minutes, 40 cycles at 95°C for 15 sec and 60°C for 1 minutes. Following this, the melt curve was generated with 15 seconds at 95°C and then 60 seconds each at 1.6°C increments between 60°C and 95°C. The primer efficiency was calculated using the LinRegPCR software. *ADP-ribosylation* factor was used as the reference gene after prior confirmation of its expression stability. Quantification of relative gene expression for e[CO_2] compared with a[CO_2] was then analysed using the method described by Pfaffl (2001). Data are presented as the log2 fold change.

Sugar analysis

A portion of the roots for each sample were removed from the -80°C freezer and dried in an oven at 65°C for three days. Dried roots were then ground into a powder with a Tissuelyser (Qiagen, Hilden, Germany) and weighed to approximately 50 mg before sugar analysis. The ground root samples were extracted twice with 80% (v/v) ethanol at 80°C. Samples were centrifuged at 12,000 × g for 5 minutes and the supernatant collected and dried in a vacuum. The remaining pellets were used for the determination of sugars by an enzymatic method using F-kits (J.K. International Co., Ltd., Tokyo, Japan).

Root proteome analysis

Protein extraction

Frozen root samples were weighed to 300mg for protein extraction. Roots were ground in liquid nitrogen using a mortar and pestle and homogenised in 500 µl of solubilisation buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF). The homogenised sample was collected into 1.5 ml Eppendorf tubes, then vortexed and sonicated three times before incubating for 30 minutes at 37°C with shaking. Tubes were centrifuged at 18000 x g for 10 minutes and the supernatant transferred to new

tubes. Samples were passed through Zeba Spin Desalting Columns (Thermo Fisher Scientific, USA) before proteins were quantified using a Microplate BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Methanol was added to the protein extract at a ratio of 100 µl sample : 400 µl methanol and centrifuged for 10 seconds at 9000 x g. 100 µl of chloroform was then added per 100 µl sample and tubes were once more centrifuged for 10 seconds at 9000 x g. Then, 300 µl water was added per 100 µl sample and centrifuged for 1 minute at 9000 x g. The water-methanol phase at the top of the tube was removed and 100 µl methanol added per 100 µl sample. Tubes were centrifuged for 2 minutes at 9000 x g and the supernatant removed. Protein pellets were then air dried. Proteins were identified by mass spectrometry analysis undertaken at La Trobe's Comprehensive Proteomics Platform at La Trobe University, Australia.

Statistical analysis

Statistical analysis to determine significant difference between mean relative expression of genes in roots grown under e[CO_2] and a[CO_2] was performed by a Paired-samples t test using the Compare Means function in the IBM SPSS Statistics ver. 23 software package (IBM Corp., 2015, Armonk, NY). Sugar content and photosynthesis responses to e[CO_2] and genotype were analysed by performing a Two-Way ANOVA by general linear model using IBM SPSS Statistics ver. 23. A Pearson product-moment correlation test was conducted using Bivariate Correlations to analyse potential correlation between sugar content change under e[CO_2] and relative gene expression. Statistical analysis for the protein fold change was performed using the statistical programming language R. The results of all analyses were regarded as significant at $P \leq 0.05$.

Results

Effect of [CO_2] and genotype on gene expression

To observe the effect of e[CO_2] on nitrogen uptake and assimilation related gene expression in roots, expression analysis was undertaken for seven genes involved in nitrogen uptake (Table 1. No. 1 – 9) and nine genes involved in nitrogen assimilation (Table 1. No. 10 – 16). Elevated [CO_2] affected gene expression for nitrogen uptake genes depending on genotype (Table 2). Greater levels of expression for all nitrate and ammonium transporter genes (*AMT1.1*, *AMT1.2*, *AMT2.1*, *NRT1.1A*, *NRT1.2*, *NRT1.3A* and *NRT2.1*) were observed in Kukri and DH_R039 under e[CO_2], however, transcript levels of *AMT1.1* in Kukri and *AMT1.1*, *AMT1.2* and *NRT2.1* in DH_R039 were relatively unchanged despite the slight increase. Transcript levels of all

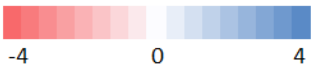
nitrogen uptake genes were lower in DH_R205 under e[CO_2], although *AMT2.1* was relatively unchanged. Transcript levels of DH_R209 were relatively unchanged for most of the genes studied under e[CO_2]. In the other genotypes, there was a mixture of responses to e[CO_2], with some genes being up-regulated and others down-regulated across the nitrate and ammonium transporters studied. No genes demonstrated consistent up- or down-regulation across all genotypes. Some genes had a greater response to e[CO_2] in individual genotypes. For example, the strongest instance of down-regulation occurred with the expression of *ASN1* in genotype DH_R120, while the strongest instance of up-regulation occurred with the expression of *GS1a* in genotype DH_R311. Expression of *NAR2.1* and *NAR2.2* was relatively unchanged for most genotypes, with exception of *NAR2.1* being up-regulated in DH_R120.

Table 1. List of nitrogen uptake and assimilation genes and the primers used in gene expression analysis.

				Primers
No.	**Acession No.**	**Gene Name**	**Orientation**	**5' - 3' sequence**
1	AY525637	Ammonium transporter, AMT (*AMT1;1*)	Fw	CTACACCGACGAGGACTCCA
			Rv	CCGACTTGAGCATGAACCCT
2	AY525638	Ammonium transporter, AMT (*AMT1;2*)	Fw	CGCGCTCTTCTACTACCTCTT
			Rv	CGAAGAAGTGCTTCCCGATAAAC
3	AY428038	Ammonium transporter, AMT (*AMT2;1*)	Fw	GAGCCGAACCTCTGCAATCT
			Rv	GTTCCACCCGATCACGAAGA
4	HF544985	Low affinity nitrate transporter, NRT1 (*NRT1.1A*)	Fw	CCTTCACCTACATCGGCCAG
			Rv	CTGACGAAGAATCCGAGCGA
5	AY587264	Low affinity nitrate transporter, NRT1 (*NRT1.2*)	Fw	ATACCTGGGGAAGTACCGGACAGC
			Rv	AGGATCTGCCCAAAGAGTCCAAGCA
6	HF544990	Low affinity nitrate transporter, NRT1 (*NRT1.3A*)	Fw	AGCCTCAACAATGACGGAAGT
			Rv	CCAGAAGAGGATGCAGGTGG
7	AF332214	High-affinity nitrate transporter, NRT2 (*NRT2.1*)	Fw	TCAAGAAAGATGGAGGTGCAGGCCG
			Rv	TGTGGGGGTTGGCGAAGGAGAA
8	AY763794	Component of high affinity nitrate transporter, NAR2 (*NAR2.1*)	Fw	TCGCCTCCCTCGCATTCTTCTTCT
			Rv	GACCGGCCAGAGAAAGCCAACA
9	AY763795	Component of high affinity nitrate transporter, NAR2 (*NAR2.2*)	Fw	CGTCGTCTCCCTCGCCTTCTTCTT
			Rv	AAGTCGCGCACGGACGAACA
10	AK333426	Putative NADH-nitrate reductase, NR (*NIA*)	Fw	CTCAAGCGCAGCACGTCTA
			Rv	CTCGGACATGGTGAACTGCT
11	FJ527909	Ferredoxin-nitrite reductase, NiR (*nir*)	Fw	AACCTCCTCTCCTCCTACATCA
			Rv	CCTAGGAAGGTTGGTGATGGC
12	AK333382	Molybdenum cofactor biosynthesis protein, MoCo	Fw	AGCGGCGTGTGGGATAAGAGGA
			Rv	ATGCACTCCGCGACAGCGTT
13	DQ124209	Glutamine synthetase, cytosolic, GS1 (*GS1a*)	Fw	AGGTCATCGTGGATGCCGTGGA
			Rv	TTTGCGACGCCCCAGCTGAA
14	AY621539	Asparagine synthetase, AS (*ASN1*)	Fw	GAACCAGCAAGAAGCCGAGGATGA
			Rv	AGAGATTGGCAAGCAGGACAGGACA
15	TC387834	Putative NADH-dependent glutamate synthase, NADH-GOGAT	Fw	GCCATTGAATCAGTTCCAGGGCCAC
			Rv	GCCAGCACCTGAGCTTTCCTGATG
16	TC394038	Putative ferredoxin-dependent glutamate synthase, Fd-GOGAT	Fw	CGGCAATGGAGGCTGAGCAACA
			Rv	TGAGCCTGCTCGATGGTCACTGT

Table 2. Heat map of relative expression of e[CO_2] compared with a[CO_2] grown plants. Genes are related to nitrogen uptake and assimilation. All data are the mean of 3 replicates and represent the log2 fold change.

	Genotype									
Gene	DH_R039	DH_R095	DH_R097	DH_R120	DH_R205	DH_R209	DH_R283	DH_R311	Kukri	RAC0875
AMT1.1	0.16	-0.06	1.24	2.31	-0.91	0.66	-1.45	0.42	0.49	1.04
AMT1.2	0.32	-0.48	-0.94	-0.93	-0.48	-0.44	-1.35	1.02	1.57	0.22
AMT2.1	2.15	-2.80	-1.11	-1.57	-0.05	-1.17	-0.43	0.43	1.53	2.16
NRT1.1A	2.71	-3.38	-1.97	-2.70	-1.18	0.77	-2.53	-1.27	3.47	1.82
NRT1.2	3.50	-1.45	1.59	-2.26	-2.47	-0.42	-0.42	1.23	2.03	-0.52
NRT1.3A	1.13	-1.62	0.10	2.30	-1.81	-0.26	0.29	0.33	3.64	-0.97
NRT2.1	0.41	2.02	-1.99	0.24	-1.10	0.97	-1.20	-0.68	1.35	1.21
NAR2.1	0.66	0.70	0.03	2.63	0.08	0.91	-0.13	0.69	-0.09	-0.35
NAR2.2	0.53	1.11	0.79	0.97	-1.34	0.80	-0.37	0.70	-0.27	0.72
NIA	-0.10	-2.00	-0.79	2.28	-2.31	-0.16	-2.95	0.26	-0.10	0.88
NiR	0.77	-2.03	-1.49	-1.32	-2.24	0.42	-1.88	-0.65	1.03	-0.39
MoCo	0.97	-1.17	1.20	-0.62	1.29	-0.33	-0.07	-0.37	-0.37	1.08
GS1a	-1.35	-2.14	-0.74	-1.13	-0.36	0.32	-0.37	4.29	1.82	2.43
ASN1	-2.08	2.99	-1.21	-4.60	-0.19	0.16	2.11	-0.11	2.42	3.47
NADH-GOGAT	1.07	-0.14	-0.95	-0.55	-2.48	-0.53	-0.89	2.92	2.36	1.52
Fd-GOGAT	0.73	-1.09	0.01	-1.11	0.91	0.47	-0.45	0.28	2.11	0.22



In addition, genes involved with nitrogen assimilation were analysed. Unlike the nitrogen uptake genes, no genotypes showed consistent up- or down-regulation across all nitrogen assimilation genes studied. Most genes were not strongly up- or down-regulated across genotypes. Expression levels in DH_R209 were relatively unchanged for all genes. Furthermore, expression levels in DH_R311 were relatively unchanged in all genes except *GS1a* and *NADH-dependent GOGAT* (*NADH-GOGAT*). In Kukri, the nitrate assimilation genes were relatively unchanged, while all of the ammonium assimilation genes were up-regulated. This was similar for RAC0875, however, *ferredoxin-depended GOGAT* (*Fd-GOGAT*) was relatively unchanged. In DH_R120, all genes were down-regulated except for *NIA*. In DH_R095, most genes were also down-regulated, although *Asparagine synthetase* (*ASN1*) was up-regulated.

Effect of [CO_2] and genotype on sugar content

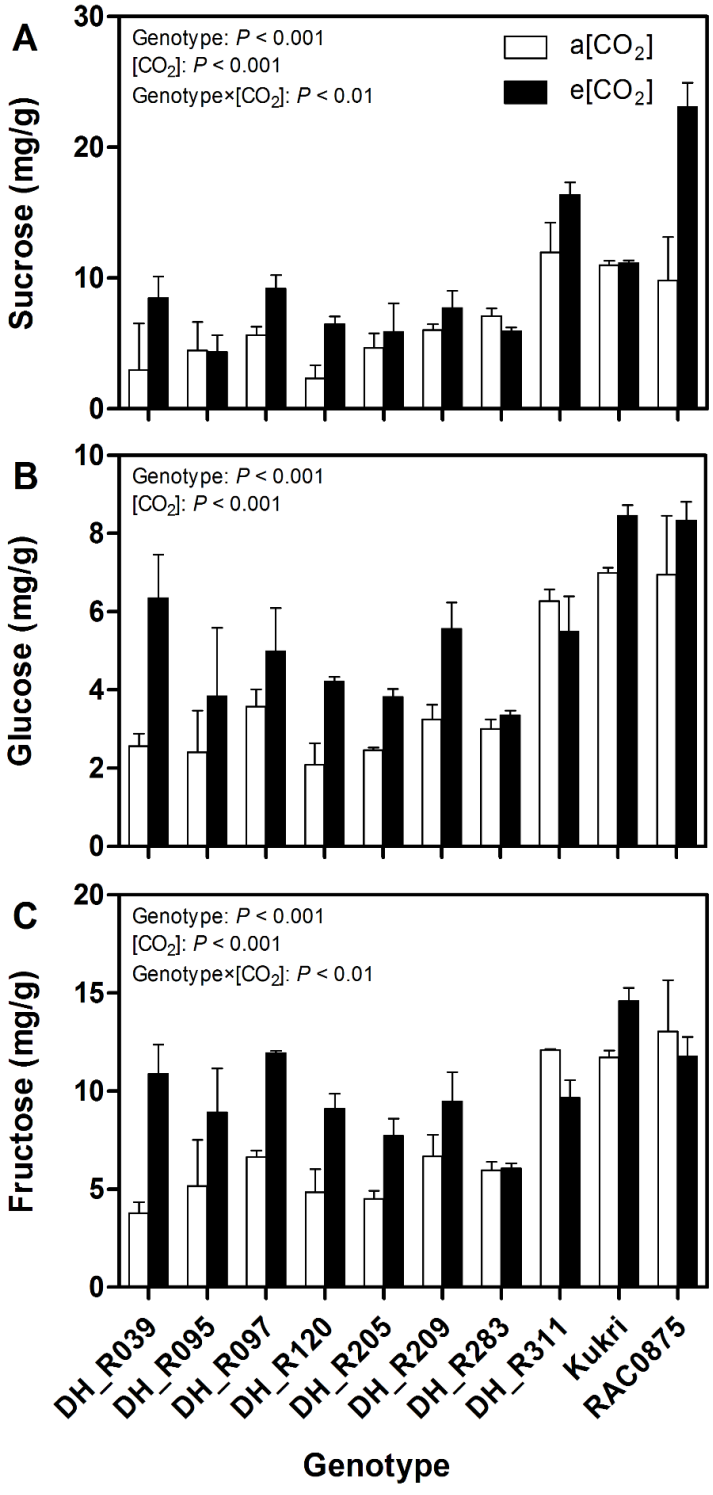
Root samples from e[CO_2] and a[CO_2] grown plants were analysed for their sucrose, glucose and fructose content. A Two-way ANOVA analysis found that e[CO_2] and genotype had a significantly positive effect on sucrose content, increasing in all genotypes except for DH_R095 and DH_R283 (Fig. 1). In addition there was a significant genotype × [CO_2] interaction ($P < 0.005$) for sucrose content. Of the two parental genotypes, RAC0875 had a

significantly greater response of root sucrose content to e[CO_2] ($P \leq 0.05$). RAC0875 had the highest root sucrose content of all genotypes under e[CO_2] as well as the greatest increase in sucrose content.

Glucose content increased in all genotypes except DH_R311 in response to e[CO_2] (Fig. 1). As such, the two genotypes which decreased in sucrose both increased in glucose content in roots under e[CO_2]. Both genotype and [CO_2] had a significant effect on root glucose content ($P \leq 0.001$), although there was no significant interaction effect. In addition, the response of both parental genotypes did not differ significantly. DH_R039 showed the greatest increase in glucose in response to e[CO_2].

As with sucrose and glucose, fructose content increased in the majority of genotypes under e[CO_2] (Fig. 1). Only two genotypes, DH_R311 and RAC0875 decreased in fructose content. Both genotype and [CO_2] had a significant effect on root fructose content ($P \leq 0.001$). Additionally, the interaction effect of genotype × [CO_2] was significant ($P = 0.008$) for fructose content. Despite the average root fructose content decreasing for RAC0875 under e[CO_2], there was no significant difference between the two parent genotype's response. In addition to glucose, DH_R039 displayed the greatest response to root fructose content under e[CO_2].

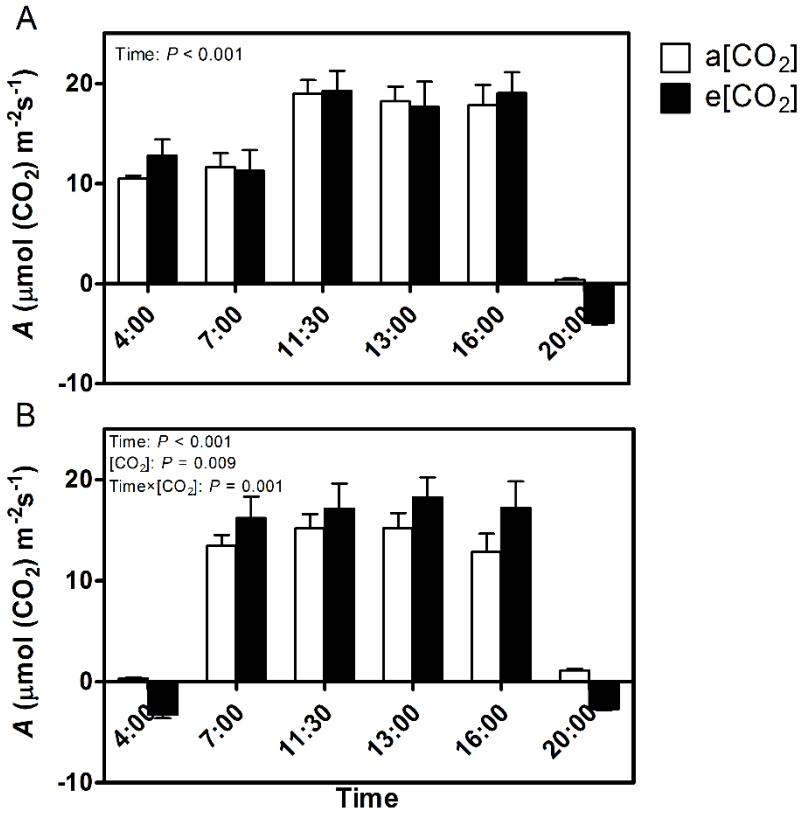
Figure 1. Sugar content of genotypes grown under e[CO_2] and a[CO_2]. A) Sucrose content, B) Glucose content and C) Fructose content. The data represents the mean and standard error of n = 3 replicates.



Effect of [CO_2] and genotype on photosynthesis

Rate of photosynthesis was measured for the parental genotypes Kukri and RAC0875 in 3-4 hour intervals over 16 hours. RAC0875 showed the greatest stimulation from e[CO_2], with all measurements increasing under e[CO_2] when taken during the light period (Fig. 2). As expected, photosynthesis ceased in RAC0875 during the dark period. The negative values seen in Fig. 2 represent respiration, rather than photosynthesis. In contrast, there was less of a stimulation seen in Kukri, with rate of photosynthesis slightly lower in e[CO_2] at two time points (7:00 and 13:00) (Fig. 2). Photosynthesis appeared to be lower in Kukri at 7:00 compared to the other measurements taken during the light period. Unexpectedly, there appeared to be photosynthetic activity at the first measurement during the dark period (4:00). A Two-way ANOVA found that the time of measurement was significant for both genotypes, while [CO_2] was only significant for RAC0875. Similarly, the interactive effect of time of measurement and [CO_2] was only significant for RAC0875.

Figure 2. Rate of photosynthesis of A) Kukri and B) RAC0875 under e[CO_2] and a[CO_2]. Data were taken at six time points over one day and represent the mean and standard error of n = 5 replicates.



Correlation of sugar content with gene expressions

The Pearson correlation method was used to determine whether any correlation exists between the change in sugar content of roots under e[CO_2] and the effect that e[CO_2] has on the gene expression of various nitrogen uptake and assimilation genes. Overall, only three correlations were found to be significant (Table 3 and Fig. 3). There was a moderately positive correlation between the change in sucrose content and *AMT2.1* expression (r = 0.654, P = 0.040). A change in glucose had a moderate negative correlation with *GS1a* (r = -0.683, P = 0.029). Furthermore, fructose content exhibited a strong negative correlation with *GS1a* (r = -0.721, P = 0.019). There were no significant correlations with any genes when compared with total sugar content.

Table 3. Coefficients of correlation (r) between the response of sugar content and gene expression to e[CO_2]. Data used were the difference between measurements from e[CO_2] grown plants and measurements from a[CO_2] grown plants for sugar content and the relative expression for each gene under e[CO_2]. Measurements were collected from 10 wheat genotypes. Significant data are indicated in bold; *, $P \leq 0.05$; **, $P \leq 0.01$.

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	AMT1.1	*AMT1.2*	*AMT2.1*	*NRT1.1A*	*NRT1.2*	*NRT1.3A*	*NRT2.1*	*NAR2.1*	*NAR2.2*	*NIA*	*NiR*	*Moco*	*Gs1a*	*Asn1*	*NADH-GOGAT*	*Fd-GOGAT*
Sucrose	0.347	0.043	**.654***	0.083	0.132	-0.221	0.011	0.024	0.329	0.389	0.067	0.453	0.267	0.328	0.220	-0.165
Glucose	0.218	-0.179	0.346	0.372	0.552	0.102	0.226	0.238	0.162	0.190	0.469	0.243	**-.683***	-0.178	-0.474	0.093
Fructose	0.189	-0.240	-0.043	0.192	0.501	0.145	0.059	0.244	0.107	0.041	0.224	0.213	**-.721***	-0.429	-0.542	0.071
Total Sugar	0.424	-0.139	0.564	0.255	0.505	-0.069	0.092	0.208	0.352	0.369	0.282	0.526	-0.344	-0.022	-0.235	-0.068

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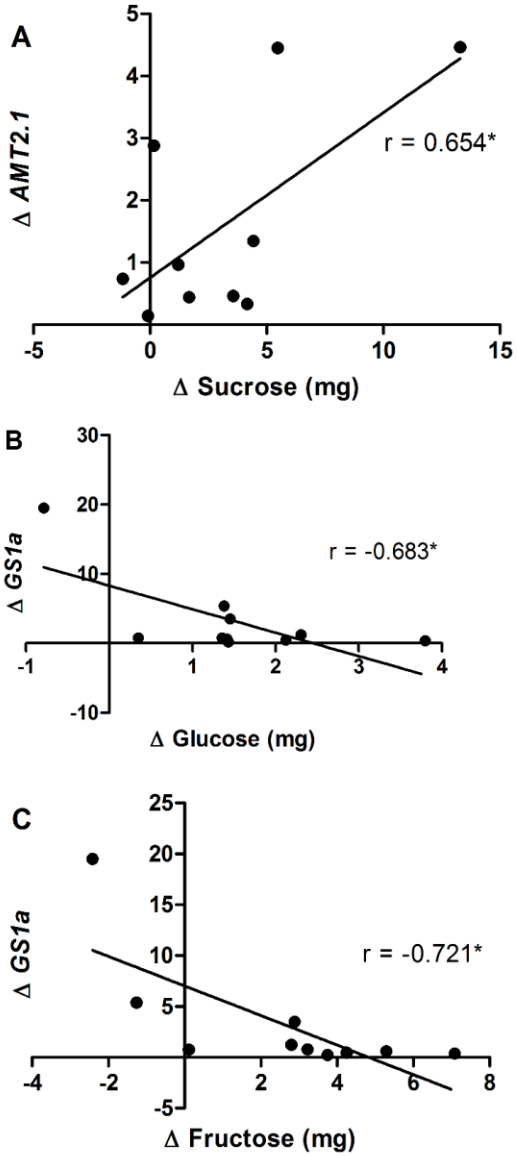
Figure 3. Correlation (*r*) of A) sucrose content and *AMT2.1* expression, B) glucose content and *GS1a* expression, and C) fructose content and *GS1a* expression. Significance is indicated as: *, $P \leq 0.05$.



Response of root proteome to [CO_2]

Root proteome analysis was undertaken for both parent genotypes, Kukri and RAC0875. This analysis looked at the effect of e[CO_2] on the root proteome of the individual genotype (Table 4 and 5).

In Kukri, 1757 proteins were observed from proteomics analysis. Of these, only 10 were involved with nitrogen uptake or assimilation (Table 4). These consisted of three ammonium

304 transporters, three glutamine synthetase accessions, three glutamate dehydrogenase accessions
305 and nitrate reductase. Of this, only one of the glutamine synthetase proteins were found to be
306 significantly affected by e[CO_2], increasing in abundance (Table 4). The other nine proteins
307 were mostly unchanged under e[CO_2], but this was not significant. Aside from glutamine
308 synthetase, 88 proteins were significantly affected by e[CO_2]. Of these proteins, none were
309 involved in nitrogen uptake or assimilation. A large portion of the proteins searched in the
310 UniProt database were labelled as uncharacterized proteins and, therefore, may contain proteins
311 associated with nitrogen uptake and transport that were previously not identified.

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Table 4. Nitrogen uptake and assimilation proteins identified by root proteomic analysis in Kukri. *P* values are regarded as significant at $P \leq 0.05$. Significant values are displayed in bold.

Accession No	Protein	Fold change	MW (kDa)	*P*
	Higher abundance proteins			
W5GVB4	Ammonium transporter	0.28	52.88	0.738
Q6RUJ2	Glutamine synthetase (EC 6.3.1.2)	1.71	38.73	**0.008**
Q45NB7	Glutamine synthetase (EC 6.3.1.2)	0.32	39.20	0.418
	Lower abundance proteins			
A0A1D5Z4Z0	Ammonium transporter	-0.02	50.50	0.974
W5BH53	Ammonium transporter	-0.13	52.30	0.840
A0A1D6AQB0	Glutamine synthetase	-0.11	29.37	0.892
E9NX12	Glutamate dehydrogenase	-0.40	44.24	0.356
A0A1D5Z9N0	Glutamate dehydrogenase	-0.40	45.32	0.625
W5BS00	Glutamate dehydrogenase	-0.14	44.52	0.719
A0A1D6AGQ1	Nitrate reductase	-0.42	97.49	0.439

In RAC0875, 1637 proteins were observed, and similar to Kukri only 10 were involved with nitrogen uptake and assimilation (Table 5). None of these proteins were significantly different between e[CO_2] and a[CO_2] grown plants. These 10 proteins were the same accessions as those identified in Kukri. Of the 1637 proteins, 82 were found to be significant, but were not involved with nitrogen uptake or assimilation. As with Kukri, there were also many uncharacterized proteins.

Table 5. Nitrogen uptake and assimilation proteins identified by root proteomic analysis in RAC0875. *P* values are regarded as significant at $P \leq 0.05$. Significant values are displayed in bold.

Accession No	Protein	Fold change	MW (kDa)	*P*
	Higher abundance proteins			
W5BH53	Ammonium transporter	0.47	52.30	0.454
W5GVB4	Ammonium transporter	0.37	52.88	0.500
A0A1D6AGQ1	Nitrate reductase	0.30	97.49	0.664
A0A1D6AQB0	Glutamine synthetase	0.63	29.37	0.279
	Lower abundance proteins			
A0A1D5Z4Z0	Ammonium transporter	-0.50	50.50	0.363
Q45NB7	Glutamine synthetase (EC 6.3.1.2)	-0.76	39.20	0.052
Q6RUJ2	Glutamine synthetase (EC 6.3.1.2)	-0.09	38.73	0.877
W5BS00	Glutamate dehydrogenase	-0.44	44.52	0.289
A0A1D5Z9N0	Glutamate dehydrogenase	-0.16	45.32	0.562
E9NX12	Glutamate dehydrogenase	-0.02	44.24	0.934

Discussion

Effect of [CO_2] on gene expression

It is well established that growth of wheat under e[CO_2] causes a decline in grain protein concentration. Currently, there is a lack of understanding as to all of the mechanisms affected by e[CO_2] that contribute to this decline. Nitrogen uptake, which is the first step needed to deliver nitrogen to wheat grain, may play an important role in determining wheat GPC. The form of nitrogen taken up (NO_3^- and NH_4^+) has been shown to affect the GPC of wheat (Fernando *et al*., 2017). Nitrate and ammonium transporters in roots are responsible for the uptake of nitrogen from soil and as such, we analysed the effect that e[CO_2] had on the

expression of some of these transporters during the early growth of ten wheat genotypes. The degree of expression for each of the genes studied may change throughout the plant's development and therefore, may not represent the expression of these genes throughout the plant's lifecycle. Even so, the early growth stage of wheat is critical for establishing the plant and may have a large impact on future growth and development. We aimed to identify whether e[CO_2] alters the expression of nitrogen uptake and assimilation genes in order to further elucidate the mechanisms behind lower nitrogen concentration in wheat grown under e[CO_2].

Elevated [CO_2] did not consistently affect any of the nitrogen uptake genes across the genotypes and any effect of e[CO_2] was likely genotype dependent. Nitrate is the dominant form of nitrogen taken up from the soil (Xu *et al*., 2012) and while the four nitrate transporter genes (*NRT1.1A*, *NRT1.2*, *NRT1.3A* and *NRT2.1*) were all up-regulated in two genotypes (RAC0875 and DH_R039), these genes were mainly down-regulated or relatively unchanged across the other genotypes. The down-regulation of these genes would likely lead to a decline in the total nitrate uptake during this growth period when compared with plants grown under a[CO_2] and ultimately result in less nitrate throughout the plant. Expression of the three ammonium transporters (*AMT1.1*, *AMT1.2* and *AMT1.3*) was mostly either downregulated or unchanged under e[CO_2] across each of the genotypes. Generally, it appeared that genotypes which were down-regulated in ammonium transporters were also down-regulated in nitrate transporters, and vice versa. There were still cases where one or two genes showed the opposite, as well as other instances where the expression of various genes was relatively unchanged. In other studies focussing on nutrient uptake, rather than gene expression, e[CO_2] led to differential effects on the uptake of nitrate compared to ammonium (Bassirirad, 2000), in some cases leading to an inhibition in nitrate uptake and having no affect in others (Jackson and Reynolds, 1996; Vicente *et al*., 2016). In addition to nitrate and ammonium transporters, we analysed the expression of two genes encoding components of high affinity nitrate transporters (NAR2.1 and NAR2.2). These genes were also relatively unchanged under e[CO_2] across each genotype. Despite the increase in expression of the nitrate transporters in Kukri under e[CO_2], the transcript levels of *NAR2.1* and *NAR2.2* were slightly less than the a[CO_2] grown plants. Vicente *et al*., (2016) analysed various nitrogen uptake and assimilation related genes of durum wheat under e[CO_2] and two nitrogen levels (low and high) and found that the genes were mostly affected by e[CO_2] when grown under high nitrogen, with most of the genes being upregulated under both e[CO_2] and high nitrogen. Our results suggest that the effect of e[CO_2] on nitrate and ammonium transporter gene expression is genotype dependent. The difference

in gene expression found in these genotypes may lead to differential growth under e[CO_2] and ultimately contribute to the nitrogen content of mature grains.

In a previous experiment (data unpublished), we analysed the total nitrogen uptake of the genotypes studied in this experiment, in addition to others from the same population. Elevated [CO_2] had a significantly positive effect on total nitrogen uptake at both anthesis and maturity. Of the genotypes studied in the current experiment, most increased in nitrogen uptake at anthesis under e[CO_2], while only half increased at maturity. The three genotypes which declined at anthesis (DH_R120, DH_R205 and DH_R209) also declined at maturity. In this experiment, the expression of nitrogen uptake genes for these genotypes was down-regulated under e[CO_2] for most genes, which may suggest a consistent down-regulation of nitrogen uptake in these genes under e[CO_2] throughout the plant's development. In addition, both parental genotypes (Kukri and RAC0875) increased in total nitrogen uptake at both developmental stages while exhibiting up-regulation in the expression of most nitrogen uptake genes. Contrasting these findings, DH_R039, which increased in expression for most genes studied, declined in total nitrogen uptake at maturity, despite its increase at anthesis. In addition, both DH_R205 and DH_R209 increased in total nitrogen at both anthesis and maturity, but showed a general down-regulation in expression of nitrogen uptake genes. Taken altogether, these findings indicate nitrogen uptake may change over the plant's development, although fluctuations in nitrogen uptake appear to be genotype specific.

The other group of genes studied related to nitrate (*NIA*, *NIR* and *MoCo*) and ammonium assimilation (*GS1a*, *NADH-GOGAT*, *Fd-GOGAT* and *ASN1*). It is thought that growth of wheat under e[CO_2] inhibits nitrate assimilation in leaves and this contributes to the decline in GPC (Bloom *et al*., 2014). Nitrate assimilation mostly occurs in shoot tissues (Xu *et al*., 2012), but whether inhibition of nitrate assimilation under e[CO_2] also occurs in roots is not understood. From our results, we found a range of responses for the nitrate assimilation genes in roots under e[CO_2]; however, for many genotypes, gene expression remained relatively unchanged. A few genotypes (DH_R095, DH_R205 and DH_R283) had a greater down-regulation of nitrate gene expression than others and it appeared that the expression of nitrate transporter genes was also mostly down-regulated in these genotypes. It is likely then, that nitrate assimilation was down-regulated due to less nitrate being available in the roots for assimilation. *MoCo* was upregulated more than both *NIA* and *NIR*; however, this gene is not directly involved in nitrate assimilation. Molybdenum cofactor biosynthesis protein, which is translated from *MoCo*, is needed for the successful functioning of nitrate reductase (Schwarz and Mendel, 2006). Therefore, while this

protein is needed for nitrate assimilation, it relies on the presence of nitrate reductase. Nitrate reductase (*NIA*) reduces nitrate to nitrite, which is the first step of nitrate assimilation before nitrite is further reduced to ammonium by nitrite reductase (*NIR*) (Tischner, 2000). The two genotypes that increased the most in transcript levels of nitrate uptake genes, Kukri and DH_R039, were both relatively unchanged in the expression of nitrate assimilation genes. As mentioned above, the majority of nitrate assimilation occurs in the shoot, and therefore, the lack of an increase in nitrate assimilation genes in Kukri and DH_R039 may suggest that most of the nitrate is being transported to the shoot. The down-regulation of nitrate assimilation genes in some of the other genotypes may be a result of lower nitrogen uptake rather than due to e[CO_2].

Lastly, genes relating to ammonium assimilation were analysed; *GS1a, NADH-GOGAT, Fd-GOGAT* and *ASN1*. Both GS and GOGAT form the GS/GOGAT cycle, whereby GS forms glutamine from ammonium and glutamate, and GOGAT converts the glutamine into two molecules of glutamate (Masclaux-Daubresse *et al*., 2010). All four ammonium assimilation genes were up-regulated in the two parent genotypes Kukri and RAC0875, although *Fd-GOGAT* was relatively unchanged in RAC0875. This was not the case for the other genotypes, in which ammonium assimilation related genes were largely either relatively unchanged or down-regulated under e[CO_2]. The genes *GS1a* and *NADH-GOGAT* increased in DH_R311, while *Asn1* increased in DH_R095 and DH_R283. Asparagine synthetase contributes to nitrogen assimilation by producing glutamate and asparagine from glutamine and aspartate (Masclaux-Daubresse *et al*., 2010). According to our results, e[CO_2] did not consistently affect any of the ammonium assimilation genes studied across each genotype. Like with the other genes studied, the effect of e[CO_2] on ammonium assimilation genes is likely genotype dependent.

Effect of [CO_2] on root sugar content and photosynthesis

In this study, growth at e[CO_2] for one month increased the content of sucrose, glucose and fructose in the roots of nearly all genotypes studied (Fig. 1). It has been well documented that when plants are grown under e[CO_2], it typically causes an increase in the rate of photosynthesis, which subsequently increases the sugar content of the leaves (Ainsworth and Long 2005). How much of this sugar is exported to the roots is not well understood. Our results showed that, on average, e[CO_2] had a significantly positive effect on the sugar content of roots.

There was a large difference in the response of root sucrose content between the parent genotypes Kukri and RAC0875. While both genotypes had similar sucrose contents under a[CO_2], the sucrose content more than doubled in RAC0875 under e[CO_2], as opposed to Kukri, which only incurred a slight increase under e[CO_2]. This difference is reflected in the photosynthesis rates of each genotype. RAC0875 increased in photosynthesis under e[CO_2] for all measurements taken during the light period, while the rate of photosynthesis for Kukri was affected less under e[CO_2] and slightly declined at two time points (7:00 and 13:00). Photosynthesis rates were similar throughout the light period for RAC0875, while photosynthesis ceased in the dark period. This was similar for Kukri, although there appeared to be photosynthetic activity during the dark period in the morning (4:00), while the first measurements taken during the light period (7:00) were less than those taken throughout the rest of the day. This likely means there was a steady production of sugars, which occurred throughout the day for both genotypes. The remaining genotypes were not assessed for photosynthesis rate due to time constraints. Overall, our results suggest that an increase in photosynthesis during the early growth stage of wheat contributes to an increased supply of sucrose to the roots; however, stimulation of photosynthesis does not occur in all genotypes under e[CO_2] and as such, e[CO_2] may not always increase the root sucrose content.

In contrast with sucrose, glucose levels were, on average, similar for both parent genotypes under a[CO_2] and e[CO_2], while on the other hand, fructose content only increased in Kukri, not RAC0875. In Kukri, most sugars transported to the roots were fructose, rather than sucrose. While sucrose content appeared to be an indicator of the response of photosynthesis to e[CO_2], the same cannot be said for either glucose or fructose. In other studies, sucrose content is usually higher than hexose content in plants grown under e[CO_2] (Grimmer *et al*., 1999; Rogers *et al*., 2004). In our study, the hexose content (glucose + fructose) was typically greater than the sucrose content. RAC0875 appeared to be the only genotype that had greater sucrose than hexoses.

Sugars can lead to changes in gene expression via sugar sensing or signalling pathways (Rolland *et al*., 2006; Thompson *et al*., 2017). The pathway that is induced, and ultimately which gene is affected, may depend on which type of sugar has initiated the pathway (Arroyo *et al*., 2003; Horacio and Martinez-Noel, 2013). Higher fructose content in Kukri may contribute to changes in gene expression that are different to those brought about by the more dominant sucrose levels in RAC0875. The current information about the role of sugar sensing in roots is limited and the effect that e[CO_2] has on these pathways even more so. While this

study does not confirm any specific pathways affected by e[CO_2] and the subsequent increase in root sugar content, it is a first look into observing any correlation between increased sugar content and changes to the gene expression of various nitrogen uptake and assimilation related genes under e[CO_2].

Correlation of sugar content with gene expression

To test whether an increase in sugar content in roots under e[CO_2] was associated with [CO_2] induced changes to expression of nitrogen uptake and assimilation related genes, we performed a correlation analysis between the sugar content of each genotype and the change in gene expression. In general, e[CO_2] increased the sucrose, glucose and fructose content of roots, but the effect on gene expression was less consistent. Sucrose only appeared to affect expression of one gene that was related to ammonium uptake, with an increase in sucrose having a moderately positive correlation with *AMT2.1* expression (Table 3 and Fig. 3). Despite this positive correlation, *AMT2.1* expression in some genotypes was downregulated under e[CO_2]. Increased sucrose in roots has been shown to induce the expression of three ammonium transporter genes (*AMT1.1*, *AMT1.2* and *AMT1.3*) in *Arabidopsis* (Lejay *et al*., 2003) and has also induced transcription of nitrate transporters (*NRT1* and *NRT2* genes) in other studies on both *Arabidopsis* and rice (Lejay *et al*., 1999; Feng *et al*., 2011). We found no significant correlation between sucrose content and expression of the four nitrate genes (*NRT1.1A*, *NRT1.2*, *NRT1.3A* and *NRT2.1*) in our study. In addition, none of the nitrogen assimilation genes appeared to have any significant correlation with sucrose content. Glucose and fructose were only significantly correlated with the expression of *GS1a* and in both cases an increase in sugar correlated with a decrease in gene expression (Table 3 and Fig. 3).

Due to a lack of significant correlations, our results cannot provide sufficient evidence of e[CO_2] controlling sugar sensing pathways which significantly alter the expression of nitrogen uptake and assimilation genes in the roots. However, we have identified two key genes involved in ammonium uptake and assimilation, that are up-regulated (*AMT2.1*) and down-regulated (*GS1a*) in conditions of increased sugar supply under e[CO_2]. Most of the genes studied displayed a variation in their expression under e[CO_2] that showed no correlation with sugar content, which may be due to other variables. It may also be that sugar sensing pathways are affected later in development. It could also be due to a difference in sampling time between genotypes, as gene expression may change throughout the day. While sampling of each plant was done as closely as possible, some plants were inevitably sampled later than others due to

the time it takes to sample a single plant. Overall, our results suggest that an increase in photosynthesis and the subsequent increase in root sugars after one month growth, does not significantly contribute to the change in nitrogen concentration of wheat under e[CO_2] at this growth stage. At this stage, nitrogen uptake and assimilation may be controlled by the plant's growth response to e[CO_2], rather than altered sugar sensing pathways. As such, sugar sensing may instead control nitrogen uptake and assimilation later in the plant's development.

Response of root proteome to [CO_2]

To confirm changes in the abundance of various nitrogen uptake and assimilation related root proteins, we performed a proteomic analysis of the roots for both parent genotypes. There is currently a lack of information regarding the effect of e[CO_2] on the root proteome of wheat, with the only literature available focusing on the grain proteome (Fernando *et al*., 2015). Other studies looking into the effect of e[CO_2] on plant proteomes focus on other species, such as rice and soybean (Bokhari *et al*., 2007; Qiu *et al*., 2008). We analysed the root proteome of wheat in order to further understand how e[CO_2] affects nitrogen uptake and assimilation in roots during early growth and help explain the decline in nitrogen concentration in the grain.

We identified 33 proteins in the roots of Kukri that were significantly affected by e[CO_2], with 21 proteins increasing in abundance. Of these, GS, an enzyme that functions in ammonium assimilation (Masclaux-Daubresse *et al*., 2010), was the only protein involved in nitrogen assimilation (Table 4). This seems to confirm the observation of increased gene expression for *GS1a* that was also seen in Kukri; but although *GS1a* had a greater increase in relative gene expression in RAC0875, GS was found to be lower in abundance from our proteomic analysis of RAC0875, although this wasn't significant (P = 0.052). Two further GS accessions were identified in both Kukri and RAC0875, however, these were not significantly affected by e[CO_2]. In barley, it was identified that *HvGS1_2* expression differs in roots based on the nitrogen concentration in the growth media (Goodall *et al*., 2013). Expression of *HvGS1_2* was higher when plants were supplied with less nitrogen, particularly nitrate. Goodall *et al*., (2013) suggested that this was due to the nitrate assimilation occurring predominantly in the leaves when more nitrogen was taken up. In the present experiment, each pot contained soil from the same source measured to contain 0.4% nitrogen, but minor differences across each pot may have affected the expression of GS. It is unlikely that these minor differences contributed significantly to the variety of responses seen across the genotypes. The change in GS abundance may instead be related to either parent's preference for assimilation of nitrate in

roots or leaves. In addition to GS, three glutamate dehydrogenase proteins were identified in roots of both parent genotypes, however, like most of the GS accessions, these were also not significantly affected by e[CO_2]. These results suggest that ammonium assimilation is unaffected by e[CO_2] in roots of these two genotypes. Our gene expression analysis, on the other hand, indicates an increase in ammonium assimilation gene expression under e[CO_2], with nitrate assimilation related genes being relatively unchanged instead (Table 2). The only nitrate assimilation related protein identified by proteomic analysis was nitrate reductase, which was not significantly affected by e[CO_2]. While nitrate reductase remained relatively unchanged in abundance in both Kurki and RAC0875, the slight decrease in abundance in Kukri and the slight increase in RAC0875 (Table 4 and 5) appears to reflect the expression of nitrate reductase in our gene expression analysis.

Proteomic analysis identified three ammonium transporters (Table 4 and 5) in root samples. Of these transporters, none were significantly affected by e[CO_2] in either parent genotype, remaining relatively unchanged in abundance. Some of these transporters slightly increased in abundance, while others slightly decreased. None of the ammonium transporters analysed by qPCR declined in expression for either Kukri or RAC0875 (Table 2), which could mean that the transporters identified by proteomic analysis were a different type than the three analysed by qPCR. In contrast to the ammonium transporters, there were no nitrate transporters identified by proteomic analysis. This could be due to the high number of uncharacterized proteins identified, which could contain nitrogen transporters uncharacterized in the UniProt database. This may also be the case for nitrogen assimilation proteins not identified by proteomic analysis as well. Results of the gene expression analysis demonstrated that these genes were transcribed and it is unlikely that no protein translation took place.

Conclusion

The results of this study confirm a general increase in sugar content in roots of the majority of genotypes studied. We aimed to identify whether an increase in sugars stimulated by e[CO_2] would lead to altered gene expression of nitrogen uptake and assimilation related genes, however, our results suggest that for most of the genes studied, there is no correlation between sugar content and gene expression. The two genes that did correlate with an increase in sugar (*AMT2.1* and *GS1a*) may indicate that an increase in sugar leads to greater ammonium uptake, but decreases ammonium assimilation. Contrasting this, sugar content did not correlate with any genes involved with either nitrate uptake or assimilation, which may indicate that

regulation of nitrate uptake and assimilation is independent of sugar sensing pathways. While we could not find strong evidence for sugar sensing pathways affected by e[CO_2], some genes were affected by e[CO_2], although this appeared to be genotype dependent.

Supplementary Table 1. Proteins identified as significantly affected by e[CO2] in Kukri.

Supplementary Table 2. Proteins identified as significantly affected by e[CO_2] in RAC0875.

Supplementary Table 3. Root proteome comparison of RAC0875 in relation to Kukri under a[CO_2].

Supplementary Table 4. Root proteome comparison of RAC0875 in relation to Kukri under e[CO_2].

Conflicts of Interest

The authors declare no conflicts of interest.

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679 **Supplementary Table 1.**

Accession No	Protein	Fold change	MW (kDa)	*p*
	Higher abundance proteins			
Q6RUJ2	Glutamine synthetase	1.71	38.73	0.008
Q93W25	Peptidyl-prolyl cis-trans isomerase	1.28	18.391	0.011
A0A1D6B2M0	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	1.49	45.184	0.014
A0A1D6SEV8	Catalase	1.37	56.55	0.017
A0A1D5YEW8	Purple acid phosphatase	1.68	51.701	0.026
W5D591	Small ubiquitin-related modifier	1.59	11.101	0.027
A0A1D5SCJ1	ATP synthase subunit beta	0.99	59.291	0.033
F1DPS1	Early salt-stress induced 2-2	2.51	31.846	0.039
A0A1D5ST71	Gamma-glutamyl hydrolase	1.13	41.489	0.039
W5BEP1	ATP synthase subunit d, mitochondrial	1.99	19.548	0.042
A0A1D5Y681	Protein disulfide-isomerase	1.08	56.016	0.047
W5B0S2	Succinate--CoA ligase [ADP-forming] subunit alpha, mitochondrial	1.71	34.283	0.048
W5C3E3	Pyruvate dehydrogenase E1 component subunit alpha	2.62	45.927	0.002
T1VYS7	Dehydrin WZY1-2	1.54	28.155	0.048
W5EME0	Eukaryotic translation initiation factor NCBP	1.80	23.005	0.016
A0A1D5TXL9	Calcium-transporting ATPase	2.02	116.815	0.019
M5AJV9	Hemoglobin 1	1.89	18.059	0.024
A0A1D5YQI6	Triticain gamma	1.58	36.787	0.037
A0A1D6CZF4	Pyruvate dehydrogenase E1 component subunit alpha	1.88	42.652	0.025
A0A1D5UU04	Peroxidase	1.91	35.388	0.027
A0A1D6CQJ4	60S ribosomal protein L27	2.43	15.583	0.027
	Lower abundance proteins			
A0A1D6AFT9	Chlorophyll a-b binding protein, chloroplastic	-2.84	43.575	0.002
A0A1D5WDA0	Peroxidase	-2.45	36.624	0.011
D8L9Q2	Glucan endo-1,3-beta-glucosidase GII,putative, expressed	-1.74	35.042	0.013
A0A1D5TXK5	Histidinol dehydrogenase, chloroplastic	-1.67	46.697	0.019
P11383	Ribulose bisphosphate carboxylase large chain	-2.45	52.851	0.008
A0A1D5UGR3	6,7-dimethyl-8-ribityllumazine synthase	-1.23	22.432	0.034
W5AS89	Peroxidase	-1.74	36.521	0.036
W5BCG4	Peroxidase	-1.41	36.661	0.037
A0A1D5W070	AP-4 complex subunit epsilon	-1.02	91.314	0.045
F8S6U2	Pathogenesis-related protein 1-12	-2.12	17.596	0.009
A0A1D5V9D5	Reticulon-like protein	-1.40	25.894	0.048
W5B1M0	Peroxidase	-1.94	32.785	0.010

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682 **Supplementary Table 2.**

Accession No	Protein	Fold change	MW (kDa)	*p*
	Higher abundance proteins			
A0A1D6DHK4	Cysteine proteinase inhibitor	1.36	11787	0.004
A0A1D6BIA4	Reticulon-like protein	1.71	25919	0.010
A0A1D6S9P2	Annexin	1.96	35372	0.010
W5CQ97	Cysteine proteinase inhibitor	1.21	26749	0.012
A0A1D5SV82	Nascent polypeptide-associated complex subunit beta	1.06	17950	0.012
W5H151	S-(hydroxymethyl)glutathione dehydrogenase	0.72	40657	0.035
Q8GVD3	Thioredoxin	0.69	12694	0.038
A0A1D6C4A1	Thiamine thiazole synthase, chloroplastic	0.73	42592	0.042
A0A1D6RR58	Glutamate decarboxylase	1.16	53899	0.044
A0A1D5VHL0	Phospholipase D	1.12	92062	0.045
A0A1D6RGU8	Importin subunit alpha	0.69	57694	0.047
D2KZ08	Aminotransferase	0.98	55546	0.047
W5ETU1	Fructose-bisphosphate aldolase	1.92	41975	0.045
A0A1D6D5D4	Plastocyanin	1.73	15603	0.033
Q9SAU8	HSP70	2.22	71031	0.018
A0A1D6AMM3	Aspartate aminotransferase	2.10	49786	0.022
	Lower abundance proteins			
A0A1D5WVK9	Carboxypeptidase	-1.11	51878	0.011
P11383	Ribulose bisphosphate carboxylase large chain	-1.51	52851	0.030
A0A1D5SQY8	Tubulin alpha chain	-0.69	45863	0.043
A0A1D5V9D5	Reticulon-like protein	-1.35	25894	0.034
Q00434	Oxygen-evolving enhancer protein 2, chloroplastic	-1.49	27270	0.034
A0A1D5XUK2	Lipoxygenase	-1.83	94108	0.046
A0A1D6AWP4	Ferredoxin--NADP reductase, chloroplastic	-1.24	40152	0.032
A0A1D5YHQ7	Chlorophyll a-b binding protein, chloroplastic	-1.92	24161	0.018
A0A1D5S805	60S ribosomal protein L36	-1.71	12557	0.035

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690 **Supplementary Table 3.**

Accession No	Protein	Fold change	MW (kDa)	*p*
	Higher abundance proteins			
A0A1D5TXK5	Histidinol dehydrogenase, chloroplastic (HDH) (EC 1.1.1.23)	1.92	46.697	0.000
A0A1D6DCS0	Ribokinase (RK) (EC 2.7.1.15)	0.53	36.971	0.001
W5EMA7	Proteasome subunit alpha type (EC 3.4.25.1)	0.28	27.436	0.001
A0A1D5VKI1	Cysteine synthase (EC 2.5.1.47)	0.15	40.934	0.001
R9W6A6	ER molecular chaperone	0.22	73.186	0.002
Q1XIR9	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside beta-D-glucosidase 1a, chloroplastic (EC 3.2.1.182) (Beta-glucosidase 1a) (TaGlu1a) (EC 3.2.1.21)	0.83	64.508	0.002
W5I774	Sucrose synthase (EC 2.4.1.13)	1.05	92.4	0.005
P38076	Cysteine synthase (EC 2.5.1.47) (CSase A) (O-acetylserine (thiol)-lyase) (OAS-TL A) (O-acetylserine sulfhydrylase)	0.33	34.114	0.006
A0A1D5YM24	Cysteine synthase (EC 2.5.1.47)	0.68	40.452	0.009
W5C3H4	Proteasome subunit alpha type (EC 3.4.25.1)	0.39	25.828	0.011
W5ERM8	Proteasome subunit beta type (EC 3.4.25.1)	0.32	23.198	0.015
A0A1D6B0C9	Aminopeptidase (EC 3.4.11.-)	0.10	98.207	0.020
A0A1D6RR58	Glutamate decarboxylase (EC 4.1.1.15)	0.07	53.899	0.021
A0A1D5SM88	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	0.08	52.795	0.023
A3KLL4	Malate dehydrogenase (EC 1.1.1.37)	0.86	35.486	0.027
A0A1D6C4Q5	60S acidic ribosomal protein P0	0.47	33.77	0.029
W5FWT6	Aldehyde dehydrogenase 7B1	0.37	54.361	0.030
Q43206	Catalase-1 (EC 1.11.1.6)	0.29	56.808	0.036
A0A1D6BEM4	Peroxidase (EC 1.11.1.7)	0.95	33.623	0.037
A0A1D5WER2	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	0.93	50.575	0.040
A0A1D5SZ32	(1,31,4) beta glucanase	0.93	31.492	0.040
Q5I7K5	Ribosomal protein P1	0.57	11.193	0.041
	Lower abundance proteins			
A0A1D6S518	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	-0.20	51.664	0.000
A0A1D6AKZ2	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	-0.25	32.197	0.000
I3RN54	Inorganic pyrophosphotase	-1.07	24.287	0.001
W5F826	Proteasome subunit beta type (EC 3.4.25.1)	-0.20	24.333	0.002
A0A1D6CV93	Peroxidase (EC 1.11.1.7)	-0.82	38.416	0.002
W5C3E3	Pyruvate dehydrogenase E1 component subunit alpha (EC 1.2.4.1)	-1.59	45.927	0.003
A0A1D5UU04	Peroxidase (EC 1.11.1.7)	-2.13	35.388	0.003
P11383	Ribulose bisphosphate carboxylase large chain (RuBisCO large subunit) (EC 4.1.1.39)	-1.28	52.851	0.005

	W5D591	Small ubiquitin-related modifier (SUMO)	-2.10	11.101	0.007
	W5D0E3	Fructose-bisphosphate aldolase (EC 4.1.2.13)	-0.62	38.895	0.007
	A0A1D5XZ43	Beta-galactosidase (EC 3.2.1.23)	-1.39	92.126	0.008
	A0A1D5ST71	Gamma-glutamyl hydrolase (EC 3.4.19.9)	-2.09	41.489	0.010
	A0A173FEH2	TSK1 protein	-1.25	19.059	0.011
	A9EEM6	Triticain beta 2	-1.10	50.417	0.015
	W5H3N4	Proteasome subunit alpha type (EC 3.4.25.1)	-0.01	31.311	0.020
	W5GBW4	S-adenosylmethionine synthase (EC 2.5.1.6)	-1.12	43.252	0.029
	Q2QKB4	Splicing factor U2af large subunit B (U2 auxiliary factor 65 kDa subunit B) (U2 small nuclear ribonucleoprotein auxiliary factor large subunit B) (U2 snRNP auxiliary factor large subunit B)	-1.52	60.586	0.031
	Q69G22	Pyridoxal kinase	-0.71	34.241	0.037
	A0A1D5XY50	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-)	-1.85	46.858	0.037

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693 **Supplementary Table 4.**

Accession No	Protein	Fold change	MW (kDa)	*p*
	Higher abundance proteins			
W5BEP1	ATP synthase subunit d, mitochondrial	0.62	19.548	0.000
A0A1D5SQY8	Tubulin alpha chain	0.63	45.863	0.000
A0A1D5ZA24	Clathrin heavy chain	0.11	189.889	0.006
A0A1D5XQ85	Alanine--tRNA ligase (EC 6.1.1.7) (Alanyl-tRNA synthetase) (AlaRS)	0.14	109.594	0.023
A0A1D6S4L2	Carboxypeptidase (EC 3.4.16.-)	-0.92	51.327	0.025
W5E2W7	40S ribosomal protein SA	0.85	33.271	0.030
A0A1D6B0C9	Aminopeptidase (EC 3.4.11.-)	0.22	98.207	0.031
A0A1D6B308	S-adenosylmethionine synthase (EC 2.5.1.6)	0.79	42.766	0.041
	Lower abundance proteins			
A0A1D5YL72	Elongation factor 1-alpha	-0.45	47.806	0.000
Q0Q0I7	Heat shock protein 90	-0.33	80.46	0.000
A0A1D6S518	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	-0.32	51.664	0.006
A0A173FEH2	TSK1 protein	-0.44	19.059	0.024
A0A1D6C4Q5	60S acidic ribosomal protein P0	-0.04	33.77	0.034
D2KZ08	Aminotransferase	-1.53	55.546	0.035
A0A1D5TM94	Carboxypeptidase (EC 3.4.16.-)	1.24	55.914	0.039
A0A1D6AQV8	Aconitate hydratase (Aconitase) (EC 4.2.1.3)	-1.75	106.306	0.047

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Chapter 6

Discussion, Conclusion and Future Directions

Wheat grown under atmospheric CO_2 concentrations predicted for the end of this century has repeatedly been shown to suffer a decline in GPC (Taub et al., 2008; Myers et al., 2014). Due to the importance of wheat as a staple food in many people's diets, and given the inevitable increase in global population, understanding the mechanisms behind GPC decline will be crucial in order to successfully maintain the nutritional value of wheat as the [CO_2] of the atmosphere increases. Progress has been made into identifying key processes involved with this decline, such as the finding that e[CO_2] inhibits nitrate assimilation in leaves (Bloom et al., 2014; Bahrami et al., 2017), however, we are yet to understand the other processes that lead to lower GPC. The most apparent cause of GPC decline is that the greater availability of CO_2 leads to an increase in grain biomass, which dilutes the protein content. However, as e[CO_2] was found to inhibit nitrate assimilation, this suggests there is more at play than only the dilution of protein in the grain.

Increasing the application of nitrogen as fertiliser has shown promise in mitigating the effect of e[CO_2], however, this is an unreasonable solution due to the increases in costs for farmers as well as nitrate leaching. Therefore, the development of wheat cultivars which are capable of responding positively to the rising [CO_2], in terms of both GPC and biomass, appears to be the best course of action. To achieve this goal it will be beneficial to understand the mechanisms responding to e[CO_2], which lead to GPC decline. To that end, this study focussed on investigating several areas likely to be linked with the control of GPC response to e[CO_2]. Firstly, we studied whether there were any different responses in GPC to e[CO_2] between three types of wheat (tetraploid, hexaploid and synthetic hexaploid). Secondly, we focussed on various nitrogen and biomass related traits of hexaploid wheat genotypes to identify which traits had the most involvement in determining the GPC response to e[CO_2], in order to further elucidate the mechanisms behind GPC decline. Finally, we investigated whether sugar content of roots increases in the early growth stage of hexaploid wheat and whether a change in sugar content affects the transcription of various nitrogen uptake and assimilation related genes. Each of these objectives contributes to the overall knowledge of the wheat GPC response to e[CO_2].

6.1 Significant findings of the study

In Chapter 3, I analysed the GPC and biomass of nineteen wheat genotypes grown under both ambient and elevated [CO_2], consisting of tetraploids, hexaploids and synthetic hexaploids.

This chapter aimed to identify whether there were separate mechanisms found in each wheat type that resulted in a different GPC in response to e[CO_2]. Unfortunately, this study found no significant difference between the GPC of each wheat type. It was observed that each wheat type contained genotypes which increased in GPC in response to e[CO_2] as well as genotypes which decreased. This indicates that the mechanisms which are affected by e[CO_2] in tetraploids may be the same as those in hexaploids and synthetic hexaploids. While this study did not confirm any wheat type-specific mechanisms, it demonstrated that some genotypes in each wheat type are capable of responding positively to e[CO_2] in regards to GPC. From the genotypes studied, it appears that the GPC response was genotype dependent, rather than wheat type dependent. Additionally, to my knowledge this study was the first to investigate the GPC of synthetic hexaploids under e[CO_2] and demonstrated that, although not significant, none of the three synthetic hexaploid genotypes analysed incurred a GPC decline as severe as some of the tetraploid or hexaploid genotypes. Biomass data were also compared across each wheat type. For most genotypes there was a larger increase in grain biomass than shoot biomass, however, this was not the case for two of the synthetic hexaploids, and this meant that while the HI increased for most genotypes, it decreased in the two synthetic hexaploids. Harvest index did not have any correlation with GPC in this study, which is in opposition to results found in Chapter 4. One of the main hypotheses for the cause of GPC decline is biomass dilution, however, these results do not support biomass dilution as the sole cause of the decline.

Chapter 4 focussed on identifying the contribution of different traits involved with nitrogen flow and plant biomass to the GPC of wheat under e[CO_2]. For this experiment, it was decided to make bread wheat the focus. Plants were grown until maturity under both ambient and elevated [CO_2] and the nitrogen content and biomass were analysed. We found that of the traits analysed, there was a clear correlation with the two aspects of NUE. On the one hand, NUtE correlated negatively with GPC, while on the other, NUpE correlated positively with GPC. In addition, HI correlated negatively with GPC. Taken together, the negative correlation between GPC and both NUtE and HI indicates the plant's biomass as a main contributor to GPC under e[CO_2]. In the previous paragraph one of the conclusions to Chapter 3 was that biomass dilution alone cannot explain the decline in GPC. The results of Chapter 4 expand on this idea by indicating that, while the grain biomass is an important factor in GPC decline, it is in how the nitrogen flow of the plant is affected that results in an increase or decrease in GPC. The positive correlation between NUpE and GPC showed that the efficiency of a plant to acquire the nitrogen from the soil will affect how much nitrogen ends up in the grain. Additionally, this

experiment supported the conclusion from Chapter 3 that the response of wheat to e[CO_2] is genotype dependent. By looking at each genotype individually it was evident that different aspects of nitrogen flow are affected in each genotype, and those that increased in GPC each did so through a different strategy. An interesting observation was that the parent genotype RAC0875 increased in GPC mainly due to a lack of increase in grain biomass, while the genotype DH_R097 appeared to increase in GPC due to a large uptake of nitrogen post-anthesis.

In Chapter 5, the focus shifted to the roots of wheat grown for one month under e[CO_2] to investigate the potential role of sugar content on regulating the uptake and assimilation of nitrogen in young wheat roots. While this experiment did not directly explore a link between sugar content in roots of young wheat plants and the GPC of mature plants, the aim was to explore potential mechanisms affected by e[CO_2] that may contribute to the overall nitrogen status of the plant, and thus the GPC. After long-term exposure, e[CO_2] appears to down-regulate genes involved with photosynthesis through sugar sensing pathways (Jang and Sheen 1994), therefore, it is likely that other sugar sensing pathways are affected by e[CO_2] as well. Our results found that of the 16 genes analysed, only two genes appeared to be potentially regulated by a change in sugar content under e[CO_2]. Elevated [CO_2] caused an increase in sugar content for all three sugars (sucrose, glucose and fructose) across almost all genotypes. The ammonium transporter gene *AMT2.1* positively correlated with sucrose content and the glutamine synthetase gene *GS1a* negatively correlated with both glucose and fructose content. This suggested that these two genes were potentially regulated by sugars, however, a definite connection could not be made from this study. The affect that these three sugars have on these genes could indicate that greater sugar content may increase ammonium uptake, but decrease ammonium assimilation in roots. On the other hand, the lack of any significant correlation between sugar content and the nitrate genes studied could indicate no involvement of sugar sensing pathways in regulating nitrate uptake and assimilation. It may be that the effect increased sugar content has on wheat roots may change throughout the plant's development. At any rate, in this study it appears that the altered gene expression observed across genotypes for most of the genes may be due to other factors affected by e[CO_2], rather than sugar content. Furthermore, little information could be gleaned from the proteomics analysis, which only identified one protein relating to nitrogen assimilation that was significantly affected by e[CO_2]. Glutamine synthetase increased in abundance in Kukri, while it decreased in RAC0875. When compared with the gene expression analysis of *GS1a*, the proteomics data

supports the increase in *GS1a* seen in Kukri, but conflicts with the decline in expression in RAC0875, although the latter result was not significant (P = 0.052). The proteomics results suggest that e[CO_2] does not significantly alter proteins involved in nitrogen uptake or assimilation and as such, cannot confirm the results observed for the gene expression analysis. From this experiment, we cannot conclude any significant impact by e[CO_2] on the regulation of nitrate uptake and assimilation after one month growth, particularly due to altered sugar sensing pathways, however, e[CO_2] may alter sugar sensing pathways related to ammonium uptake and assimilation. At this early stage of growth, nutrient acquisition may be regulated mostly by the plant's growth rather than sugar sensing. Nitrogen uptake and assimilation may be affected by sugar sensing pathways further along in the plant's development, which should be investigated in future experiments.

6.2 Future directions

The experiments discussed in this thesis were each undertaken in order to further elucidate the mechanisms behind the response of GPC to e[CO_2]. Prior research in this area has provided some insight into how e[CO_2] affects wheat GPC, however, more work needs to be done in order to understand this process and develop genotypes suitable for future CO_2 conditions.

Our study indicated that wheat type had no noticeable effect on the GPC response of wheat to e[CO_2], however, we acknowledge that the number of genotypes studied was inconsistent across each wheat type and was insufficient to make this conclusion. Our study was limited to the genotypes available at the time of the experiment, and therefore, it would be beneficial to screen a larger number of genotypes for each wheat type, not only to contribute to the determination of wheat type specific responses, but also to identify more genotypes which respond positively to e[CO_2] in terms of GPC. Identifying genotypes with a variety of responses to e[CO_2] may be important in order to further understand the mechanisms of GPC decline and will identify genotypes that could be targets of breeding programs aimed at developing high protein wheat varieties under e[CO_2].

The results of the second study indicated that the mechanisms which lead to a change in GPC under e[CO_2] may be affected to varying degrees across different genotypes. As such, future studies should take this into account when investigating the mechanisms of GPC decline. For example, although some studies have found nitrate assimilation to be inhibited by e[CO_2] (Bloom et al., 2014; Bahrami et al., 2017), this may not be the case in all genotypes. The genotype DH_R097 could be further explored to gain a better understanding of why this

genotype was able to increase both GPC and grain biomass under e[CO_2]. As post-anthesis nitrogen uptake appeared to be important for increasing GPC, future studies could look at the gene expression of nitrogen transporters throughout the lifecycle of the plant. It may also be important to identify which form of nitrogen (nitrate or ammonium) is most dominantly utilised by the plant, as the ratio of nitrate to ammonium taken up by the plant appears to contribute to GPC under e[CO_2] (Fernando et al. 2017). As nitrogen remobilisation is the main contributor to GPC, the timing of senescence should also be recorded in future studies as this may help to determine the GPC response in some genotypes. As the response of GPC appears to be genotype dependent, future research should focus on identifying how gene expression is differentially expressed across a range of genotypes with differing GPC responses. From there, we need to identify what is signalling the up- or down-regulation of these genes in response to e[CO_2].

The third experiment in this study explored sugars as the potential signalling molecule to initiate a change in gene expression in wheat roots. While this study did not provide sufficient evidence for gene expression being altered by increased sugar content, sugar sensing could still play a role in determining GPC under e[CO_2]. This experiment could be expanded upon by firstly being repeated at different stages throughout the plant's lifecycle as well as in other organs, such as the leaves. Also, it's known that sugars can affect gene expression by altering the regulation of plant hormones (Thompson et al. 2017). Therefore, in addition to measuring sugar content and nitrogen metabolism related gene expression, hormone levels could also be analysed. Furthermore, while analysing the expression of genes in roots, it would be beneficial to determine the ratio of nitrate to ammonium taken up. This would help to support the gene expression data related to nitrate and ammonium transporters. Each of these further experimental steps will contribute to elucidating the role of sugar sensing in controlling the nitrogen status of the plant and ultimately the GPC. The gene expression work of this study could further be expanded to include an investigation of the expression of genes in the developing grain, such as genes involved with protein synthesis, but also other genes responsible for grain development and filling. These results could be compared with the GPC of the genotype to identify the core genes affected by e[CO_2] during the development of the grain. Finally, another route that could be taken is protein engineering as a method to alter the ability of gluten-forming proteins to form dough. As dough strength and bread volume decreases under e[CO_2] (Fernando *et al.* 2015), it may be worth targeting the proteins responsible for these qualities in addition to breeding wheat with higher GPC under e[CO_2].

6.3 Conclusion

Understanding how e[CO_2] controls the GPC of wheat is crucial in order to prevent the nutritional decline in wheat predicted to occur before the end of the century. The results of Chapter 3 indicate that, when it comes to the extent that e[CO_2] affects GPC, it will vary depending on genotype, and thus it will be important in the future to determine which wheat genotypes will respond in a positive manner. These genotypes can then be used in breeding programs to develop improved varieties that increase in GPC under future environments. Chapter 4 described how the GPC response to e[CO_2] is the result of a combination of various traits, which each respond differently to e[CO_2] depending on genotype. For example, increased PANU may improve GPC in some genotypes, while increased nitrogen remobilisation may improve GPC in others. Conversely, some genotypes may decline in GPC due to lower PANU or nitrogen remobilisation. This study then found in Chapter 5 that, while sugar sensing does not appear to play a significant role in regulating the complete nitrogen uptake and assimilation pathway during the early growth stage of wheat development, change in expression of *AMT2.1* and *GS1a* was associated with increased sugar content in roots. Sugar sensing should be investigated at later stages, as the regulation of the whole nitrogen uptake and assimilation pathway may change throughout plant's development. This project contributes to the current knowledge gap in our understanding of the mechanisms behind GPC response to e[CO_2], however, much work remains to be done to achieve sufficient understanding of the effect of e[CO_2] on wheat and prevent the nutritional decline predicted for the future.

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