New insights into the impacts of elevated CO₂, nitrogen, and temperature levels on the regulation of C and N metabolism in durum wheat using network analysis

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Abstract

The use of correlation networks and hierarchical cluster analysis provides a framework to organize and study the coordination of parameters such as genes, metabolites, proteins and physiological parameters. In this work, we analyze 142 traits from primary C and N metabolisms, including biochemical and gene expression analyses, in a range of 32 different growth conditions (various [CO₂] levels, temperatures, N supplies, growth stages and experimental methods). To test the integration of primary metabolism, specially under climate change, we investigated which C and N metabolic traits and transcript levels are correlated in durum wheat flag leaves using a correlation network and a hierarchical cluster analysis. There was a high amount of positive correlations between traits involved in a wide range of biological processes, suggesting a close and intricate coordination between C-N metabolisms at biochemical and transcriptional levels. Transcript levels for genes related to N uptake and assimilation were especially coexpressed with genes belonging to the respiratory pathway, highlighting the coordination between the synthesis of organic N compounds and provision of energy and C skeletons. Also involved in this coordination were Rubisco and nitrate reductase activities, which play a key role in the regulation of plant metabolism. Carbohydrate accumulation was linked with a down-regulation of photosynthetic and N metabolism genes and nitrate reductase activity. Based on the amount of connections between nodes, network exploration facilitated the identification of some traits that can be biologically relevant during plant abiotic stress tolerance, as most of them are involved in limiting steps of plant metabolism.

Keywords

Carbon metabolism; climate change; coexpression; durum wheat; correlation network; nitrogen metabolism

1 Introduction

2
3 Plant responses to changes in environmental growth conditions include complex biological processes and
4 involve a large number of physiological and metabolic components. A powerful tool to understand the
5 mechanisms controlling such processes operating in plant metabolism dealing with a large amount of data is the
6 use of correlation networks. These are rapidly spreading because of the technological advances in the last
7 decades that allow the generation of a huge amount of data with omic approaches [1] and high-throughput
8 phenotyping platforms [2]. Additionally, they can be easily constructed in three basic steps: the calculation of all
9 pairwise correlations, the selection of significant correlations based on a threshold and the visualization of the
10 data using different softwares, such as R environment [3] or Cytoscape [4]. Hierarchical cluster analysis is also a
11 valuable resource to complement network analysis because it defines groups of observations that are similar [2].
12

13 Most of the network studies have evaluated the correlations between one type of biological data, such as genes,
14 metabolites, proteins or physiological parameters [5-7], but few of them have integrated different data, i.e.
15 physiological parameters and gene expression [8], enzyme activities and metabolite levels [9], or quantitative
16 trait loci (QTLs) and sensory and metabolic traits [10]. Such research is currently best done in model plants, like
17 Arabidopsis, rice or poplar [1, 7, 11], but much less effort has been dedicated to crop species as maize [12, 13],
18 barley [14], bread wheat [6] and durum wheat [15]. This cereal is one of the most widespread crops in the
19 Mediterranean basin used mainly for the production of pasta and accounting for approximately 8% of the global
20 wheat production [16]. The Mediterranean climate is already constraining durum wheat yield by both low
21 seasonal rainfall and high temperatures, especially at late growth stages [16], and climate change will probably
22 aggravate these limitations. According to climate change projections, temperature is predicted to rise in a range
23 depending on the scenario, in association with higher water limitation in the Mediterranean basin [17].
24 Moreover, [CO₂] is expected to increase in a range from 794-1142 $\mu\text{mol mol}^{-1}$ by the end of the 21st century
25 [17]. While above optimal temperatures could inhibit photosynthesis and plant growth, elevated [CO₂] is known
26 to stimulate CO₂ assimilation by an inhibition of photorespiration and an increase of Rubisco's substrate for
27 carboxylation, [18]. Furthermore, plant responses to both factors are influenced by N supply during the crop
28 cycle, being negatively affected by N deficiency [18, 19]. In this context, the performance of C and N
29 metabolisms, which constitute the backbone of primary metabolism under climate change in crops such as
30 durum wheat, is a matter of concern.
31

32 In this paper, we focus on identifying connections of C and N metabolic parameters and transcript levels in flag
33 leaves of durum wheat grown under different environmental conditions, anticipated with climate change, in
34 order to improve the understanding how the future climatic scenario acts on primary metabolism. For this aim,
35 we examine the changes in expression of C and N related genes and biochemical parameters in three different
36 experiments, to perform a network and a hierarchical cluster analyses. Thus, we provide new evidence about the
37 coordination between C and N metabolism in durum wheat with special emphasis on future climate change and
38 suggest several candidate regulatory traits.
39

Material and methods

Experimental setup

The present study utilized new and already published data from three experiments as is detailed below (**Table A.1**). The experiments were carried out with durum wheat plants (*Triticum durum* Desf. cv. Regallo) provided by Agromonegros S.A. (Spain) under various growth conditions, which included two hydroponic cultures and a field trial varying levels of atmospheric [CO₂], temperature and N supply, and different growth stages. The experimental design of each study is described in Vicente *et al.* [18] and summarized in **Fig. 1**.

Briefly, the first hydroponic culture was carried out in two controlled environment chambers, one for ambient CO₂ (390 $\mu\text{mol mol}^{-1}$) and the other for elevated CO₂ (700 $\mu\text{mol mol}^{-1}$), maintaining a daily cycle of 16 h at a photon flux density of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 60% relative humidity and a day/night temperature of 20/15 °C. Wheat seedlings were transferred to 10-L opaque plastic pots with aerated nutrient solution containing 0.5 or 5.0 mM NO₃⁻. The nutrient solution was renewed periodically (one, two or three times per week depending on the plant growth) and the pH adjusted daily. Flag leaves were harvested at anthesis and grain filling stages after 24 h of incubation on a fresh nutrient solution. A second hydroponic culture was conducted with a similar procedure as that described for the first experiment. A more severe nitrate deficiency was imposed by decreasing the nitrate concentration in the nutrient solution (0.25 and 2.5 mM NO₃⁻) and by changing the solution less frequently than in the previous hydroponic experiment. Flag leaves were harvested after 4 or 24 h of incubation on a fresh nutrient solution at anthesis.

The field trial was conducted at the Muñovela IRNASA farm, Salamanca, Spain (40°95'N, 5°5'W) in a clay-sand soil under Mediterranean climate conditions during the crop season 2007/2008 using temperature-gradient chambers. Plants were grown under ambient (370) and elevated CO₂ (700 $\mu\text{mol mol}^{-1}$), ambient temperature and 4 °C warmer, and two N levels (15 and 140 kg N ha⁻¹ supplied as Ca(NO₃)₂). At ear emergence and anthesis flag leaves of different plants per factor combination were harvested.

In summary, new measurements carried out in this study correspond to the second hydroponic culture after incubating the plants for 4 h in 0.25 and 2.50 mM NO₃⁻ nutrient solution as well as for the field trial at ear emergence, while the other factor combinations were evaluated in preceding studies [18, 20, 21] (**Table A.1**).

Biochemical and gene expression analyses

Different biochemical parameters and transcript levels were determined in this study and in the preceding ones using the same protocols and equipment. The list of the traits analyzed and their abbreviations are included in **Table A.2**. N content was determined in dry samples of flag leaves after Kjeldahl digestion and a specific enzymatic method [22]. The rest of the measurements were carried out in flag leaves frozen in liquid N₂ during the sampling and kept at -80 °C until the analyses. Glucose, fructose, sucrose, fructans, starch and total soluble carbohydrates (TSC), calculated as the sum of hexoses in all of them, were measured spectrophotometrically

according to Morcuende *et al.* [23]. Chlorophylls were assessed according to Pérez *et al.* [24]. Total amino acids were analyzed after ethanol/water extraction using the ninhydrin method [25]. Soluble and ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) protein contents and Rubisco activity were determined according to Pérez *et al.* [26], while nitrate reductase (NR) activity was measured as described in Morcuende *et al.* [23]. Gene expression of C and N metabolism-related genes was evaluated by using the qRT-PCR platform described by Vicente *et al.* [18]. There were four biological replicates per treatment combination in both hydroponic cultures and six in the field trial for biochemical parameters, and three for gene expression analysis.

Construction of correlation network and hierarchical cluster analysis

All data generated in the three experiments were used to construct a correlation network. Firstly, data were processed by Z-score normalization using R v3.3.1 environment [3]. A correlation matrix between all trait pairs was generated using the package *gplots* and the Spearman method, while a plot for the sampling distribution of Spearman's r was made in GenStat v6.2 (VSN International Ltd, UK). The significance threshold for correlations between traits was set at $r > 0.7$ for positive correlations and $r < -0.7$ for negative correlations, with a P value < 0.001 in both cases. Graphical visualization of the correlation network (considering only the significant correlations) was performed in Cytoscape v3.4.0 software [4] in which a node corresponds to a trait and an edge between two nodes corresponds to a significant correlation. Hierarchical cluster analysis of all traits was performed in R environment using the *pvclust* package with a number of bootstrap replications of 1,000. Clusters were considered statistically significant at $P < 0.05$.

Results and discussion

A set of 32 factor combinations from three experiments, including different $[\text{CO}_2]$, temperatures, N supplies, growth stages and culture methods, were evaluated (**Fig. 1** and **Table A.1**). We analyzed in each factor combination the content of different C and N-rich compounds, Rubisco and NR enzyme activities, as well as the transcript levels of 125 genes involved in C and N metabolism, in flag leaves of durum wheat (**Table A.2**). Results from previous studies [18, 20, 21] were complemented with new analyses in order to include more data of different growth conditions. Moreover, all data used in this manuscript have been obtained with the same protocols and equipment in our group. Initially, a correlation matrix of 142 traits was built in R environment, obtaining a total of 10,011 correlations based on Spearman's rank correlation coefficient (**Fig. 2**). The distribution of the correlation coefficients obtained showed that most of them were positive rather than negative (**Fig. 2**). Based on Spearman's r and P value, we selected a total of 1,230 significant correlations: 1,164 positive (95%) and 66 negative (5%). These were represented as edges in the network using Cytoscape software, while nodes represented the 125 traits which were significantly correlated with at least another trait (**Fig. 3**). In the next paragraphs we detail the most relevant associations found between parameters of C and N metabolism.

N uptake and assimilation is integrated with C metabolism, including photosynthesis, photorespiration and respiration [19, 27]. This coordination is necessary for an efficient synthesis of organic N compounds using C skeletons, energy provided by respiration and inorganic N from the soil. In our study an overview of the

network (**Fig. 3**) showed that most of the traits considered in this experiment that belong to C and N metabolism were positively correlated, including biochemical parameters and transcript levels. Indeed, each trait is often associated with a high number of traits from different pathways. This fact clearly highlights that both C and N metabolisms are tightly coordinated at biochemical and transcriptional level [6, 7, 27], even considering the environmental conditions anticipated for climate change such as elevated CO₂ and high temperatures.

In general, genes that belong to the same pathway or collaborate in a shared function were coexpressed (**Fig. 3**), in agreement with Wei *et al.* [5] in *Arabidopsis thaliana*. Particularly, the genes that encode enzymes associated with primary N assimilation, such as NR, nitrite reductase (NiR; see **Table A.2** for a glossary of abbreviations), glutamate synthase (GOGAT) and glutamine synthetase (GS), were coexpressed with each other and with some N transporters, given the existing coordination between soil N uptake and subsequent assimilation into organic N compounds [28]. Among these genes, GOGAT isoforms (NADH- or ferredoxin-dependent) had a significantly higher number of correlations than other N metabolism-related genes. Expression of both isoforms was correlated with parameters of different biological functions (N metabolism, light reactions, carbohydrate metabolism, etc.), suggesting key roles for these isozymes. Furthermore, NADH-dependent GOGAT was correlated with NR activity and seems to be negatively regulated by carbohydrate metabolism.

Relative to N uptake, some ammonium and nitrate transporters were related among them and with several metabolic pathways, although the low affinity nitrate transporter NRT1.1 (isoforms A and B) was largely the most connected node (**Fig. 3**). It is an orthologous gene of *NRT1.1* in *A. thaliana*, which encodes a protein that acts as dual-affinity nitrate transporter and a nitrate sensor [29]. The relationships between the transcript levels of this transporter with those involved in photosynthesis, carbohydrate and N metabolisms suggest a central role in leaves of durum wheat for this transporter, which could be involved in the induction of N metabolism genes, in agreement with other studies [29, 30]. A surprising result is the high number of negative correlations (12) of transcripts levels for the ammonium transporter AMT2.1 with transcripts for Calvin-Benson cycle, carbohydrate metabolism, respiration, as well as with total Rubisco activity. Although we have observed a regulatory crosstalk between C and N metabolisms, this transporter could be related with NH₄⁺ transport within the plant more than with N uptake [31]. In our study, the Dof1 transcription factor was coexpressed with 21 genes related to N metabolism, photosynthesis and respiration. The Dof1 transcription factor has been reported to be involved in the activation of C and N metabolism-related genes in wheat [32]. Yanagisawa *et al.* [33] also disclosed a central role for Dof1, linking the up-regulation of the maize Dof1 gene in transgenic Arabidopsis to an enhancement of N assimilation. This finding could be relevant in future climate change scenario since elevated CO₂ often leads to a decline in plant N [18, 21].

It is well known that NR is regulated at transcriptional and post-translational level by many signals [34]. Although transcript levels for NR were only coregulated with those for NiR (**Fig. 3**), the NR activity was positively coordinated with many factors, especially with transcript levels for N-metabolism enzymes. Also NR activity positively correlated with the amino acid content and with some genes for photosynthetic light reactions, Calvin-Benson cycle, carbohydrate metabolism and TCA cycle. Similar to NR activity, positive correlations were found between transcript levels for genes linked to N and respiratory metabolisms, i.e.

glycolysis and tricarboxylic acid (TCA) cycle. Taking together all results concerning N metabolism traits, we observed a high coordination between all components of N metabolism at the gene expression level, enzyme activity and metabolic products for an efficient N uptake and assimilation, in accordance with previous studies [18, 28, 35], and with respiratory metabolism [36]. We propose key roles in N metabolism for genes encoding GOGAT isoforms, NRT1.1 and Dof1, at least under the growth conditions used in this study. NR activity, more than the expression of the enzyme, was coordinated with many genes of primary metabolism, thus adjusting amino acid biosynthesis to the supply of C skeletons provided by photosynthesis.

In this study, Calvin-Benson cycle was the pathway with a higher number of significant correlations at transcript level (**Fig. 3**), similar to earlier results in *A. thaliana* [5]. The genes involved in the Calvin-Benson cycle were also highly connected with other pathways, particularly carbohydrate metabolism (19.4% of the total genes correlated with Calvin-Benson cycle genes), N metabolism (19.4%), glycolysis (17.9%), light reactions and energy (16.4%), and photorespiration (13.4%). The activation state of Rubisco is regulated by the chloroplastic protein Rubisco activase (RCA) [37]. Transcript levels for RCA were closely correlated with transcript levels for enzymes involved in Calvin-Benson cycle and photorespiration. Rubisco catalyzes the first and key step of both pathways [38]. Therefore, the activation of Rubisco by RCA could play a significant role, with a special emphasis on future climate conditions, due to the observed changes in *RCA* expression reported under heat stress [37]. The assembly of Rubisco subunits requires coordinated control by chloroplastic and cytosolic processes, in which proteins known as chaperonins are involved, especially the chaperonin 60 (*cpn60*) [39]. We observed a coexpression between *cpn60* and *rbcL*, although *cpn60* expression was also related to chloroplastic genes, confirming that this protein could be involved in the folding of a large number of chloroplast proteins [40]. Two Calvin-Benson enzymes, phosphoribulokinase (PRK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), form a multi-enzyme complex with the protein CP12 in darkened leaves, which is rapidly dissociated at light [41]. In this study, *CP12* was coexpressed with the genes encoding GAPDH, PRK and other pathway neighbours. These results highlight the central position occupied by Calvin-Benson cycle in plant metabolism and the transcriptional coordination of this pathway.

As expected, we found a strong positive correlation between N content (as a percentage of total dry matter) and soluble protein content, in particular Rubisco (**Fig. 3**). Rubisco protein content was also correlated with its activity and the gene encoding ADP-glucose-pyrophosphorylase (AGPase) large subunit. Moreover, Rubisco activity was correlated with a large group of genes that encode proteins involved in photosynthesis and respiration, among other pathways. The activation state of the Rubisco enzyme was negatively correlated with its total activity. Moreover, the genes encoding Rubisco small and large subunits were coexpressed with a large number of genes involved in a wide range of metabolic pathways, mainly Calvin-Benson cycle, photorespiration, and carbohydrate and N metabolisms. The Rubisco amount, activity and transcript levels are positively correlated with a large group of genes, mostly involved in photosynthesis and respiration. This underlines the relevance of Rubisco as a key protein in the photosynthetic process.

Fructan content was highly correlated with transcript levels for all fructosyltransferases (**Fig. 3**), which are the enzymes responsible for its synthesis, in agreement with results reported by Kawakami and Yoshida [42] with

hardened wheat. Additionally, fructan content was correlated with TSC, reflecting that fructans are the predominant carbohydrates in durum wheat together with sucrose, especially under elevated CO₂ and N deficiency [18, 21]. Fructose content was positively correlated with glucose content and with transcript levels for enzymes involved in carbohydrate metabolism (hexokinase, glucose-6-phosphate/phosphate translocator and trehalose-6-phosphate synthase). Sugar signaling has been extensively studied, and it is well known that sugar signals can repress photosynthetic gene expression and activity by the activation of hexokinase-dependent or -independent pathways [43, 44], although not too much attention has been paid to its relation with N metabolism. There were many negative correlations between carbohydrate content (glucose, fructose, sucrose and fructans) and photosynthetic transcript levels (glutamyl-tRNA reductase, GluTR; and photosystem II chlorophyll a-b binding protein, LHCII), N-rich compounds (chlorophyll and amino acid content), NR activity and N metabolism-related transcript levels (nitrate transporters, GOGAT and GS). Relative to N transporters, we observed a significant negative correlation between fructose and fructan contents with *NAR2.1* and *NAR2.2* expression. These genes are components of the high affinity nitrate transporters NRT2 which are essential for nutrient uptake [45]. Overall, we found a close relationship between carbohydrate content and expression of C metabolism-related genes, while the accumulation of carbohydrates, mainly fructans, might inhibit photosynthesis and be modulated by N assimilation, either directly or indirectly, as a strategy to adapt the metabolic status to the plant demand.

Regarding other important relationships, the transcription factor NF-YB3 correlated with NR activity and the expression of certain photosynthetic genes, particularly those encoding GluTR and LHCII (**Fig. 3**). Stephenson *et al.* [46] reported that overexpression of *NF-YB3* in transgenic wheat was associated with an up-regulation of *GluTR* and photosynthetic genes such as those for chlorophyll a-b binding proteins. Hence, NF-YB3 was involved in the regulation of photosynthetic genes, in agreement with Stephenson *et al.* [46], and a novel relationship was found between NF-YB3 and the activity of the key enzyme of N metabolism, NR. Several carbonic anhydrase genes were correlated to each other and with different photosynthetic and respiratory genes. This could be due to the wide range of functions attributed to carbonic anhydrases in carboxylation or decarboxylation reactions in photosynthesis and respiration [47]. Furthermore, gene expression of *HSP90*, which encodes the heat shock protein (HSP) 90 involved in cellular signaling, protein folding, translocation and degradation under heat stress [48], was clearly correlated with expression of genes from the photorespiratory pathway. This could highlight that the predicted higher photorespiratory rate [49] and HSP content [448] under future temperature increment will also be coordinated at transcriptional level.

To complement the relationships found in the network between all trait pairs, we performed a hierarchical cluster analysis in R to identify groups of traits that are coordinated (**Fig. 4**). Eleven modules were clustered at a threshold of $P < 0.05$. Both *NAR2* proteins were grouped in cluster I, although they did not cluster with *NRT2* transporters, with whom they integrate the high affinity nitrate transporters. Cluster II indicated a relationship between transcript levels of photosystem I chlorophyll a-b binding protein, catalase and adenosine diphosphate glucose pyrophosphatase, an enzyme that competes with starch synthase for substrate [50]. Clusters IV and VIII showed the close correlation between carbohydrate contents, fructans and TSC in cluster IV and glucose and fructose in cluster VIII. Cluster V revealed the high degree of coordination between all fructosyltransferases,

indicating that these enzymes operate simultaneously in flag leaves of durum wheat, as has been reported in bread wheat stems [51]. Cluster VI grouped transcript levels of a variety of metabolic processes. We highlight the coordination of alkaline and cell wall-invertases with fructan 1-exohydrolase, all of them involved in sugar hydrolysis (sucrose and fructans, respectively), providing C and energy for the synthesis of numerous compounds [52]. The integration of C and N metabolisms was also evident by this analysis in clusters III, VII, IX, X and XI. These clusters grouped a wide range of transcripts for genes linked to both metabolisms, clusters III and VII being especially relevant due to associations between biochemical parameters and gene expression. Thus, metabolic compounds as chlorophylls and amino acids were related with NR activity, sucrose and major transcripts for N assimilation, carbohydrate metabolism and respiration in cluster III. In cluster VII, key roles for transcript levels for phosphoenolpyruvate carboxylase, fructan 6&1-exohydrolase, 2-oxoglutarate dehydrogenase complex, and asparagine synthetase were suggested because they are related with N, Rubisco and soluble protein contents, and Rubisco activity. The importance of these transcripts resides in their coding for enzymes involved in C supply and amino acid biosynthesis, which showed significant changes in gene expression and activity under elevated CO₂ and/or high temperature [18, 21, 53, 54]. Therefore, valuable information was extracted from cluster analysis that complements the associations discussed in network analysis.

Correlations networks can also be a useful tool for identifying traits that may be biologically relevant in the regulation of primary metabolism. In addition to the key roles suggested for some traits described above, the selection of candidate regulatory traits can be based on the number of correlations that a parameter has with others [7, 8]. In this sense, we underlined the top most connected nodes, corresponding either to gene expression or to biochemical parameters (**Table 1**). Relative to gene expression, we suggested potential regulatory functions for AGPase, both large and small subunits, serine hydroxymethyltransferase (SHMT), ferredoxin-NADP(H) oxidoreductase (FNR), the Rieske iron-sulfur subunit of cytochrome b₆f complex (Cytb6f), RCA, CP12 protein, inorganic pyrophosphatase (IPP), serine:glyoxylate aminotransferase (SGAT), and cytosolic fructose-1,6-bisphosphatase (cFBPase). RCA and CP12 protein were described above, while AGPase is known to catalyze the limiting step of starch biosynthesis and is allosterically regulated [43]. The genes of two photorespiratory enzymes, SHMT and SGAT, were highly interconnected and could play essential functions in photorespiration and amino acid metabolism [55] or in controlling cell damage provoked by several abiotic stresses [56]. FNR and Cytb6f are involved in the photosynthetic electron transport chain. Changes in gene expression for both proteins were observed under future climate change scenario in durum wheat [18, 57], which could affect energy provision for photosynthesis and N assimilation in the chloroplast. IPP is an enzyme that hydrolyzes pyrophosphate into two orthophosphate molecules, which could affect the reversibility of a variety of reactions. Finally, cFBPase could play important regulatory roles in carbohydrate metabolism, because it catalyzes the first irreversible reaction in sucrose synthesis and is allosterically regulated [58]. In the case of biochemical parameters, a high number of interactions were found for Rubisco and NR activities, as well as fructose and fructan contents, which were discussed above. In general, most of the candidate regulatory traits correspond to highly regulated limiting steps of plant metabolism, while others are novel candidates.

Conclusions

In conclusion, network analysis integrated metabolic responses of the plant to the environment and the regulation mechanisms underlying these responses. The high amount of positive correlations between traits at biochemical and transcriptional level, together with hierarchical cluster analysis, demonstrated the close coordination between C and N metabolisms in durum wheat plants in response to different environmental conditions, including those anticipated for climate change, for the maintenance of the metabolic homeostasis. Our study has also shown that this tight coordination involves all metabolic pathways studied and suggests many to our knowledge novel relationships between metabolic traits in the non-widely studied durum wheat. Thus, the photosynthetic light reactions and N assimilation were highly controlled by end-product inhibition (carbohydrates), and new relationships were found between carbohydrate content and N-related traits (transcripts, enzyme activities and compounds). Furthermore, we propose that genes encoding NRT1.1 and GOGAT isoforms play a major role in N metabolism. The activities of key enzymes, such as Rubisco and NR, seem to modulate primary metabolism tightly, both of them controlling the expression of many genes and influencing the accumulation of relevant end-products. We highlight that the transcriptional control of Calvin-Benson cycle might be relevant to plant growth and development under different environmental scenarios, since their genes are linked with most of the pathways of primary metabolism. It is worth noting the important role played by transcription factors such as NF-YB3 and Dof1 on the regulation of gene expression, but further efforts are necessary in this area. Additionally, network exploration could facilitate the identification of potential core metabolic processes and factors regulating metabolic switches, which may be targets for genetic improvement against different abiotic stresses.

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Legends

Table 1 Candidate regulatory traits based on the number of interactions with adjacent nodes.

Figure 1 Schematic representation of the experiments carried out for data acquisition.

Figure 2 Clustered heat map of the correlation matrix for 142 traits of C and N metabolism (left) and sampling distribution of Spearman's r (right). Each point of the correlation matrix is a Spearman correlation coefficient between two traits (blue, positive correlation; red, negative correlation).

Figure 3 Overview of the correlation network for biochemical parameters and gene expression related to carbon and nitrogen metabolism in durum wheat under various growth conditions. Edge color represent positive correlations between traits in blue (Spearman's $r > 0.7$; $P < 0.001$) and negative correlations in red (Spearman's $r < -0.7$; $P < 0.001$). The different traits were classified by node fill color in different metabolic pathways (see legend).

Figure 4 Hierarchical cluster analysis of biochemical and molecular traits analyzed in durum wheat grown under various growth conditions with a bootstrap replications of 1,000. Values at branches are approximately unbiased probability values (AU, p-values, in red), bootstrap probability values (BP, in green) and cluster labels (in grey).

Hydroponic culture 1

$\text{CO}_2 \times \text{Nitrogen}$

Treatments

AL
AH
EL
EH

Growth stages:

- Anthesis
- Early grain filling

Hydroponic culture 2

$\text{CO}_2 \times \text{Nitrogen}$

Treatments

AL
AH
EL
EH

Time points:

- 4 h of incubation
- 24 h of incubation

Field experiment

$\text{CO}_2 \times \text{Temperature} \times \text{Nitrogen}$

Treatments

AIL
AIH
AFL
AFH
EIL
EIH
EFL
EFH

Growth stages:

- Ear emergence
- Anthesis

BIOCHEMICAL PARAMETERS

GENE EXPRESSION

Table 1 Candidate regulatory traits based on the number of interactions with adjacent nodes.

Trait	Description	Identifier	Pathway	Edge count
Gene expression	ADP-glucose-pyrophosphorylase large subunit	AGPase.L	Carbohydrate metabolism	52
Gene expression	Serine hydroxymethyltransferase	SHMT	Photorespiration	49
Gene expression	Ferredoxin-NADP(H) oxidoreductase	FNR	Light reactions	48
Gene expression	Cytochrome b ₆ f complex Rieske iron-sulfur subunit	Cytb6f	Light reactions	47
Gene expression	Rubisco activase	RCA	Reductive pentose phosphate pathway	46
Gene expression	CP12 protein	CP12	Reductive pentose phosphate pathway	45
Gene expression	Inorganic pyrophosphatase	IPP	Carbohydrate metabolism	44
Gene expression	ADP-glucose-pyrophosphorylase small subunit	AGPase.S	Carbohydrate metabolism	44
Gene expression	Serine:glyoxylate aminotransferase	SGAT	Photorespiration	43
Gene expression	Fructose-1,6-bisphosphatase, cytosolic	cFBPase	Glycolysis	42
Biochemical parameter	Total Rubisco activity	TRA	CO ₂ assimilation	14
Biochemical parameter	Maximum nitrate reductase activity	mRNA	Nitrogen assimilation	14
Biochemical parameter	Fructose content	fructose	Carbohydrate metabolism	13
Biochemical parameter	Fructan content	Fructans	Carbohydrate metabolism	11
Biochemical parameter	Selective nitrate reductase activity	sRNA	Nitrogen assimilation	10

New insights into the impacts of elevated CO₂, nitrogen, and temperature levels on the regulation of C and N metabolism in durum wheat using network analysis

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Appendix A. Supplementary Data

Table A.1 Experiments and growth conditions used for data acquisition.

Table A.2 List of the biochemical parameters and transcripts analyzed and abbreviations used.

Table A.1 Experiments and growth conditions used for data acquisition.

Experiment	System	Growth stage	[CO ₂]	Temperature	N supply	Incubation	Reference
1	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.50 mM NO ₃ ⁻	24 h	Vicente <i>et al.</i> [20]
1	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	5.00 mM NO ₃ ⁻	24 h	
1	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.50 mM NO ₃ ⁻	24 h	
1	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	5.00 mM NO ₃ ⁻	24 h	
1	Hydroponic	Early grain filling	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.50 mM NO ₃ ⁻	24 h	
1	Hydroponic	Early grain filling	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	5.00 mM NO ₃ ⁻	24 h	
1	Hydroponic	Early grain filling	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.50 mM NO ₃ ⁻	24 h	
1	Hydroponic	Early grain filling	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	5.00 mM NO ₃ ⁻	24 h	
2	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.25 mM NO ₃ ⁻	4 h	This study
2	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	2.50 mM NO ₃ ⁻	4 h	
2	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.25 mM NO ₃ ⁻	4 h	
2	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	2.50 mM NO ₃ ⁻	4 h	
2	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.25 mM NO ₃ ⁻	24 h	Vicente <i>et al.</i> [21]
2	Hydroponic	Anthesis	390 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	2.50 mM NO ₃ ⁻	24 h	
2	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	0.25 mM NO ₃ ⁻	24 h	
2	Hydroponic	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	15 °C / 20 °C	2.50 mM NO ₃ ⁻	24 h	
3	Field	Ear emergence	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	15 kg N ha ⁻¹		This study
3	Field	Ear emergence	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	15 kg N ha ⁻¹		
3	Field	Ear emergence	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	140 kg N ha ⁻¹		
3	Field	Ear emergence	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	140 kg N ha ⁻¹		
3	Field	Ear emergence	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	15 kg N ha ⁻¹		Vicente <i>et al.</i> [18]
3	Field	Ear emergence	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	15 kg N ha ⁻¹		
3	Field	Ear emergence	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	140 kg N ha ⁻¹		
3	Field	Ear emergence	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	140 kg N ha ⁻¹		
3	Field	Anthesis	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	15 kg N ha ⁻¹		
3	Field	Anthesis	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	15 kg N ha ⁻¹		
3	Field	Anthesis	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	140 kg N ha ⁻¹		
3	Field	Anthesis	370 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	140 kg N ha ⁻¹		
3	Field	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	15 kg N ha ⁻¹		
3	Field	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	15 kg N ha ⁻¹		
3	Field	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Ambient	140 kg N ha ⁻¹		
3	Field	Anthesis	700 $\mu\text{mol}\cdot\text{mol}^{-1}$	Amb. + 4 °C	140 kg N ha ⁻¹		

Table A.2 List of the biochemical parameters and transcripts analyzed and abbreviations used.

Trait	Identifier	Trait	Identifier
BIOCHEMICAL PARAMETERS			
Carbon-rich compounds			
Glucose	glucose	Fructose	fructose
Sucrose	sucrose	Fructans	fructans
Total soluble carbohydrates	TSC	Starch	starch
Nitrogen-rich compounds			
Chlorophylls	chl	Amino acids	amino.acid
Rubisco protein	Rubisco	Soluble protein	sol.prot
Percentage of nitrogen	%N		
Enzyme activities			
Initial Rubisco activity	IRA	Total Rubisco activity	TRA
Rubisco activation state	actRubisco	Maximum nitrate reductase activity	mNRA
Selective nitrate reductase activity	sNRA	Nitrate reductase activation state	actNR
TRANSCRIPTS			
Light reactions & energy			
Photosystem I chlorophyll a-b binding protein	LHCI	Photosystem II chlorophyll a-b binding protein	LHCII
Photosystem I P700 chlorophyll a apoprotein A1	PsaA	Photosystem I P700 chlorophyll a apoprotein A2	PsaB
Photosystem II protein D1	D1	Photosystem II protein D2	D2
Cytochrome b ₆ f complex Rieske iron-sulfur subunit	Cytb ₆ f	Plastocyanin	PC
Ferredoxin	Fd	Ferredoxin-NADP(H) oxidoreductase	FNR
ATP synthase β-subunit, plastidial	pATPase	ATP synthase β-subunit, mitochondrial	mATPase
ATP synthase B1 subunit, vacuolar	vATPase	Glutamyl-tRNA reductase	GluTR
Reductive & oxidative pentose phosphate pathway			
Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit	rbclL	Ribulose-1,5-bisphosphate carboxylase oxygenase small subunit	rbclS
Rubisco activase	RCA	Rubisco subunit-binding protein cpn60 subunit α	cpn60
Carbonic anhydrase, plastidial	CA1	Carbonic anhydrase, plastidial	CA2
Carbonic anhydrase, plastidial	CA3	Carbonic anhydrase, plastidial	CA4
Phosphoglycerate kinase, plastidial	pPGK	Glyceraldehyde-3-phosphate dehydrogenase, plastidial	GAPDH
Triose phosphate isomerase, plastidial	pTPI	Fructose-1,6-bisphosphate aldolase, plastidial	pFBA
Fructose-1,6-bisphosphatase, plastidial	pFBPase	Transketolase, plastidial	pTK
Sedoheptulose-1,7-bisphosphatase	SBPase	Ribulose-5-phosphate 3-epimerase, plastidial	pRPE
Ribulose-5-phosphate 3-epimerase, cytosolic	cRPE	Ribose-5-phosphate isomerase, plastidial	pRPI
Ribose-5-phosphate isomerase, cytosolic	cRPI	Phosphoribulokinase	PRK
CP12 protein	CP12		
Photorespiration			
Phosphoglycolate phosphatase	PGLP	Glycolate oxidase	GO
Catalase-1	CAT	Glutamate:glyoxylate aminotransferase	GGAT
Serine:glyoxylate aminotransferase	SGAT	Glycine decarboxylase complex P subunit	GDC.P
Glycine decarboxylase complex H subunit	GDC.H	Serine hydroxymethyltransferase, mitochondrial	SHMT
Hydroxypyruvate reductase, peroxisomal	HPR1	Glycerate kinase	GLYK
Carbohydrate metabolism			
Inorganic pyrophosphatase	IPP	ADP-glucose-pyrophosphatase	AGGPase
ADP-glucose-pyrophosphorylase large subunit, plastidial	AGPase.L	ADP-glucose-pyrophosphorylase small subunit, plastidial	AGPase.S
Starch synthase isoform I	SSI	Starch synthase isoform III	SSIII
Starch synthase isoform IV	SSIV	Isoamylase 1	ISO1
Isoamylase 3	ISO3	Triose phosphate/phosphate translocator	TPT
Glucose-6-phosphate/phosphate translocator	GPT	Fructose-1,6-bisphosphatase, cytosolic	cFBPase
Nucleoside diphosphate kinase 1, cytosolic	NDK	UTP-glucose-1-phosphate uridylyltransferase	UGPase
Sucrose-phosphate synthase 1	SPS	Sucrose-phosphate phosphatase 1	SPP
Sucrose synthase type 1	SUS	Alkaline invertase, cytosolic	AINV
Sucrose:sucrose 1-fructosyltransferase	SST1	Sucrose:fructan 6-fructosyltransferase	SFT6
Fructan:fructan 1-fructosyltransferase isoform A	FFT1A	Fructan:fructan 1-fructosyltransferase isoform B	FFT1B

Table A.2 (Continued)

Trait	Identifier	Trait	Identifier
TRANSCRIPTS			
Glycolysis			
Fructan 1-exohydrolase	FEH1	Fructan 6&1-exohydrolase	FEH6.1
6-kestose exohydrolase	KEH6	Trehalose-6-phosphate synthase	TPS
Trehalase	TRE	Cellulose synthase	CESA
Cell wall invertase	CWINV	Hexokinase	HXK
Phosphoglucumutase, plastidial	pPGM	Phosphoglucumutase, cytosolic	cPGM
Phosphoglucose isomerase, plastidial	pPGI	Phosphoglucose isomerase, cytosolic	cPGI
Pyrophosphate-fructose-6-phosphate	PFP	Putative 6-phosphofructokinase 1-phosphotransferase	PFK
Fructose-1,6-bisphosphate aldolase, cytosolic	cFBA	Fuctose-2,6-bisphosphatase/6-phosphofructo-2-kinase	F2KP
Triose phosphate isomerase, cytosolic	cTPI	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPC
Glyceraldehyde-3-phosphate dehydrogenase (non-phosphorylating), cytosolic	GAPN	Phosphoglycerate kinase, cytosolic	cPGK
Phosphoenolpyruvate carboxylase	PEPC	Phosphoglycerate mutase, PGAM	PGAM
		Putative pyruvate kinase, PK	PK
Tricarboxylic acid cycle			
Pyruvate dehydrogenase complex E1 component α -subunit, mitochondrial	PDC	Putative NADP-dependent isocitrate dehydrogenase, cytosolic	ICDH
Putative NAD-dependent isocitrate dehydrogenase, mitochondrial	IDH	Putative 2-oxoglutarate dehydrogenase complex E1 subunit	OGDC
Putative citrate synthase, mitochondrial	CS		
Nitrogen metabolism			
Ammonium transporter AMT1;2	AMT1.2	Ammonium transporter AMT2;1	AMT2.1
Low affinity nitrate transporter NRT1.1A	NRT1.1A	Low affinity nitrate transporter NRT1.1B	NRT1.1B
Low affinity nitrate transporter NRT1.3A	NRT1.3A	Low affinity nitrate transporter NRT1.5B	NRT1.5B
Low affinity nitrate transporter NRT1.7B	NRT1.7B	Low affinity nitrate transporter NRT1.8B	NRT1.8B
High affinity nitrate transporter NRT2.2	NRT2.2	Component of high affinity nitrate transporter NAR2.1	NAR2.1
Component of high affinity nitrate transporter NAR2.2	NAR2.2	NADH-nitrate reductase	NR
Molybdenum cofactor biosynthesis protein	MOCO	Ferredoxin-nitrite reductase	NIR
Glutamine synthetase, cytosolic	GS1a	Glutamine synthetase, cytosolic	GSr1
Glutamine synthetase, plastidial	GS2a	Glutamine synthetase, plastidial	GS2b
NADH-dependent glutamate synthase	N.GOGAT	Ferredoxin-dependent glutamate synthase	F.GOGAT
Glutamate dehydrogenase	GDH	Aspartate aminotransferase, cytosolic	cAAT
Aspartate aminotransferase, plastidial	pAAT	Asparagine synthetase	AS
Chloride channel 1	CLC		
Stress & transcription factors			
Heat shock protein HSP90.1-A1	HSP90	Heat shock protein HSP70	HSP70
Myb transcription factor MYB13-1	MYB13.1	Dof transcription factor Dof1	DOF1
NF-Y transcription factor NF-YB3	NF.YB3		