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1 **Comparative analysis based on transcriptomics and metabolomics data reveal differences**
2 **between emmer and durum wheat in response to nitrogen starvation**
3
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29 **Summary**

30 Mounting evidence indicates the key role of Nitrogen (N) on diverse processes in plant, including not
31 only yield but also development and defense. Using a combined transcriptomics and metabolomics
32 approach, we studied the response of seedlings to N starvation of two different tetraploid wheat
33 genotypes from the two main domesticated subspecies, emmer (*Triticum turgidum* ssp. *dicoccum*)
34 and durum wheat (*Triticum turgidum* ssp. *durum*). We found that durum wheat exhibits broader and
35 stronger response in comparison to emmer as evidenced by the analysis of the differential expression
36 pattern of both genes and metabolites and gene enrichment analysis. Emmer and durum wheat showed
37 major differences in the responses to N starvation for transcription factor families. While emmer
38 showed differential reduction in the levels of primary metabolites to N starvation, durum wheat
39 exhibited increased levels of most metabolites, including GABA as an indicator of metabolic
40 imbalance. The correlation-based networks including the differentially expressed genes and
41 metabolites revealed tighter regulation of metabolism in durum wheat in comparison to emmer, as
42 evidenced by the larger number of significant correlations. We also found that glutamate and GABA
43 had highest values of centrality in the metabolic correlation network, suggesting their critical role in
44 the genotype-specific response to N starvation of emmer and durum wheat, respectively. Moreover,
45 this finding indicates that there might be contrasting strategies associated to GABA and Glutamate
46 signaling modulating shoot *vs* root growth in the two different wheat subspecies.

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47 **Introduction**

48 Availability and uptake of nitrogen (N) is considered a major driver of growth (Lea and Azevedo,
49 2006). Indeed, N is an essential nutrient for all organisms, including plants, and is required for the
50 biosynthesis of macromolecules, such as proteins, nucleic acids, and chlorophyll, and for the synthesis
51 of many secondary metabolites with different roles in adaptation and signaling (Miller *et al.*, 2007).
52 As a result, N deficiency (limited availability) and starvation (complete absence) dramatically affects
53 plant growth and metabolism (Obata and Fernie, 2012).
54 However, only 30–50% of supplied N is taken up by crops (Raun and Johnson, 1999), and the
55 remainder is lost by denitrification or leaching into terrestrial ecosystems, causing eutrophication and
56 contamination of drinking water (Cassman *et al.*, 2003). Therefore, plant breeding efforts should be
57 combined with improvement of crop management towards a more efficient use of N also to limit the
58 use of fossil energy and environmental pollution (Ayadi *et al*., 2014; Ruisi *et al*., 2015). Towards this
59 key objective, it is necessary to understand how plants react and cope with low N availability and
60 identify the molecular basis of the natural genetic variation for adaptation to low N conditions.
61 Understanding the molecular mechanisms underlying the variation in traits responsible for the
62 phenotypic plasticity in crop and wild species is a key step in addressing the challenges of modern
63 agriculture, such as resilience to climate changes (Godfray *et al.*, 2010). In particular, understanding
64 the genetic variation in N metabolism in major crop species, such as wheat, is expected to provide
65 novel strategies for crop improvement (Kant *et al*., 2011; Xu *et al*., 2012; Hawkesford, 2017).
66 In an increasing number of model and crop species, transcriptome studies have highlighted the
67 complexity of the regulatory mechanisms involved in the control of leaf or root gene expression under
68 both N-limiting and non-limiting conditions (Krapp *et al*., 2011; Humbert *et al.,* 2013; Simons *et al.*,
69 2014; Curci *et al*., 2017). In addition, studies about the response of several cereal (e.g. rice, barley,
70 sorghum, and wheat) to N starvation have highlighted differentially expressed genes (DEGs) involved
71 in the response (Curci *et al*., 2017; Zuluaga *et al*., 2017; Yang *et al*., 2015, Gelli *et al*., 2014; Guo *et*
72 *al.*, 2014). For instance, Gelli *et al.* (2014) compared transcriptomic levels in four tolerant and three
73 sensitive sorghum genotypes to low N condition. Furthermore, Chen *et al.* (2011) and Hao *et al.*
74 (2011) compared gene expression changes in response to N stress in two maize and soybean
75 genotypes with contrasting low N tolerance.
76 Several works reported the combination of different 'omics' approaches in the evaluation of different
77 crops responses to N starvation (Scheible *et al*., 2004; Amiour *et al*., 2012; Bielecka *et al*., 2015;
78 Vicente *et al.*, 2016; Yu *et al*., 2017). Nevertheless, a limitation in these studies was the focus on a
79 single genotype.

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80 The analysis of gene expression can be complemented and expanded by using data on metabolite
81 levels and their joint investigation with the help of network analysis approaches. The latter approaches
82 have been useful in highlighting the role of metabolites in particular processes, but also for
83 understanding the structure and regulation of the underlying metabolic and gene regulatory processes
84 (Hirai *et al*., 2005; Caldana *et al*., 2011; Toubiana *et a*l., 2012, 2016; Beleggia *et al*., 2016).
85 The aim of this study was to investigate and compare the transcriptomic and metabolomics responses
86 of two genotypes of tetraploid wheats (one emmer landrace and one elite durum wheat cultivar) to N
87 starvation at the vegetative stage (seedling growth) that showed phenotypic responses to differences
88 in N availability. Tetraploid wheats, (*Triticum turgidum* L. 2n=4x=28; AABB genome), alongside
89 with einkorn and barley, were domesticated in the Fertile Crescent, and durum wheat derived from
90 domesticated emmer (*Triticum turgidum* ssp. *dicoccum*) through a rather long human-driven selection
91 process, including distinct and sequential domestication bottlenecks and continuous gene flow from
92 wild emmer (*Triticum turgidum* ssp. *dicoccoides*) (Nesbitt and Samuel, 1998; Tanno and Willcox,
93 2006; Luo *et al*., 2007; Nevo, 2014). Here we obtained transcriptomics and metabolomics data from
94 one emmer and one durum wheat genotype—the parents of a RIL population developed at CREA-CI
95 Foggia (Russo *et al*., 2014). Our integrative analyses facilitated an in-depth molecular
96 characterization and the comparison of tetraploid wheats responses to N starvation.

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97 **Results**

98 **Morphological and physiological differences under the two N conditions**

99 First, we investigated the effect of N-starvation on plant growth by the evaluation of 13 complex
100 traits, namely 12 morphological traits, including: Total leaf number (TLN); Total leaf area (TLA);
101 Shoot fresh weight (SFW); Primary visible root length (PRL); Lateral visible root length (LRL); Total
102 visible root length (TRL); Visible root system depth (RSD); Visible root system width (RSW); Root
103 dry weight (RDW); specific root length (SRL); Total visible root length/total leaf area ratio
104 (TRL/TLA); Lateral visible root length/ Primary visible root length ratio (LRL/PRL), as well as one
105 physiological trait, Leaf chlorophyll content (SPAD) in emmer (Molise Sel. Colli) and durum wheat
106 (Simeto). Table 1 shows the significant changes according to a two-way ANOVA due to genotype
107 (G), N treatment (N) and their interaction (GxN). The traits TLA and SFW showed significant
108 differences due to G (higher values in durum wheat) and N effect (higher values under optimal N
109 condition). There were three measured traits, namely TLN, RDW and TRL/TLA which were
110 significantly affected by N starvation. Finally, for SRL and SPAD, a significant effect due to the GxN
111 interaction was observed. For instance, emmer at optimal N exhibited the largest value of SRL in
112 comparison to emmer at N starvation and durum wheat in both N conditions. The opposite held for
113 SPAD, for which durum wheat showed the highest value at optimal N compared to durum wheat at -
114 N and for both treatments of emmer.

115

116 **Transcriptomic differences between the two N conditions**

117 A global transcriptome analysis for the comparison of the two analyzed tetraploid wheat genotypes
118 was performed using RNA-Seq Illumina technology resulting in 9.9 to 19.5 million reads per
119 genotype (Table S1). These numbers were reduced after additional processing steps (see Methods)
120 by 4.3 to 7.5%, depending on the sample. The cleaned reads were mapped on the bread wheat
121 reference covering, on average, 70% of all reads in the analyzed genotypes (Table S1).
122 We used the mapped reads to assess the DEGs in each genotype between the two N conditions, i.e.
123 N starvation and optimal N condition. The total number of genes expressed in emmer and durum
124 wheat were 27,792 and 28,812, respectively. The number of significant DEGs for emmer was 1,788,
125 while in durum wheat it was 3,129. The number of DEGs specific to durum wheat was ~3.2-fold
126 larger than in emmer, and the number of DEGs common to the two genotypes was 1,095 (Figure 1A).
127 In addition, the number of the up-regulated DEGs specific to durum wheat was 2.5-fold larger than
128 those specific to emmer, while the number of down-regulated DEGs specific to durum wheat was
129 3.5-fold larger than those specific to emmer (Figure 1B). Therefore, we found a stronger
130 transcriptional response in durum wheat to the change in N availability in comparison to emmer.

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131 **Functions of DEGs in emmer and durum wheat between the two N conditions**

132 The functional annotation of DEGs either common or specific to one of the genotypes, were reported
133 in Table S2. Several DEGs were directly involved in N metabolism and transport. The key DEGs
134 involved in nitrate assimilation, i.e. the gene coding for asparagine synthetase and aspartate
135 aminotransferase, were up-regulated in both genotypes. In durum wheat, the gene coding for nitrate
136 reductase was up-regulated, while the genes orthologous to *Arabidopsis* glutamine synthetase and
137 glutamate dehydrogenase family were down-regulated in response to N-starvation (Table S2). A
138 similar result was reported by Curci *et al*. (2017) for the response of durum wheat leaves to N chronic
139 starvation during grain filling. One nitrate transporter and two ammonium transporters were found
140 among the DEGs in emmer (Table S2). Interestingly, other DEGs associated with the translocation
141 of other nutrient (potassium (8 genes), phosphate (1- [PhO1]), sulfate (1), zinc (1), calcium (8), copper
142 (2), magnesium (3) and ABC transporter (6)) also changed under N starvation (Table S2).

143 A general alteration was observed for genes participating in carbon metabolism, especially for those
144 involved in glycolysis, tricarboxylic acid cycle (TCA), photosynthesis and photorespiration,
145 particularly in durum wheat (Table S2). Notably, gene coding for Phosphoglycerate kinase (PGK),
146 Pyruvate kinase (PK), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and Fructose
147 bisphosphate aldolase were up-regulated and specific to durum wheat, while Pyruvate dehydrogenase
148 E1-component subunit alpha (PDHA), Pyrophosphate--fructose 6-phosphate 1-phosphotransferase
149 subunit alpha (PFP-ALPHA), and ATP-dependent 6-phosphofructokinase (PFK1) were up-regulated
150 and specific to emmer. Concerning the Pentose phosphate pathway, one DEG encoding for glucose-
151 6-phosphate dehydrogenase (G6PD) was up-regulated in both genotypes, while two orthologs to
152 ribose-5-phosphate-isomerase (Rpi) were up-regulated only in durum wheat. Notably, orthologues to
153 RuBisCO (5 DEGs) and ferrodoxin (3 DEGs) were up regulated only in durum wheat.

154 Transcription factors from the ARFs (5 DEGs) and NF-Y (3 DEGs) families were found to be down-
155 regulated in both genotypes, while the MYB family (1 DEGs) was up-regulated in durum wheat and
156 PTACs (5 DEGs) families were up-regulated in both genotypes (Table S2). In addition, 35 protein
157 kinases (PKs) were identified as DEGs, of which 13 were common to the two genotypes, while six
158 and 16 were found as DEGs specific to emmer and durum wheat, respectively. Generally, N starvation
159 causes several stress responses. About two thirds of DEGs common or specific to each genotype were
160 up-regulated, and among them there were several antioxidant enzymes encoding genes, such as:
161 superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxiredoxin (Prx),
162 and lipoxygenases (LOXs), as well as enzymes of the ascorbate-glutathione cycle, such as:
163 glutathione reductase (GR) or those involved in the biosynthesis of secondary metabolites (Table S2).
164

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165 **GO enrichment analysis of DEGs**

166 To investigate the transcriptomic changes in leaves of emmer and durum wheat under the two N
167 conditions, we assessed the GO enrichment in the set of DEGs (see Methods).
168 The GO terms identified were categorized into 21 and 23 categories for emmer and durum wheat,
169 respectively (Figure 2, Table S3). In both genotypes, the highest number of DEGs up-regulated were
170 included in the categories 'cellular process', 'metabolic process', 'binding' and 'catalytic' while those
171 that were differentially down-regulated were principally grouped into 'binding' and 'catalytic'
172 categories. Differences between the two genotypes were observed with respect to the molecular
173 function category 'transcription regulator' which was enriched in both the down- and up-regulated
174 DEGs in emmer and durum wheat, respectively, and in the GO terms associated with biological
175 process categories 'regulation of biological process' and 'reproductive process', which were only
176 enriched in durum wheat for up-regulated DEGs.
177 Extended list of over represented GO terms with the p-value of at most 10^{-5} for emmer and durum
178 wheat is reported in Table S4**.** Notably, all GO terms of the categories 'cellular process', 'metabolic
179 process', 'binding' and 'catalytic' (e.g. those involving the nitrogen) which were enriched in emmer
180 were also found in durum wheat. Durum wheat showed also specific over-represented GO terms in
181 several categories; for instance, these included the cellular amino acid, oxoacid or organic acid
182 metabolic processes, or the metabolic/biosynthetic process of isopentenyl diphosphate (Table S4). In
183 addition, regarding the categories 'binding' and 'catalytic activity', durum wheat showed different
184 over-represented GO terms among the DEGs differentially up- and down-regulated. For example,
185 GO terms of oxidoreductase, ligase, hydrolase (on glycosyl bond or O-glycosyl compounds), lyase
186 and transferase activity were not enriched in the down-regulated DEGs, while GO terms of kinase,
187 protein kinase, protein serine/threonine kinase and phosphotransferase activity were not enriched on
188 the up-regulated DEGs (Table S4).
189

190 **Metabolic differences between the two N conditions**

191 A total of 46 metabolites were identified and quantified using GC-MS (see Methods). These included
192 41 polar and five non-polar compounds, divided into the following compound classes: amino acids,
193 organic acids, sugars and sugar alcohols, fatty acids, polycosanol and phytosterols. The data were
194 analyzed using two-way ANOVA and significant differences ($P \leq 0.05$) for 23 metabolites including
195 the TCA cycle intermediates, some sugars, shikimic and quinic acids, several amino acids and GABA
196 were reported (Table S5). A higher content of metabolites was found in emmer under optimal N
197 condition in comparison to durum wheat (see Figure 3 for illustrative comparison). For all
198 metabolites, a strong significant effect due to the interaction of the genotype and N treatment (GxN)

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199 was observed. Five metabolites (glutamic acid, aspartic acid, citric acid, saccharic acid and maltitol)
200 showed also significant differences due to the effect of genotype (G) and treatment (N). Tryptophan
201 showed only the genotype effect while aconitic and shikimic acids showed significant differences due
202 to G apart from the GxN interaction.
203

204 **Network analysis of combined data sets**

205 In general, the correlation structure among the combined data sets (transcripts and metabolites) of
206 each genotype can be represented by a network, where a node denotes a transcript, or a metabolite
207 and an edge stand for the presence of significant Pearson correlation between the data associated to
208 the nodes. Overall, durum wheat showed 2.8-fold more significant correlations in comparison to
209 emmer (Table 2). The intersection of the networks from the two genotypes included ~397,000 edges,
210 of which 99.3% did not demonstrate significant differences between the two networks (using Fisher's
211 z-transformation, see Methods). The latter set of edges (with no significant differences between the
212 networks obtained for each genotype; by applying Fisher's z transformation) is said to comprise the
213 common network between the two genotypes that represents the 31.5% and 11.2% of the total
214 correlations in the networks of emmer and durum wheat, respectively.
215 Because we are interested in understanding if the differences in correlation could reflect the
216 differences in regulation of transcripts and metabolites, we considered only the significant
217 correlations between DEGs and significantly altered metabolites under the two N conditions; the
218 number of such correlations in durum wheat was 2.3-fold larger than in emmer. Focusing the attention
219 only on those metabolites that showed differential behavior between the two N conditions, as reported
220 above, we observed that for emmer GABA is involved in the smallest number of edges (12), while
221 maltitol participates in the largest number of edges (1,667). In durum wheat we find almost
222 contrasting situation, isomaltose was involved in the smallest number of edges (28), while GABA
223 exhibited the largest number of edges (2,954) (Table S6).
224 The effect of the observed differences between the correlation structures (i.e. networks) obtained for
225 both genotypes can be investigated for each node and can be summarized by its centrality in the
226 network. In this context, we selected those nodes showing the centrality measures (i.e., degree and
227 betweenness) greater than the corresponding mean values in each genotypic-specific network.
228 Considering the nodes of the two genotype-specific networks, those with a central role included 260
229 and 479 genes in emmer and durum wheat, respectively (Table 2). In durum wheat also the
230 metabolites: myo-inositol, quinic acid and valine showed high values for both centrality measures.
231 To refine the network, we next included only DEGs with high values of centrality and only
232 metabolites that were significantly contrasted between the two N conditions. In general, the total

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233 number of edges decreased of about 79% and 85% for emmer and durum wheat, respectively (Table
234 S6). The total number of edges between central DEGs and differentially behaved metabolites in
235 durum wheat is higher than those in emmer by 3.6-fold for alanine and 479-fold for GABA. In
236 contrast, in emmer, the number of edges between central DEGs and significantly contrasted
237 metabolites: glutamic acid, isocitric acid, isomaltose, saccharic acid, serine, succinic acid, and
238 threonine, were higher than those in durum wheat. Noteworthy, with aspartic acid, citric acid, fumaric
239 acid and maltitol the number of edges was the same in both genotype-specific networks.
240
241 **Function of DEGs having a central role in the networks**
242 To evaluate the common or specific responses to N starvation in the two genotypes, we looked for
243 the annotated functions of the DEGs shared between the two genotype-specific networks with a
244 central role in at least one of the two networks (Table S7). Several DEGs related to photosynthesis
245 were expressed in both genotypes but in some cases, they showed a central role only in emmer-
246 specific network (e.g. Chlorophyll synthase (CHLG)) while, in contrast Carboxyl-terminal-
247 processing peptidase 3 (CTPA3), Cytochrome c biogenesis protein (CCS1), and magnesium-chelatase
248 subunit ChlD (ChlD) were found to have a central role in the durum wheat-specific network. In the
249 network specific to emmer the most central nodes coded for Pyruvate phosphate dikinase 1 (PPDK)
250 and Pyruvate dehydrogenase E1component subunit alpha-3 (PDH-E1 ALPHA) which were down-
251 and up-regulated, respectively.
252 In durum wheat-specific network, DEGs related to proteolysis as well as the synthesis of the cofactor
253 FMN, that were up-regulated, had a central role in the network, and at the same time, Allantoinase
254 (ALN), a key enzyme for biogenesis and degradation of allantoin and its degradation derivatives,
255 essential in the assimilation, metabolism, transport, and storage of nitrogen in plants, was among the
256 central nodes.
257 In both genotype-specific networks, different DEGs involved in the chloroplast development showed
258 central roles (Table S7). Among the central DEGs, there were several genes related to detoxification
259 and plant stress responses caused by N starvation. Only one DEG (Traes_2BL_CCD296233, down-
260 regulated) encoding for the Stress Enhanced Protein 2 [SEP2], showed a central role in both genotype-
261 specific networks (Table S7).
262 To highlight the differences between emmer and durum wheat, we also considered the putative
263 annotation of the central DEGs in each genotype-specific network (see Table S8). In emmer, several
264 genes involved in C metabolism or related to stress conditions responses were up-regulated; at the
265 same time a DEG related to carbonic anhydrase (EC 4.2.1.1), involved in N metabolism, was down-
266 regulated. In contrast, in durum wheat, several DEGs related to photosynthesis were differently

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267 regulated, i.e. chlorophyll synthase and the ferritin were up-regulated while the ferrochelatase was
268 down-regulated. Importantly in durum wheat-specific network, there is also a central DEG
269 (Traes_3AS_3CB8A9C01) for glutamate decarboxylase [GAD] which was up-regulated.
270 Figure 4 represented the genotype-specific networks of DEGs-metabolites reported in Table S7 and
271 Table S8 for emmer (A) and durum wheat (B), respectively. As illustrated, the network structure was
272 different between the two genotypes; consistently emmer-specific network showed a higher number
273 of negative correlations between DEGs and metabolites while durum wheat-specific network has
274 higher number of positively correlated DEGs and metabolites pairs. Of note, glutamic acid and valine
275 were the metabolites highly connected to the other nodes in emmer-specific network while GABA,
276 quinic acid, *myo*-inositol and valine were highly connected to the rest of the nodes in durum wheat-
277 specific network.
278

279 **DEGs position on the genome**

280 We have also considered the position of DEGs in both genotypes on the physical map. In general, for
281 each chromosome durum wheat showed a higher number of DEGs compared to emmer. In both
282 genotypes, the larger number of DEGs was located on chromosome 2A, 2B, 4A, 5A and 5B, while
283 lower number of genes was found in the chromosome 3B. Few genes were in chromosome 6B in
284 emmer (Figure S1).
285 Figure 5 illustrates the location of down- and up-regulated DEGs with central role in the
286 corresponding genotype-specific networks. Observing the results, the higher number of central nodes
287 in the emmer-specific network was located on chromosome 2B, 4B, and 5A, while for durum wheat-
288 specific network the higher number of central DEGs was located on chromosome 2A, 2B, 4A and
289 5B.

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290 **Discussion**

291 In a preceding work we found that emmer and durum wheat showed contrasting phenotypic responses
292 associated to N starvation (Gioia *et al*., 2015). Here we present the results of gene expression and
293 metabolites levels of emmer and durum wheat using two representative genotypes which were part
294 of the previous investigation. Indeed, a striking result showed by our study is the major differences
295 in the response to N starvation between our emmer and durum wheat genotypes based on their gene
296 expression and metabolite levels. Emmer responded to the stress condition by slowing down all the
297 metabolic functions, probably limiting his energy expenditure. On the contrary, durum wheat
298 responded to the stress condition by activating a much larger number of genes (e.g. triggering more
299 defense responsive pathways) and mechanisms resulting in an accumulation of metabolites in the
300 investigated tissues (leaves) most likely associated to a metabolic imbalance. Moreover, evaluating
301 the differences in plant growth, a significant growth variation under N starvation was observed in
302 both genotypes according to the results reported by Gioia *et al*. (2015) which was more evident in the
303 aerial part in durum wheat and in the below-ground part in emmer.

304 Durum wheat responded to N starvation with a much higher number of DEGs up-regulated. Some of
305 these genes, directly involved in N metabolism, were differentially expressed exclusively in durum
306 wheat (i.e. NR (up-regulated), GS and GDH (down-regulated)). In addition, the results of gene
307 enrichment analysis indicate that emmer and durum wheat adapt to nitrogen starvation by a
308 reprogramming of transcription. Transcription factors are important for controlling the expression of
309 other genes in plant exposed to limited N condition or in complete starvation (Krapp *et al*., 2011;
310 Yang *et al*., 2015; Curci *et al*., 2017) and, accordingly, our results showed as the regulation of
311 transcripts was highly different and, in some case, with an opposite trend between emmer and durum
312 wheat. In addition, some GO categories were only enriched for the DEGs in durum wheat, such as:
313 the cellular amino acids, oxoacid or organic acids metabolism which were also highlighted by Huang
314 *et al.* (2016) in their study on the transcriptomic evaluation in response to the imbalance of carbon:
315 nitrogen ratio in rice seedling.

316 Moreover, the levels of metabolites showed significant differences in response to the N starvation in
317 both emmer and durum wheat. In general, in stressed conditions a reduction in plant growth and
318 photosynthesis is expected (Shaar-Moshe *et al*., 2018) and, consequently, this should lead to a
319 decrease in monosaccharides content. Nevertheless, an increase in the starch and soluble sugars
320 content was reported in the shoot of Arabidopsis thaliana under N starvation (Krapp *et al*., 2011).
321 Accordingly, an increase of total sugars in both genotypes was observed, with a pronounced effect in
322 durum wheat (which also showed a significant decrease of photosynthetic efficiency).

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323 Consistently to the differences observed at transcriptomic level, the content of amino acids, under N
324 starvation was lower in emmer (fold change = -2.7), while in durum wheat a higher accumulation
325 (fold change= 1.7) of these metabolites was observed. Tschoep *et al*. (2009) showed that when
326 Arabidopsis plants were grown under continuous N limitation, the total amino acids levels were found
327 to be higher than under high N condition due to a metabolic imbalance. The results obtained in our
328 conditions suggest a reduced use of amino acids for protein synthesis and growth in durum wheat that
329 links with the reduction of photosynthetic activity under N starvation. On the other hand, the lower
330 accumulation in emmer may indicate an earlier phase of the N starvation syndrome which could result
331 in a drastically reduced, but still efficient, metabolism.

332 In this sense, it is also important to discuss carefully the behaviors of both glutamic acid and
333 GABA, both altered in response to the N starvation condition in emmer and durum wheat. GABA is
334 synthesized mainly from glutamate, closely associated with the TCA cycle, and having a signaling
335 role (Bouchè and Fromm, 2004; Fait *et al*., 2008, Caldana *et al*., 2011). Two studies have suggested
336 a signaling role of GABA during the nitrate uptake in both *Brassica napus* root (Beuve *et al*., 2004)
337 and *Arabidopsis thaliana* (Barbosa *et al*., 2010). Moreover, Sulieman (2011) reported the important
338 role of GABA in increasing of the efficiency of symbiotic N_2 fixation in legumes. Michaeli and
339 Fromm (2015), proposed that the metabolic and signaling functions of GABA has been evolved to be
340 functionally entwined under nutrient starvation. Thus, it seems that GABA levels increase during
341 plant nutrient starvation and energetically demanding stresses (Carillo, 2018), aspect that could be
342 supported, from our data, by the negative correlation between the SPAD values (indicating reduced
343 chlorophyll content) and the GABA content in durum wheat ($r = -0.86$; $P = 0.0061$). On the other
344 hand, Forde and Lea (2007) reported the possible long-distance signaling role of glutamate between
345 shoot and root as part of a network of N signaling pathways that enable the plant to monitor and adapt
346 to changes in N status. In their model, when the shoot-derived glutamate arrive at the root tip, is
347 sensed by plasma membrane glutamate receptors enabling meristematic activity in the root tip to
348 respond to changes in the N/C status of the shoot. In our study the positive correlation in emmer
349 between shoot glutamate and SRL ($r = 0.97$; $P = 0.0001$) could support this suggestion. In addition,
350 the increase of the root morphological parameters in emmer under N starvation could be also
351 sustained by a greater remobilization of the amino acids from the shoot to the root. The key role of
352 GABA and glutamate is also supported by the results of the correlation-based network analysis
353 integrating the information from both metabolites and transcripts. Indeed, durum wheat-specific
354 network was characterized by the role of GABA that was associated to many (479) DEGs while in
355 emmer-specific networks the glutamate was highly connected to many (201) other DEGs. This
356 finding, on one hand, underlies their important role as signaling metabolites in stress conditions as

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357 those occurring during nitrogen starvation and, on the other hand, it may suggest the occurrence of
358 two contrasting strategies based on GABA and glutamate signaling that appear associated to shoot
359 and root growth, respectively.
360 The genotype-specific networks of the two tetraploid wheats showed different structures. Overall,
361 only one DEG (down-regulated) common to both emmer and durum wheat showed a central role in
362 the corresponding networks (i.e. Stress Enhanced Protein 2 -[SEP2]) which is a light-inducible gene
363 as showed in *Arabidopsis thaliana* and rice (Umate, 2010). A previous work reported that the
364 regulation of SEP gene expression by light stress is very specific while other physiological stresses,
365 such as: cold, heat, wounding, desiccation, salt or oxidative stress, did not promote accumulation of
366 SEP transcripts indicating that they were not triggered by photooxidative damage itself (Heddad and
367 Adamska, 2000). Therefore, based on our results we can speculate that SEP2 is inducible by both
368 light and N starvation.
369 Among the genes having a central role in the durum wheat-specific network, there were some
370 transcription factors (i.e. DEAD-box ATP-dependent RNA helicase 3[DEAD-box RH3] and the
371 MIKC-type MADS-box transcription factor) as well as some stress responsive genes (i.e. peroxidase
372 and protein detoxification). For example, as well documented, the DEAD-box RNA helicases are
373 involved in RNA metabolism and have important roles in diverse cellular functions (e.g. plant growth
374 and development, and in response to biotic and abiotic stresses (Vashisht and Tuteja, 2006; Li *et al.*,
375 2008; Linder and Jankowsky, 2011; Zhu *et al.*, 2015). Recently, Gu *et al.* (2014) demonstrated the
376 relevant role of the chloroplast DEAD-box RH3 on the growth and stress response in *Arabidopsis*
377 *thaliana*. Interestingly, it is reported that in bread wheat the MIKC-type MADS-box TFs have key
378 roles in plant growth (Ma *et al.*, 2017; Li *et al.*, 2018); however, even if one of these transcription
379 factors (Traes_5AL_13E2DEC48) was a central node in the durum wheat-specific network, it was
380 down-regulated under N starvation in comparison to the optimal N condition.
381 Moreover, several studies reported that in wheat, grown in either in field or greenhouse conditions,
382 activities of many enzymes in the antioxidant defense system (i.e. SOD, CAT, GPX, GR, Prx and
383 LOX) are altered to control the oxidative stress induced by other factors and to maintain the balance
384 between ROS production and detoxification which avoid potential damage to cellular components,
385 metabolism, development and growth system (Mittler *et al.*, 2004; Caverzan *et al.*, 2016 and reference
386 therein). For example, Kumar *et al.* (2013) reported an increase of SOD transcript in wheat in
387 response to heat shock treatment that may indicate greater tolerance to environmental stresses. Also,
388 in this study, many important genes related to the antioxidant defense system were up-regulated in
389 both genotypes but with a ratio of 1:2 between emmer and durum wheat.

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390 The up-regulation of genes involved in the defense-system and the increase in the content of
391 metabolites under starvation observed in durum wheat suggest that a possible mechanism of response
392 to the starvation may be linked to the autophagy. This process is inducible in different and multiple
393 stress condition or development stages, and it is defined as a non-specific degradation process for the
394 recycling of intracellular material that might be used as building blocks to temporarily overcome the
395 absence of nutrients (Liu and Bassham, 2012; Pérez-Pérez *et al*., 2012). Nutrient limitation also
396 increases ROS production, which in turn may stimulate autophagy functioning as signaling molecules
397 as suggested by Liu *et al*. (2009). Taken together, these findings indicate that the absence of nutrients
398 is a primary signal leading to autophagy activation in eukaryotes, but this stress signal is tightly
399 associated with the production and accumulation of ROS. Because in plants the chloroplasts are
400 primary source of ROS, their degradation through autophagic processes may be highly possible as
401 also reported under carbon-limited conditions (Wada *et al*., 2009).
402 To face environmental constrains, according to the plant-life history (the distribution of
403 resources between growth, reproduction and defense), plants can combine acclimation mechanisms
404 from different strategies defined as escape or resistance (Shaar-Moshe *et al*., 2018 and references
405 therein). In this sense, probably, emmer as adaptive strategy to N starvation relied mainly on the
406 below-ground part while the durum wheat reacts on the up-ground part. Although our experiment did
407 not analyze the transcriptomic and/or metabolomics responses of the roots, it provides important
408 information with respect to differential response on the level of gene and metabolites involving in the
409 efforts of this crop to retain homeostasis under nutrient stress conditions. Indeed, the responses of
410 emmer appear more plastic with enhanced activation of root growth under N starvation then durum
411 which trigger to maintain growth rate even in absence of available N.
412
413 **Experimental Procedures**
414 **Plant materials and experimental design**
415 Two genotypes of *Triticum turgidum* were considered: one emmer (*T. turgidum* ssp. *dicoccum*)
416 named 'Molise Selezione Colli', a pure line selected from a local population, and one modern durum
417 wheat cultivar (*T. turgidum* ssp. *durum*) named 'Simeto' (derived from Capeiti/Valnova), released in
418 ltaly in 1988. They showed many contrasting traits including differences in grain yield (GY), heading
419 date (HD), plant height (PH), test weight (TW), thousand kernel weight (TKW), protein content (PC),
420 yellow index (YI), gluten index (GI), roots and shoot morphological parameters (De Vita *et al*., 2006,
421 2007; Iannucci *et al*., 2017).
422 Both genotypes were previously purified by two cycles of Single Seed Descent (SSD). The samples
423 were part of a larger four-week-long experiment conducted in 2012 under N-optimal and N-starvation

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424 conditions in the PhyTec Experimental Greenhouse at the Institute of Biosciences and Geosciences
425 (IBG-2): Plant Sciences Institute, Forschungszentrum Julich GmbH, Germany (50°54'36''N,
426 06°24'49''E) which included 12 genotypes for each tetraploid wheat subspecies. The resulting 36
427 genotypes were grown under two different N conditions with two replicates per genotype in two
428 subsequent growing conditions. Thus, for each treatment genotypes were replicated four times using
429 two plants per replicate with overall 8 plants per genotype per treatment. Each rhizobox contained
430 two different genotypes of the same subspecie, each represented by two plants arranged to avoid
431 contacts between roots of different genotypes. This means that the two genotypes considered here
432 were grown in four different rhizoboxes for each N condition. Before sowing, for each genotype,
433 grains of uniform size were visually selected, surface sterilized (1% NaClO (w/v) for 15 min and
434 rinsed 10 times with deionized water), pre-germinated and then transplanted into the soil-filled
435 rhizoboxes. The soil used to fill the rhizoboxes (volume of ~18 l) was a 'Typ 0' manually sieved peat
436 soil (Nullerde Einheitserde; Balster Einheitserdewerk, Frondenberg, Germany), which provided low
437 nutrient availability, with a pH of 6.1, and the available phosphate, potassium, magnesium,
438 ammonium nitrogen, and nitrate nitrogen concentrations of 7.0, 15.0, 98.0, <1.0, and <1.0 mg l^{-1},
439 respectively. All plants were watered regularly twice a day with 400 ml of tap water and supplied
440 three times per week with 200 ml of modified Hoagland solution (Hoagland and Arnon, 1950) with
441 or without added nitrogen. For the optimal nitrogen condition, the stock solution included 5 mM
442 KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, plus trace elements while for the nitrogen-
443 starvation solutions, 1 mM KNO_3 and 5 mM $Ca(NO_3)_2$ were replaced by 2.5 mM K_2SO_4 and 5 mM
444 $CaCl_26(H_2O)$, respectively. The experiments were carried out under natural lighting in a greenhouse,
445 with the air temperature kept between 18 and 24 °C, and the relative humidity between 40 and 60%.
446 For more details concerning the experiment and growth conditions see Gioia *et al*. (2015). At the end
447 of the experiment, for each replicate, leaves of the two plants were pooled and immediately frozen in
448 liquid nitrogen to obtain leaves tissues for RNA and metabolites extraction.
449

450 **Phenotypic traits**

451 The following traits were scored for both genotypes: the total leaf area (TLA), the total number of
452 leaves (TLN), and the principal parameters of the root system architecture, such as: visible primary
453 root length (PRL), visible lateral root length (LRL), total root length (TRL) of all visible roots, root
454 system depth (RSD), and root system width (RSW). At the end of the experiment, at 28 days after
455 sowing (DAS) (Zadoks stage 14–18 for optimal N; Zadoks stage 12–14 for N starvation; Zadoks *et*
456 *al.*, 1974), the chlorophyll content (SPAD units) was estimated with a SPAD-502 chlorophyll meter
457 (Minolta Corp., Ramsey, NJ, USA). In addition, wheat plants were harvested to determine the shoot

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458 fresh weight (SFW) and the root biomass (root dry weight; RDW) after a careful washing and oven
459 drying. More details of each determination were reported in Gioia *et al*. (2015).
460
461 **Transcriptomic analysis**
462 RNA extraction was performed using 100 mg of frozen ground tissue (leaves) and treated with
463 RNase-Free DNase by the On-Column DNase I Digestion Set (Sigma-Aldrich). For the subsequent
464 analysis only RNA samples with integrity greater than 8.0 were used. Library construction and RNA
465 sequencing were carried out at the Montpellier Genomix (http://www.mgx.cnrs.fr) sequencing
466 facility. Libraries quantification, RNA-Seq data filtering and processing used in this study were
467 essentially as those described previously by David *et al.* (2014). The bread wheat chromosome survey
468 sequence for the cv. Chinese Spring (http://plants.ensembl.org/triticum_aestivum) generated by the
469 International Wheat Genome Sequencing Consortium (IWGSC) was used as the reference assembly.
470 The Biomart package of EnsEMBL were used to acquire the transcripts, and the physical genomic
471 location of the 66,307 genes was predicted from the IWGSC on the genome A and B (Ensembl release
472 22, http://plants.ensembl.org/biomart/martview/). Since these sequences were obtained by separately
473 sequencing each bread wheat chromosome arm, the bread wheat reference helped to distinguish
474 paralogous durum wheat copies.
475 RNA-Seq reads were mapped on the bread wheat reference transcriptome using BWA (Li and Durbin,
476 2009) while allowing 3 errors (-n 3 in the alignment step). Picard tools (http://picard.sourceforge.net)
477 were used to remove PCR and optical duplicates. Rough read counts were computed at all sites for
478 each individual using the idxstats function of the Samtools.
479
480 **Metabolite Profiling**
481 After collection, part of the frozen leaves of each replicate were freeze-dried and successively milled
482 using a Pulverisette 7 Planetary Micro Mill (Classic Line, Fritsch) with an agate jar and balls, and
483 stored a -20°C until analysis.
484 A total of 30 mg dry weight (dw) of each replicate was used for the extraction, derivatisation and
485 analysis by gas chromatography–mass spectrometry (GC-MS) of the polar and non-polar metabolites
486 as previously described (Beleggia *et al*., 2013). Metabolites were identified by comparing the mass
487 spectrometry data with those of a custom library obtained with reference compounds and with those
488 of the National Institute of Standards and Technology (NIST 2011) database. The chromatograms
489 and mass spectra evaluation and quantification were performed using the Mass Hunter software.
490 The standards and all the chemicals used were HPLC grade (Sigma-Aldrich Chemical Co.,
491 Deisenhofen, Germany).

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492 **Statistical analysis**

493 Analysis of variance (ANOVA) was carried out with respect to each morphological trait and
494 metabolite detected in the shoot of emmer and durum wheat lines considered. Mean discrimination
495 between emmer and durum was performed applying Tukey's test and statistically significant
496 differences were determined at the significance level of $\alpha = 0.05$. Statistical analysis of the data was
497 performed using the JMP software (SAS Institute Inc., Cary, NC, USA version 8).

498

499 **Bioinformatics analysis and network construction**

500 *Data preprocessing*

501 First, genes for which the count per million (cpm) for a single sample was smaller than one and the
502 sum of cpms across all samples was smaller than the total number of samples were filtered out. Raw
503 counts were first normalized using trimmed mean of M-values normalization method (R package
504 edgeR (Robinson *et al*., 2010) and then voom normalized using the R package limma (Smyth, 2005).

505 *Analysis of differential expression*

506 Analysis of differential expression was conducted on the data after data preprocessing. DEGs were
507 determined between N starvation and a control with optimal N level for the following scenarios: (*i*)
508 for each genotype and (*ii*) between the two genotypes. For the two scenarios, a linear model was
509 employed to determine differential behavior. To this end, we applied the R package limma (Smyth,
510 2005).

511 *GO enrichment analysis*

512 Annotations were extracted from EnsemblPlants (Kinsella *et al*., 2011)
513 (http://plants.ensembl.org/biomart/martview/2ace56daacae40bad4af00cc25d51e4f) and agriGO
514 (http://bioinfo.cau.edu.cn/agriGO/download.php) (Du *et al*., 2010). We used hypergeometric test
515 (Kachitvichyanukul and Schmeiser, 1985) to identify enriched terms in the list of DEGs. The cut-off
516 value for significance level was considered as 0.05 after FDR correction.

517 *Network analysis*

518 Co-expression networks were extracted by applying Pearson correlation on all pairs of data profiles,
519 resulting in a similarity matrix $S_{m \times m}$. We then build a network $G = (V, E)$ with m nodes,
520 corresponding to the DEGs; there is an edge between two nodes $i, j \in V(G)$ if and only if the
521 entrys_{ij} of S is significant at the level of 0.05, after FDR correction.

522 Co-expression networks are separately reconstructed for the data from each genotype (*i.e.*, Molise
523 Sel. Colli and Simeto), by identifying significant correlation coefficients between each pair of genes
524 in the network (p value<0.05, FDR corrected). For each pair of genes in the network, the Fisher Z-
525 score test is used to assess the significance of the difference between the correlation coefficients

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526 obtained from emmer and durum wheat data. The edge between a pair of genes is referred to as a
527 *significantly different edge* if the obtained p-value from Fisher Z-score test is smaller than 0.05 after
528 FDR correction. The degree and the betweenness centralities of all nodes (i.e. DEGs) in co-expression
529 networks and the differential networks were calculated using the R package igraph (Csardi and
530 Nepusz, 2006). The same analysis was repeated for metabolite data, and integration of metabolite and
531 transcript data; however, the entire metabolite profiles were used in these cases.
532 To find the nodes (i.e., genes and metabolites) which capture the differences between the two
533 genotypes, we scored the nodes by the number of correlations of value larger than τ (τ was considered
534 to be 0.6 and 0.8) present in emmer but not in durum wheat co-expression network and vice versa.
535

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541

542 **Author Contributions**

543 RB and RP conceived and designed the study. TG, FF, US carried out the experiments and performed
544 the morphological analysis; RB and FN performed the metabolite analyses. YH and JD performed
545 the RNAseq analysis; NO and ZN performed the bioinformatics and network analyses. RB, RP, NO
546 and ZN analyzed data and wrote the paper. NP and PDV reviewed the manuscript. All authors read
547 and approved the final manuscript.
548

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554

555 **Conflict of Interest**

556 The authors declare no conflict of interest.
557
558
559

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Table 1. Summary statistics and differential behavior for 12 morphological and one physiological trait in emmer and durum wheat under two N conditions: N starvation (-N) and optimal N (+N) condition. Data are reported as mean ± SE

	G			N			G x N				
	emmer	**durum wheat**	**P value**	**-N**	**+N**	**P value**	**emmer x (-N)**	**emmer x (+N)**	**durum wheat x (-N)**	**durum wheat x (+N)**	**P value**
TLN	3.88±0.54	4.81±0.48	n.s.	**3.50±0.19^{b}**	**5.19±0.59^{a}**	**0.0173**	3.00±0.00	4.75±0.92	4.00±0.00	5.63±0.80	n.s.
TLA (cm^{2})	**24.85±5.66^{b}**	**41.92±8.39^{a}**	**0.0304**	**19.98±1.43^{b}**	**46.79±8.35^{a}**	**0.0023**	17.22±1.21	32.48±10.45	22.74±1.75	61.10±8.94	n.s.
SFW (g)	**0.63±0.19^{b}**	**1.17±0.30^{a}**	**0.0453**	**0.42 ±0.05^{b}**	**1.39 ±0.28^{a}**	**0.0017**	0.34±0.04	0.93±0.32	0.50±0.07	1.85±0.35	n.s.
PRL (cm)	155.58±26.79	172.72±25.42	n.s.	191.72±21.60	136.59±26.47	n.s.	189.27±31.72	121.89±39.82	194.16±34.17	151.28±39.25	n.s.
LRL (cm)	20.49±7.00	10.89±4.04	n.s.	15.28±5.19	16.10±6.70	n.s.	18.60±8.34	22.38±12.53	11.96±6.99	9.83±5.13	n.s.
TRL (cm)	176.07±32.17	183.62±27.96	n.s.	206.99±24.99	152.69±31.39	n.s.	207.87±39.64	144.27±50.83	206.12±36.65	161.11±44.36	n.s.
RSD (cm)	62.84±5.38	65.34± 3.66	n.s.	70.28±2.54	57.90±5.04	n.s.	68.69±4.09	56.98±9.76	71.86±3.41	58.82±4.74	n.s.
RSW (cm)	23.31±3.26	22.82±3.22	n.s.	24.79±3.08	21.34±3.27	n.s.	28.72±3.47	17.89±4.25	20.87±4.68	24.78±4.89	n.s.
RDW (g)	0.02±0.01	0.03±0.00	n.s.	**0.03±0.00^{a}**	**0.02±0.00^{b}**	**0.0228**	0.03±0.01	0.01±0.00	0.03±0.01	0.02±0.00	n.s.
SRL (m g^{-1})	**107.31±15.77^{a}**	**67.69±5.99^{b}**	**0.0094**	**70.31±5.93^{b}**	**104.69±16.65^{a}**	**0.0201**	**75.75±7.06^{b}**	**138.87±21.15^{a}**	**64.87±9.72^{b}**	**70.52±8.21^{b}**	**0.0449**
TRL/TLA (cm cm^{-2})	7.92±1.65	5.98±1.62	n.s.	**10.57±1.28^{a}**	**3.34±0.45^{b}**	**0.0002**	11.68±1.71	4.17±0.54	9.46±1.98	2.50±0.43	n.s.
LRL/PRL	0.11±0.04	0.06±0.02	n.s.	0.07±0.02	0.10±0.04	n.s.	0.09±0.04	0.14±0.07	0.06±0.03	0.05±0.02	n.s.
SPAD	**26.55±1.22^{b}**	**34.91±2.24^{a}**	**<0.0001**	**26.91±1.24^{b}**	**34.55±2.41^{a}**	**0.0001**	**24.35±0.91^{b}**	**28.75±1.72^{b}**	**29.48±1.40^{b}**	**40.35±1.31^{a}**	**0.0353**

TLN: Total leaf number; **TLA**: Total leaf area (cm^{2}) Calculated on all the leaf as leaf length. maximum width.0.858 (Kalra and Dhiman. 1976); **SFW**: Shoot fresh weight (g); **PRL**: Primary visible root length (cm); **LRL**: Lateral visible root length (cm); **TRL**: Total visible root length (cm); **RSD**: Visible root system depth (cm); **RSW**: Visible root system width (cm); **RDW**: Root dry weight (g); **TRL/TLA**: Total visible root length/total leaf area (cm cm^{-2}); **SRL**: specific root length (defined as TRL/RDW); **SPAD**: Leaf chlorophyll content (SPAD units).Values annotated in bold are significantly different (Tukey's test) and the character ‘a’/’b’ implies the higher/lower observed value for each significant change between the genotypes, the two N conditions and their interaction.

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Table 2. Networks of emmer, durum wheat, intersection and common networks of transcripts and metabolites data.

	emmer	**durum wheat**	**(durum wheat /emmer)**	**intersection**	**Common** (accepting the Fisher *z* test NULL hypothesis)
Number of edges in total	1,249,637	3,500,971	2.8	396,571	393,779
Number of edges DEG-DEG	1,237,748	3,473,768	2.8	394,015	393,719
Number of edges metabolite-metabolite	185	157	0.85	65	60
Number of edges DEG-metabolites	11,704	27,046	2.3	2,491	0
Number of nodes	1,829	3,167	1.7	1,129	1,127
Number of central nodes	260	479	1.8	367	398
Number of edges to the central nodes: DEGs – significantly behaved metabolites*	1,898	4,590	2.4	1,217	0

*significantly behaved metabolites considering the effect of G, N and GxN of the ANOVA model.

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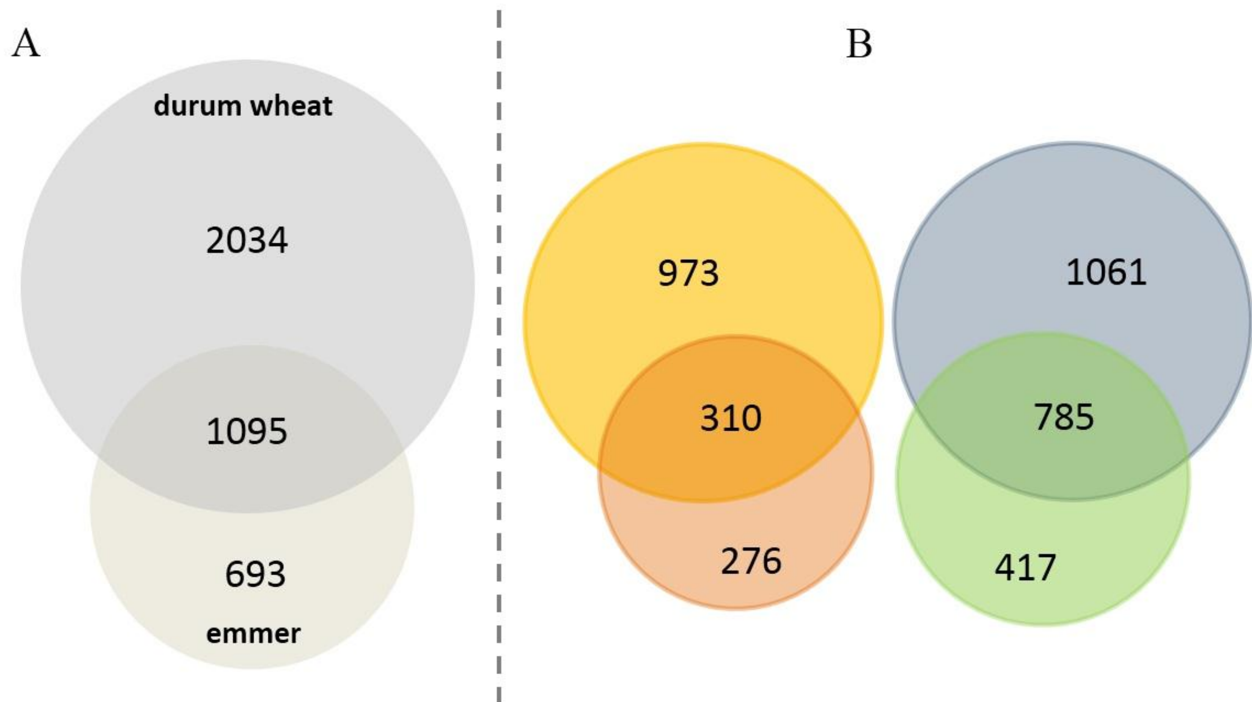
Figure 1. Differentially expressed genes between N starvation (-N) and optimal N (+N) conditions in durum wheat and emmer.



A: total DEGs between -N and +N for durum wheat and emmer; **B**: DEGs down regulated between -N and +N for durum wheat ( ) and emmer ( ); DEGs up regulated between -N and +N for durum wheat( ) and emmer ( )

Figure 2. Comparison of Gene Ontology classifications of DEGs in emmer and durum wheat. Blue and red color indicates the number of up- and down-regulated DEGs, respectively. All DEGs are categorized into 21and 23 functional groups based on GO classification for emmer and durum wheat, respectively.

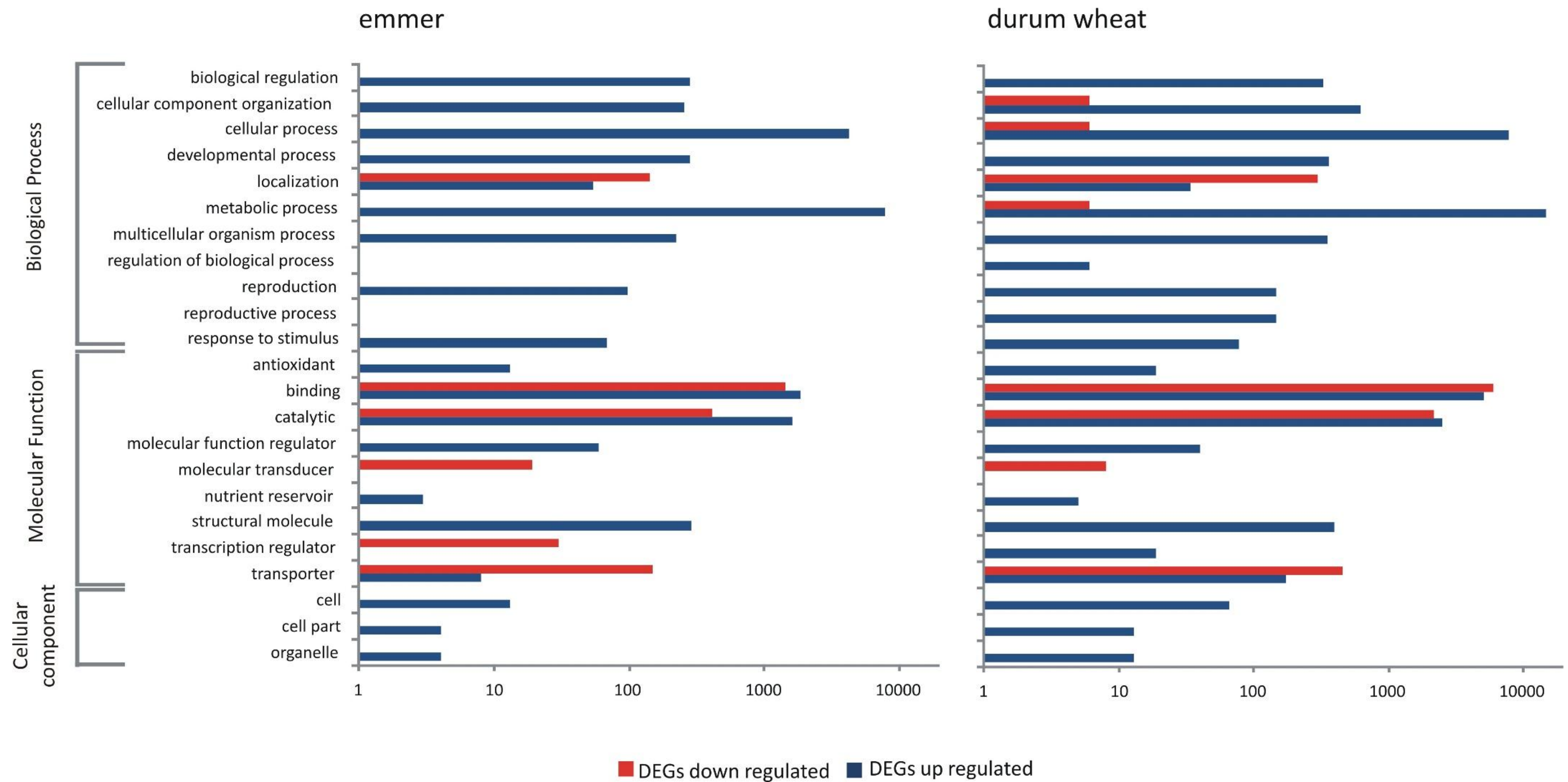


Figure 3. Metabolites exhibiting significant variation for emmer (red bars) and durum wheat (blue bars) under starvation (-N) and optimal (+N) levels due to the effect of GxN interaction. Bars with different letters are significantly different ($p < 0.05$)

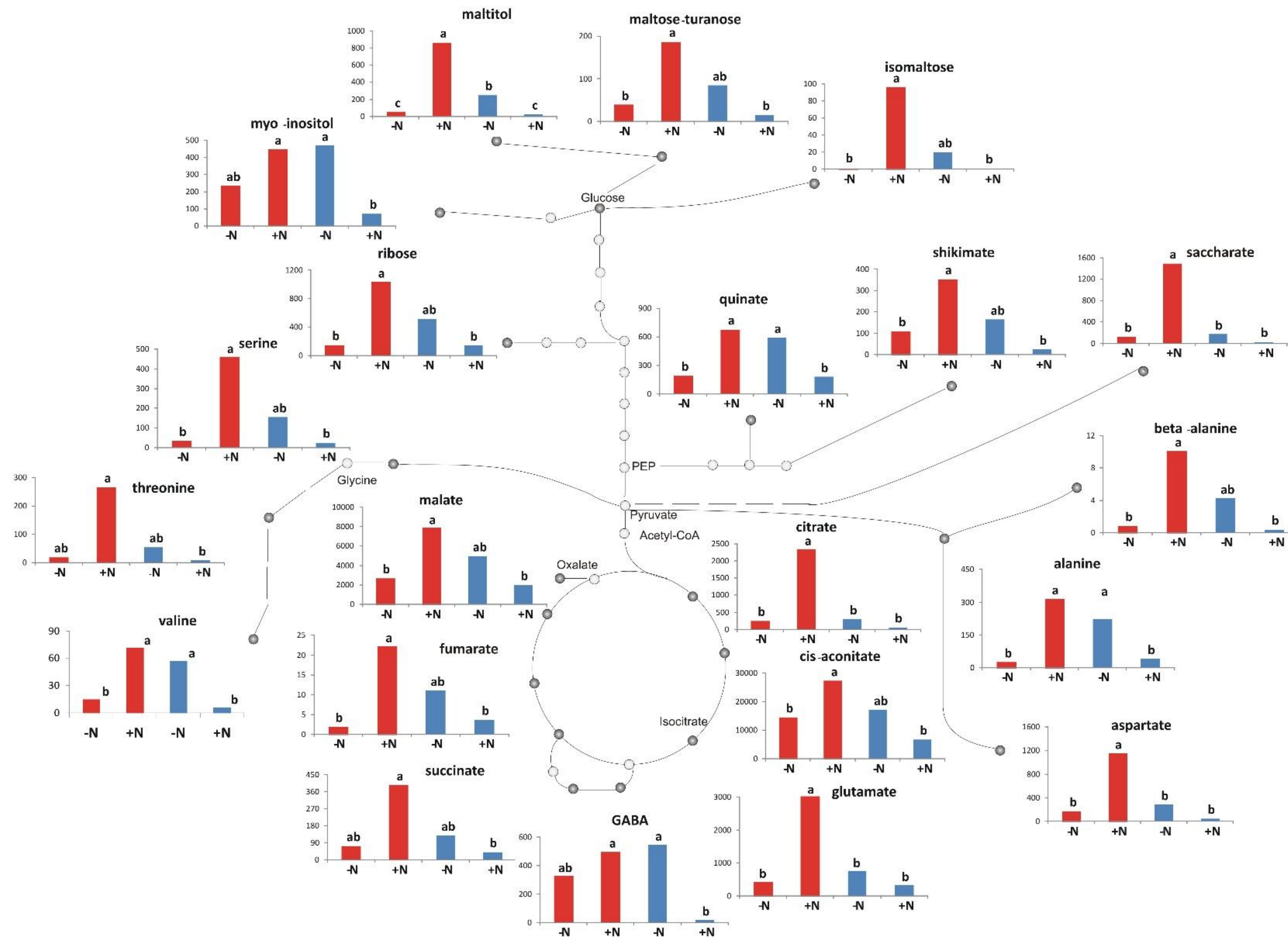


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Figure 4. Genotype-specific DEGs-metabolites correlation networks, emmer (A) and durum wheat (B).

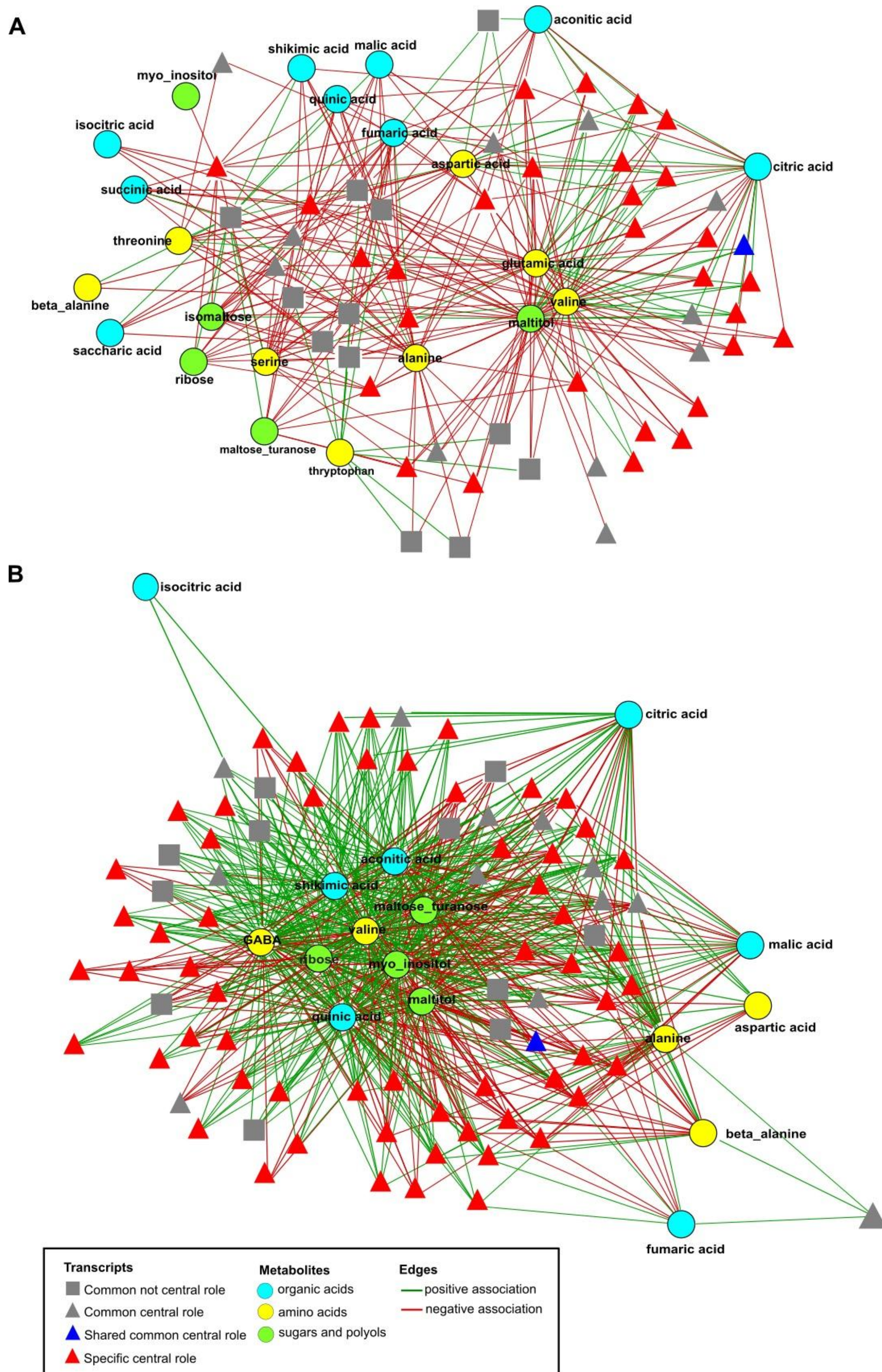

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Figure 5. Position of the central DEGs in emmer- and durum wheat-specific networks on the physical map. DEGs having putative annotations were shown with filled triangles.