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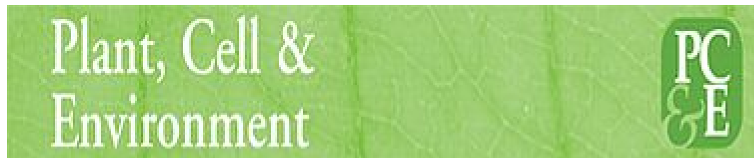

DIURNAL PATTERNS OF GROWTH AND TRANSIENT RESERVES OF SINK AND SOURCE TISSUES ARE AFFECTED BY COLD NIGHTS IN BARLEY

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Abstract:	Barley is described to mostly use sucrose for night carbon requirements. To understand how the transient carbon is accumulated and utilized in response to cold, barley plants were grown in a combination of cold days and/or nights. Both daytime and night cold reduced growth. Sucrose was the main carbohydrate supplying growth at night, representing 50-60% of the carbon consumed. Under warm days and nights, starch was the second contributor with 26% and malate the third with 15%. Under cold nights, the contribution of starch was severely reduced, due to an inhibition of its synthesis, including under warm days, and malate was the second contributor to C requirements with 24-28% of the total amount of carbon consumed. We propose that malate plays a critical role as an alternative carbon source to sucrose and starch in barley. Hexoses, malate, and sucrose mobilisation and starch accumulation were affected in barley elf3 clock mutants, suggesting a clock regulation of their metabolism, however without affecting growth and photosynthesis. Altogether, our data suggests that the mobilisation of sucrose and malate and/or barley growth machinery are sensitive to cold.

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1 **DIURNAL PATTERNS OF GROWTH AND TRANSIENT RESERVES OF SINK**
2 **AND SOURCE TISSUES ARE AFFECTED BY COLD NIGHTS IN BARLEY**
3

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29

30 **Keywords**: barley growth, cold, diurnal metabolism, carbon metabolism, sucrose, starch,

31 fructans, malate, *EARLY FLOWERING 3*, circadian clock

32 **ABSTRACT**

33 Barley is described to mostly use sucrose for night carbon requirements. To understand

34 how the transient carbon is accumulated and utilized in response to cold, barley plants were

35 grown in a combination of cold days and/or nights. Both daytime and night cold reduced

36 growth. Sucrose was the main carbohydrate supplying growth at night, representing 50-60%

37 of the carbon consumed. Under warm days and nights, starch was the second contributor with

38 26% and malate the third with 15%. Under cold nights, the contribution of starch was severely

39 reduced, due to an inhibition of its synthesis, including under warm days, and malate was the

40 second contributor to C requirements with 24-28% of the total amount of carbon consumed.

41 We propose that malate plays a critical role as an alternative carbon source to sucrose and

42 starch in barley. Hexoses, malate, and sucrose mobilisation and starch accumulation were

43 affected in barley *elf3* clock mutants, suggesting a clock regulation of their metabolism,

44 however without affecting growth and photosynthesis. Altogether, our data suggests that the

45 mobilisation of sucrose and malate and/or barley growth machinery are sensitive to cold.

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49 ## 1 INTRODUCTION

50 Plants are growing during both day and night (Walter, Silk & Schurr, 2009), but can
51 reduce CO_2 to produce carbohydrates only in the light during photosynthesis. It is yet not clear
52 how plants can orchestrate these two major fundamental processes. Growth and photosynthesis
53 are partly temporally distinct and they are also spatially separated between source and sink
54 tissues (Ludewig & Sonnewald, 2016, Schnyder, 1993, Wang & Tillberg, 1996). Thus, to fully
55 understand the cross-talk between photosynthesis and growth, it is necessary to analyse sink
56 and source tissues separately, and also gather temporal information. The storage of
57 photoassimilates is spatially separated in source and sink tissues, not only for daily/night
58 requirements for growth and maintenance, but also over long time for e.g. supplying flowering
59 and grain development (Schnyder, 1993, Smouter & Simpson, 1991). Changes in carbohydrate
60 metabolism are vital to overcome abiotic stresses (Pommerrenig, Ludewig, Cvetkovic,
61 Trentmann, Klemens *et al.*, 2018) and as such the partitioning of photoassimilates is affected
62 by environmental factors but also developmental processes. The most studied and common
63 transient carbon (C) storage in plants is starch. However, barley, wheat and some grasses, might
64 not primarily use starch like *Arabidopsis*, *Brachypodium* or maize as a transient C store for
65 night usage, but can also use sucrose and possibly fructans (Farrar & Farrar, 1985, Nagaraj,
66 Altenbach, Galati, Luscher, Meyer *et al.*, 2004, Nagaraj, Riedl, Boller, Wiemken & Meyer,
67 2001).

68 *Arabidopsis* C reserves and diurnal growth are highly controlled by circadian clock
69 genes (Graf, Schlereth, Stitt & Smith, 2010). However, in *Zea mays* and *Oriza sativa,* growth
70 is stable over the diurnal cycle and strongly affected by temperature regimes, in contrast with
71 dicotyledonous species (Poire, Wiese-Klinkenberg, Parent, Mielewczik, Schurr *et al.*, 2010).
72 Thus, monocots and dicots might have different sensitivities regarding the respective

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73 influences of the clock and the environment on the growth patterns, and thus likely C reserves
74 utilisation. In other words, clock genes are conserved amongst dicotyledons and
75 monocotyledons; however, they might not to have the same importance in the control of diurnal
76 growth and transient C reserves amongst these groups (Müller, von Korff & Davis, 2014).

77 Previous studies on photoassimilates in barley showed that the main one is sucrose,
78 with low amounts of starch and fructans (Gordon, Ryle, Mitchell & Powell, 1982, Gordon,
79 Ryle & Powell, 1977, Gordon, Ryle & Powell, 1979, Gordon, Ryle, Powell & Mitchell, 1980a).
80 However, the analysis of starch in barley and more generally fructan-accumulating plants has
81 been largely neglected, so it is not yet clear how they compete for photosynthates. Moreover,
82 no large quantitative metabolite studies over a diurnal time course have been performed, so
83 some other important metabolites cannot be excluded. Fructans are known to play an important
84 role on cold tolerance in fructan accumulating species (Abeynayake, Etzerodt, Jonaviciene,
85 Byrne, Asp *et al.*, 2015, del Viso, Puebla, Fusari, Casabuono, Couto *et al.*, 2009, Jeong &
86 Housley, 1990, Meguro-Maoka & Yoshida, 2015, Rao, Andersen, Dionisio & Boelt, 2011,
87 Tamura, Sanada, Tase & Yoshida, 2014, Tarkowski & Van den Ende, 2015). However, little
88 is known about the diurnal regulation of fructan levels and their potential role as transient
89 storage of C for night usage (Schnyder, 1993). In barley, fructan mobilization at night has been
90 suggested (Farrar & Farrar, 1985) and the accumulation at the base of young leaves has been
91 hypothesised to supply growth of new leaves (Roth, Luscher, Sprenger, Boller & Wiemken,
92 1997). Sucrose has been described previously as a transient carbon store in grasses and it is
93 also the substrate for fructan synthesis (Nagaraj *et al.*, 2004, Nagaraj *et al.*, 2001, Ritsema,
94 Brodmann, Diks, Bos, Nagaraj *et al.*, 2009). Even though fructan synthesis is correlated to
95 increase in sucrose levels (Nagaraj *et al.*, 2001), in another study (Jin, Fei, Rosenquist, Jin,
96 Gohil *et al.*, 2017), the authors described a mechanism linking fructan and starch synthesis
97 through a single gene that encodes two transcription factors named SUSIBA (sugar signalling

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98 in barley). These transcription factors have different lengths and respond to different sucrose
99 concentrations, acting in an antagonistic and auto-regulatory way, which result in the control
100 of the rates of starch and fructan synthesis in barley.

101 A common strategy of plants to acclimate to cold is the accumulation of water soluble
102 carbohydrates, the type of sugars accumulated varying between species (Ruelland, Vaultier,
103 Zachowski & Hurry, 2009). The recommended sowing period for spring barley in Ireland is
104 from late February to March. Thus, spring barley faces at a very early stage low temperature
105 during the day and night in early spring that later changes to warmer temperatures and longer
106 daylength through late spring and summer. Sowing date can be a determinant of the final yield
107 of cereal crops (Conry, 1995, Conry, 1998, Potterton & McCabe, 2018). If they undergo higher
108 temperature at tillering stage, they transition faster between developmental stages (Kirby,
109 Appleyard & Fellowes, 1982) and may produce smaller leaves and less tillers which could
110 impact the number of ears and consequently lower yield. Studies on sowing date for spring
111 barley show that the earlier the sowing, the higher the yield (Conry, 1995, Conry, 1998, Kirby
112 *et al.*, 1982, Kumar, Singh, Hooda, Sewhag & Chaudhary, 2017, Photiades &
113 Hadjichristodoulou, 1984, Potterton & McCabe, 2018). Thus, although spring varieties are less
114 exposed than winter varieties to cold, they still experience cold at early stage, which seems to
115 be crucial in regulating their development (Kirby, Appleyard & Fellowes, 1985) and thus a full
116 understanding of the response of spring barley to cold could help increase growth rates and
117 subsequently yields. Cold and freezing tolerance in plants are achieved by a combination of
118 increased protein content, sugars and other soluble metabolites such as compatible solutes (*e.g.*
119 proline, betaines, sugar alcohols) or flavonoids (Al-Hamdani & Thomas, 2001, Bourion,
120 Lejeune-Henaut, Munier-Jolain & Salon, 2003, Hurry & Huner, 1992, Janmohammadi, Mock
121 & Matros, 2014, Lorenzo, Assuero & Tognetti, 2015, Oquist, Hurry & Huner, 1993, Savitch,
122 Harney & Huner, 2000, Trischuk, Schilling, Low, Gray & Gusta, 2014, Tyrka, Rapacz, Fiust,

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123 Wójcik-Jagła & Rognli, 2015, Visioni, Tondelli, Francia, Pswarayi, Malosetti *et al.*, 2013).
124 Freezing and cold tolerance are mainly orchestrated by C-REPEAT-BINDING FACTOR
125 (CBF) genes (Cook, Fowler, Fiehn & Thomashow, 2004, Pare, Gilmour, Grumet &
126 Thomashow, 2018, Shi, Ding & Yang, 2018, Thomashow, 1999, Thomashow, 2010). The
127 circadian clock also seems to be part of the pathway regulating cold acclimation in Arabidopsis,
128 with a number of metabolites involved in cold acclimation showing circadian oscillations under
129 free running cycles in the cold, and clock mutants exhibiting impaired freezing tolerance
130 (Espinoza, Degenkolbe, Caldana, Zuther, Leisse *et al.*, 2010). Among clock mutants, *EARLY*
131 *FLOWERING 3* (*elf3*) has been involved in growth and temperature responses (Box, Huang,
132 Domijan, Jaeger, Khattak *et al.*, 2015, Ford, Deng, Clausen, Oliver, Boden *et al.*, 2016).
133 To obtain a better understanding of the temporal and spatial mobilization of transient C
134 stores to supply growth at night when spring varieties are still at early developmental stages,
135 and analyse the effects of cold treatments, we grew barley seedlings in three thermo regimes,
136 warm days and nights (22ºC:18ºC), warm day and cold nights (22ºC:4ºC) and cold days and
137 nights (10ºC:4ºC). We characterised photosynthetic traits and growth of the plants. Sink and
138 source tissues above ground were harvested during a 24 h time course and analysed for their
139 content in primary metabolites. Because the involvement in cold tolerance by the clock has
140 been suggested, we also included in our study *elf3* spring barley mutants.

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141

142 # 2 MATERIAL AND METHODS

143 ## 2.1 Plant material, growth conditions and harvest

144 Barley seeds (*Hordeum vulgare* L.) of spring variety Propino were germinated in
145 darkness at 24 ºC for 3 days on dampened paper. One seedling was transferred per pot, filled
146 with Bord na Móna potting substrate plus⁺ (Bord na Móna Horticulture Ltd., Ireland, and all
147 pots were transferred to a growth chamber (LED-36HVL LT, Percival Scientific, Inc., USA).

148 Plants were submitted to three temperature conditions: warm day and night at 22 ºC:18 ºC as
149 control, cold day and night at 10 ºC:4 ºC and cold only at night at 22ºC:4ºC; under 500 µmol
150 photons and a photoperiod of 12h:12h light:dark for all conditions. Plants were harvested when
151 they reached 3 leaf stage, with the third leaf – youngest leaf – being 3 to 5 cm above the ligula.
152 The middle section of sheaths and blades of each leaf were harvested separately. Three
153 replicates were harvested at five timepoints covering a period of 24 h, each replicate consisting
154 of the pooled sheaths or blades from three different plants. Samples were frozen in liquid
155 nitrogen, grinded to fine powder and then stored at -80 ºC for metabolic analyses

156 Seeds of the spring barley cv. Bowman and introgression lines 289 and 290 in this
157 cultivar, that carries introgression of the *eam8.k* allele, were germinated in dark at 24 ºC for 3
158 days on dampened paper and then transferred to growth chamber equipped with LED lights
159 (C75-NS1, C75-AP67, Valoya, Finland) into pots with Bord na Móna potting substrate plus^{+}
160 (Bord na Móna Horticulture Ltd., Ireland). The *eam8.k* allele is characterized by a base-pair
161 mutation leading to a premature stop codon in *HvELF3*, which is orthologous to *ELF3* in
162 Arabidopsis (Faure, Turner, Gruszka, Christodoulou, Davis *et al.*, 2012). Each introgression
163 line was grown with WT at 500µmol photons m^{-2}, 22 ºC:18 ºC, 10 ºC:4 ºC and 22ºC:4ºC
164 day:night; and a photoperiod of 12 h:12 h light:dark. Genotypes were randomly distributed in
165 the chamber and three replicates were harvested at five timepoints covering a period of 24 h,
166 each replicate consisting of 3 pooled sheaths or blades from different plants with third leaf –
167 youngest leaf – being 3-5 cm above the ligula. Samples were freeze-dried, grinded to fine
168 powder and then stored in container with silica gel.

169 Crowns of five plants were harvested at end of light and dark periods of all temperature
170 combinations for Propino, Bowman WT and introgression lines, frozen in liquid nitrogen,
171 grinded to fine powder and then stored at -80 ºC for metabolic analyses.

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172 **2.2 Elongation rate and chlorophyll fluorescence parameters**

173 Second and third blades were marked at the base of the blade at 0 h, then at end of night
174 period (12 h) were marked again at the base of the blade and lastly at end of day (24 h). The
175 elongation rate was calculated by the difference of each period's measurement divided by the
176 duration in hours of the period: 12 h for night and 12 h for day.

177 **2.3 Chlorophyll fluorescence parameters and gas exchange**

178 Chlorophyll fluorescence parameters were taken using a PAM-2500 (Heinz Walz
179 GmbH, Germany). The maximum photochemical quantum yield of PSII (F_v/F_m) and the
180 effective photochemical quantum yield of PSII (Y(II)) were determined at steady state of
181 chlorophyll fluorescence with a saturation pulse of 8.000 µmol $m^{-2}s^{-1}$ (Genty, Briantais &
182 Baker, 1989, Kitajima & Butler, 1975). ETR was calculated according to PAM-2500 handbook
183 guidelines.

184 The net photosynthesis (A_N), the stomatal conductance (g_s), sub-stomatal CO_2
185 concentrations and transpiration (E) were measured in open system infra-red gas exchange (LI-
186 6400XT, LI-COR, Lincoln, NE, EUA). The temperature of the chamber was kept at 22 °C for
187 warm day plants and 10 °C for plants under cold day, the gas chamber being temperature
188 controlled. The vapour pressure deficit (VPD) was kept around 1.1 kPa, the CO_2 concentration
189 was set at 400 ppm, light flux set to 500 µmol photons.$m^{-2}.s^{-1}$ of photosynthetic active radiation
190 (PAR). Measurements were taken on the second blade for all temperature conditions.

191

192 **2.4 Metabolites determination**

193 For metabolic analyses, 20 mg of frozen powder was submitted to ethanolic extraction.
194 Sequential extractions with ethanol concentrations of 98%, 80% and 50% were performed and
195 between each step the samples were incubated at 85ºC for 20 min and centrifuged at 3220 *g*

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196 for 10 min . The ethanolic phase was used to determinate soluble sugars and malate while starch
197 and proteins were determined in the pellet. Glucose and fructose were determined according to
198 with minor modifications. We used 0.6 U.µl^{-1} NAD^{+} dependant G6PDH and the determination
199 of sucrose was performed using 0.25 U.µl^{-1} α-glucosidase (E-MALTS, Megazyme u. c.,
200 Ireland). The production of NADH was determined at 340 nm using a spectrophotometer model
201 ELx800™ (BioTek Instruments, Inc., USA).

202 Fructans were determined after completion of sugar analyses, using the same
203 determination plate. The NADH and enzymes used for sugar analyses present in the wells were
204 hydrolysed by addition of 10 µl HCl 1 M and the plate was sealed and incubated at 95 ºC for
205 30 min. Then the plate was cooled on ice and extracts neutralized with 10 µl NaOH 1 M. To
206 each well, 7 µl of acetate buffer 0.1 M pH 4.9 were added to the plate and 1 µl of a mix
207 containing 0.1 U.µl^{-1}. endo-inulinase and 0.1 U.µl^{-1} exo-inulinase (respectively E-ENDOIAN,
208 E-EXOIAN, Megazyme u. c., Ireland). The plate was then sealed and incubated overnight at
209 37 ºC. To determine fructans, 75 µl of Hepes buffer 0.5 M pH 7 containing 3 mM ATP and 1.3
210 mM NAD was added in each well. After obtention of a sTable baseline at 340 nm, 1 µl of 0.6
211 U.µl^{-1} glucose-6-phosphate dehydrogenase, 1 µl 0.9 U.µl^{-1} hexokinase and 1 µl 0.3 U.µl^{-1}
212 phosphoglucose isomerase were added sequentially for the determination of glucose and
213 fructose molecules present in fructans. Starch was determined as previously described by
214 Hendriks, Kolbe, Gibon, Stitt and Geigenberger (2003). Malate was determined according to
215 Cross et al. (2006). Proteins were determined by the method described by Lawry et al. (1951),
216 adapted to 96-well plate.

217 **2.5 Water content and carbon content estimations**

218 The water content was determined on the second blade. Five leaf discs per blade were
219 collected on six plants. The 30 discs were excised and immediately weighed. Then the discs

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220 were dried in a drying cabinet at 70 ºC for 72 h and weighed again. Then the difference was
221 used to calculate the percentage of water and dry matter per gram of fresh weight.
222 For the calculation of carbon accumulation and consumption, we used the metabolite
223 content determined at end of day and end of night in the different plant organs, multiplying the
224 concentration of metabolite by the number of carbon atoms present in each molecule, i.e. 6 for
225 glucose, fructose, sucrose (equivalent glucose), fructans (equivalent glucose), starch
226 (equivalent glucose) and 4 for malate. Then, C concentration at end of day (µmol $C.g^{-1}$ FW)
227 and C consumption at night (µmol $C.g^{-1}$ FW) were estimated at whole plant levels by taking
228 into account the respective weights of each organ per plant. The carbon consumption at night
229 was estimated by the difference between content found at the first, last (end of night, EN) and
230 third time point (end of day, ED).

231 **2.6 Statistical analysis**

232 For the comparisons between ED and EN for elongation rates and metabolite levels,
233 independent t-test were carried out, using six replicates. For comparison of temperature
234 treatments and genotypes, , ANOVA was applied followed by Tukey test, using 3-6 replicates.
235 All tests were conducted on IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY,
236 IBM Corp. Means were considered significantly different at $P<0.05$.

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237 **3 RESULTS**

238 **3.1 Growth is reduced by cold, proteins are only affected by daytime cold, and**
239 **chlorophyll fluorescence parameters are only marginally affected**

240 Barley grown under 10 ºC:4 ºC temperature regime presented a different physiological
241 response compared to plants grown under 22 ºC:18 ºC and 22 ºC:4 ºC temperature regimes,
242 with a significant decrease in their height (Figure S1a) despite a similar biomass (Figure S1b).
243 Moreover, plants grown under 10 ºC:4 ºC showed a lower leaf 2 water content (Table 1)

244 compared to other conditions. For all conditions the fluorescence parameters were similar
245 (Table 1), at the exception of a significant small decrease in F_v/F_m, Y(II) and ETR observed in
246 plants grown under cold day and night. Highest protein contents were observed in blades of all
247 leaves and the sheath of the third leaf for all treatments (Figure 1). Protein levels did not show
248 any diurnal turnover. In warm daytime and cold night, the protein levels were similar to those
249 observed under warm daytime and night (Figure 1a-b). However, when plants were grown in
250 cold daytime and night, protein levels increased in all tissues (Figure 1c). All plants were
251 harvested at the same stage of development, with the third leaf being exposed by 3cm at least
252 and not more than 5 cm. Plants grown in 22 ºC:18 ºC reached this stage 15 days after sowing
253 (DAS), while plants under 10 ºC:4 ºC took 42 DAS, and 22 ºC:4 ºC plants 20 DAS. Elongation
254 rates at night were lower than during daytime for all treatments. Second leaves presented lower
255 elongation rate (Figure 2a) than third leaves (Figure 2b) in all conditions, suggesting that
256 second leaves were reaching maturity. Plants grown under 10 ºC:4 ºC showed on average a
257 75% reduction in the elongation rates of their second and third leaves compared to control
258 condition (Figure 2). Plants submitted to cold only at night showed similar elongation rates to
259 the 22 ºC:18 ºC treatment during the daytime, but at night elongation rates were comparable to
260 plants grown under 10ºC:4ºC.

261 **3.2 Diurnal patterns of soluble sugars, fructans, starch and malate are affected by**
262 **temperature**

263 Blades contained very low concentrations of both glucose and fructose, but sheaths
264 contained higher levels. Glucose and fructose predominantly accumulated in the youngest
265 sheath (leaf 3) for the three temperature regimes. However, glucose and fructose were only
266 almost fully consumed under the 22 ºC:18 ºC treatment (Figure S2a and Figure S3a, all
267 metabolic data in Table S1) and their turnover was strongly inhibited by the cold night plants
268 faced in the 10 ºC:4 ºC and 22 ºC:4 ºC temperature regimes (Figure S2b-c and Figure S3b-c).

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269 When cold was present during day and night, glucose and fructose turnover was totally
270 abolished in leaves while a small turnover was only observed for glucose when plants were
271 grown under the 22 ºC:4 ºC temperature regime.

272 Sucrose content in sheaths of plants grown in the three conditions was low and almost
273 no turnover was observed (Figure 3). In contrast, sucrose predominantly accumulated in old
274 blades, and a high turnover was observed for all three temperature regimes. Under the 22 ºC:18
275 ºC temperature regime, higher sucrose and starch levels were observed at ED2 compared to
276 ED1, in particular for the youngest leaf, likely due to the blades getting mature. Temperature
277 had an effect on sucrose accumulation, with the blades of plants grown under the 10 ºC:4 ºC
278 temperature regime exhibiting almost twice the concentration of sucrose observed in the blades
279 of the plants grown under 22 ºC:18 ºC (Figure 3a and 3c). Interestingly, when the cold was
280 applied only at night (22ºC:4ºC), the accumulation and turnover of sucrose in the blades was
281 very similar to those of plants grown under the 22 ºC:18 ºC temperature regime (Figure 3b).

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282 Fructan levels were not affected in the same manner as sucrose (Figure S4). Plants
283 grown under warm day and night conditions and those which experienced only cold nights
284 accumulated very low amounts of fructans in both blades and sheaths, and no turnover was
285 observed (Figure S4a-b). A small accumulation of fructans was observed in the oldest blade
286 and youngest sheath of plants grown under the 10 ºC:4 ºC treatment (Figure S4c). It is different
287 from sucrose that accumulated predominantly in older blades, but not in sheaths. Despite the
288 low levels of fructans accumulated in first blade and third sheath, the turnover of fructans
289 observed in these tissues was about 50%.

290 Plants grown under warm days and nights showed the highest starch accumulation,
291 mainly in the second and third blades (Figure 4a) and starch was nearly exhausted by the end
292 of the night. In the presence of cold treatment, either both in daytime and night or solely at
293 night, starch accumulation was reduced to less than 25% of the levels observed in blades of

294 plants grown under 22 ºC:18 ºC (Figure 4b-c). However, starch was still almost fully consumed
295 at night for both cold treatments.

296 Malate showed different patterns according to the temperature in which plants were
297 grown. Plants under 22 ºC:18 ºC showed increased malate concentration in the third sheath and
298 blade as well as a small turnover (Figure 5a), with a small accumulation in other tissues. Under
299 warm daytime and cold nights, plants showed an intermediary accumulation of malate in the
300 first and second leaves compared to 22 ºC:18 ºC and 10ºC:4ºC, and highest levels in both blades
301 and sheaths of the third leaves, so in the youngest tissues (Figure 5b). When plants were
302 submitted to 10 ºC:4 ºC, high levels of malate were again observed in youngest tissues, but also
303 in the blade of leaf 2, malate being consumed in blades at night (Figure 5c).

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304 **3.3 Metabolite accumulation in the daytime and their consumption at night are**
305 **modified in source tissues to supply growth in cold nights**

306 Plants grown under 22 ºC:4 ºC accumulated the highest amounts of metabolites at the
307 end of the day while plants under 10 ºC:4 and 22 ºC:18 ºC ºC accumulated similar levels (Table
308 4). Sucrose followed by starch and malate were the main metabolites accumulated during the
309 light period for warm day and nights, but malate was second major metabolite for plants grown
310 under warm days and cold nights and cold days and nights. Starch accumulation was drastically
311 reduced in plants grown under the 10 ºC:4 ºC (67%) and 22 ºC:4 ºC (43%) temperature regimes
312 compared to 22ºC:18ºC, and this decrease was compensated by an increase in malate content
313 compared to 22 ºC:18 ºC (Table 4).

314 The consumption of C at night by 22 ºC:18 ºC control plants was around 94% of the
315 total C accumulated during the day, while for plants grown under 10 ºC:4 ºC the consumption
316 was 74% and significantly reduced for plants grown under 22 ºC:4 ºC with 64% (Table 4). The
317 proportion of each metabolite consumed under the three temperature regimes was very similar
318 to the proportion of metabolites accumulated at the end of the day, with sucrose being the main

319 contributor. Starch was the second contributor under warm days and nights, but malate was
320 more used at night under the two cold treatments, being particularly important under 22 ºC:4
321 ºC, contributing with 28% of the total carbon consumed, despite a stark decrease in the turnover
322 of malate (Table 4). Indeed 84% of the malate accumulated at ED was consumed during 22
323 ºC:18 ºC, but only 53% during cold nights in the 22ºC:4ºC temperature regime.

324 In general, sheaths had a low contribution to the supply of carbon for night use, at the
325 exception of the sheaths of third leaf where malate and glucose were the main providers of
326 carbon (Figure 6). Under 22 ºC:18 ºC, the oldest blade provided predominantly sucrose, while
327 the second blade provided almost equally sucrose and starch and the third blade sucrose, starch
328 and malate (Figure 6a). However, when plants are submitted to 10 ºC:4 ºC, all blades primarily
329 provided sucrose, followed by malate (Figure 6c). Interestingly, in this condition, the old blades
330 contributed more than the youngest blade to the provision of C at night, in stark contrast to
331 warm days and nights where it is the opposite (Figure 6c). Unexpectedly, plants under 22 ºC:4
332 ºC presented a very irregular pattern of carbon consumption. Sheaths showed a slight sucrose
333 accumulation during the night, and the third sheath provided malate. Blades still consumed
334 more carbon than sheaths and relied mostly on sucrose and malate from the first and second
335 blades, while the youngest blade consumed mainly starch (Figure 6b).

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336 3.4 Cold nights affect mobilization of carbohydrates in *elf3* introgression lines without 337 affecting photosynthesis and leaf elongation rates

338 The involvement of the circadian clock, particularly the *elf3*, in temperature responses and
339 sugar metabolism has been described for Arabidopsis (Box *et al.*, 2015, Flis, Mengin, Ivakov,
340 Mugford, Hubberten *et al.*, 2019). However, it has been proposed that the growth and C
341 metabolism of monocots may not be affected on the same extent by circadian clock (Poire *et*
342 *al.*, 2010). Therefore, to evaluate the effect of *elf3* on the partitioning of C compounds under
343 cold in barley, Bowman WT and introgression lines 289 and 290 were grown under the three

344 growth conditions tested for Propino. When grown in 22°C:4°C, all genotypes took 20 days to
345 reach same stage of development with third leaf developing and did not present differences in
346 leaf elongation rates (Table 2). We measured net photosynthesis, stomatal conductance,
347 internal concentration of CO_2, transpiration and water use efficiency (Table 3). No significant
348 differences were observed between Bowman WT and *elf3* mutants. However, photosynthesis
349 was decreased when cold day and nights were applied for all genotypes. Under only cold night
350 condition, Propino showed a significantly decreased rate of photosynthesis compared to warm
351 days and nights, other lines showing a non-significant tendency of decrease in photosynthesis
352 rate (Table 3). The stomatal conductance and transpiration rate were increased under 10 ºC:4
353 ºC for all genotypes. This resulted in a lower WUE to all genotypes under 10 ºC:4 ºC (Table
354 3).

355 Primary metabolites were determined in blades and sheaths of WT and *elf3*
356 introgression lines grown under 22 ºC:4 ºC at end of day and end of night period. As expected,
357 cv. Bowman (WT) showed very similar metabolite patterns as those observed for Propino
358 (Table S2).

359 Sheaths of third leaves contained more glucose than other tissues at end of day, and its
360 content was lower in introgression lines than WT. Also introgression lines accumulated less
361 glucose at end of day in the second sheath and blade (Table S2), which resulted in less C
362 available for use at night. Fructose mostly accumulated in youngest sheath, and in all tissues
363 its levels either remained stable or even increased during the night. No consistent significant
364 differences were observed amongst genotypes for fructose levels except for the youngest leaf
365 which contained less fructose at the end of the night and second and third sheaths at end of the
366 day (Table S2). Sucrose was the second most accumulated C reserve in all genotypes, and the
367 highest levels were found at end of day in the first and second blades. Most tissues showed
368 partial consumption of sucrose at night in all genotypes (Table S2). Both introgression lines

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369 presented slightly higher content of sucrose in first and second blades at end of night compared
370 to WT (Table S2), an indication of less carbon consumed at night due to impaired function of
371 *elf3*. Starch was not highly accumulated in blades or sheaths but was consumed at night in all
372 tissues of all genotypes. Second and third blade accumulated more starch at end of day than
373 other tissues in all genotypes under 22 ºC:4 ºC (Figure 7). However, introgression lines
374 accumulated less starch at the end of day and also presented slightly higher level of starch than
375 WT at end of night (Figure 7, Table S2). Blades of the first leaf accumulated more fructans
376 than other tissues, with no significant difference between genotypes (Table S2). Despite low
377 levels accumulated at end of day, mobilization of fructans was observed in the blade of first
378 and second leaf for all genotypes. Malate was the most accumulated C compound in both blades
379 and sheaths of all tissues and genotypes grown under 22ºC:4ºC. Malate was slightly mobilized
380 in all blades at night in WT, but it was not observed in introgression lines (Table S2). Also
381 malate levels were decreased in introgression lines in the first and second blades in the
382 introgression lines, but increased the sheath of first leaf at end of the night, compared to WT.
383 The proteins content was similar to all genotypes, although higher content was observed in
384 blades of first and second leaves and third leaf parts with no significant mobilization at night
385 (Table S2).

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386 3.5 Crown has little participation in C supply for shoot growth under cold with 387 incomplete C consumption in *elf3* introgression lines

388 To evaluate the contribution of the crown in the partition of carbohydrates and supply
389 of growth under cold, we harvested 1 cm of crown tissue at end of day and end of night of all
390 the genotypes grown under 22ºC:18ºC, 22 ºC:4 ºC and 10ºC:4ºC. The crown region comprises
391 all meristematic tissues from which the apical meristem originates. The content of C
392 compounds in crowns was much lower than in the shoot for all temperature conditions and
393 genotypes, below 6 µmol g^{-1} FW for glucose, fructose and starch in all growth conditions.

394 Under warm days and nights, malate and fructans were the main metabolites with up to 20 and
395 µmol g^{-1} FW, both compounds decreasing at night. Under warm days and cold nights, fructans
396 and malate were again the main metabolites, with similar levels, but their levels did not
397 decrease at night, and even increased for the fructans. Under cold days and nights, sucrose,
398 fructans and malate were the major metabolites, reaching up to 26 µmol g^{-1} FW (Table S3).
399 Protein content was very similar for all genotypes, with little variation between temperature
400 conditions.

401 Under 22 ºC:18 ºC, Propino contained slightly more C compounds in crowns than
402 Bowman WT. The *elf3* mutants presented similar levels of carbohydrates compared to WT,
403 although lower levels of starch and malate. Under 22 ºC:4 ºC, only fructans at ED were lower
404 than WT in *elf3* mutants. No consistent difference between WT and the *elf3* mutants were
405 observed for all metabolites in crowns of plants grown under 10ºC:4ºC (Table S3).

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406 4 DISCUSSION

407 4.1 Growth of barley is sensitive to both day and night cold

408 Barley reached three leaf stage at 15, 20 and 42 days when grown under 22ºC:18ºC, 22
409 ºC:4 ºC and 10ºC:4ºC, respectively. Thus, low temperatures both during the day and the night
410 have a negative impact on the growth of young barley, which is in agreement with previous
411 studies performed on barley and other monocotyledons (Poire *et al.*, 2010, Walter *et al.*, 2009).
412 This is in stark contrast with Arabidopsis where growth is largely insensitive to cold night
413 temperatures (Müller, Gol, Jeon, Weber, Davis *et al.*, 2018, Pyl, Piques, Ivakov, Schulze,
414 Ishihara *et al.*, 2012). The maintenance of the growth in Arabidopsis was explained by an
415 insensibility of starch degradation machinery to temperature, allowing C resources to be
416 available even when temperature dropped, and an apparent excess in the growth machinery at
417 optimal temperatures, via e.g. an incomplete mobilisation of the ribosomes for translation at
418 warm temperatures, thus allowing the plants to mobilise this excess growth capacity when

419 temperature was dropping. Interestingly, in barley, the protein contents of all blades and
420 sheaths, as well as the water content of the leaf 2 blade, were the same for both 22 ºC:18 ºC
421 and 22 ºC:4 ºC temperature regimes (Figure 1a-b), in contrast to the plants grown under 10
422 ºC:4 ºC where protein levels were increased (Figure 1c). It suggests that cold night temperatures
423 in barley, similar to Arabidopsis, do not lead to cold acclimation, which is characterised by an
424 increase of the protein content (Guy, 1990, Pyl *et al.*, 2012) as well as an accumulation of
425 sugars and other osmolytes (Alberdi & Corcuera, 1991, Bourion *et al.*, 2003, Trischuk *et al.*,
426 2014), thicker cell walls and then a lower water content (Gorsuch, Pandey & Atkin, 2010,
427 Strand, Hurry, Henkes, Huner, Gustafsson *et al.*, 1999).

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428 Thus, if the growth inhibition observed at night in barley (Figure 2) is not linked to
429 extra costs incurred by elevated protein levels, it could be explained by (1) an inhibition of the
430 transport of C resources from the source leaves to the sink leaves due to cold inhibition of
431 phloem sap flow; (2) cold inhibition of the activities of enzymes involved in the degradation
432 of C stores; and/or (3) cold inhibition of the activities of enzymes involved in the growth
433 machinery. An inhibition of C transport from source to sink tissues is unlikely because cold
434 nights mostly affected C mobilisation in the youngest growing blades, the consumption of C
435 compounds during the night being the same for both temperature regimes in the oldest leaf (See
436 Figure 3 for sucrose, Table S1 for all metabolites). Thus, an inhibition of the flow of phloem
437 sap due to low temperatures leading to an unavailability of C for night growth is unlikely.

438 The second hypothesis is that cold driven inhibition of the activities of enzymes
439 involved in the degradation of C stores explains the growth inhibition observed at night. In that
440 case, we would expect only a partial degradation of the stores accumulated at ED, and
441 potentially an increase in the C accumulated at ED if C assimilation in warm days was
442 unaffected by cold nights. We observed a moderate inhibition of CO_2 assimilation for Propino
443 plants growing under 22ºC:4ºC compared to 22ºC:18ºC, but Bowman and the *elf3* mutants did

444 not show significant changes (Table 3). The total amount of C accumulated at ED in 22ºC:4ºC
445 was higher than at 22ºC:18ºC by around 20%, despite a major drop in starch content (43%),
446 and was mostly explained by a major increase in malate content (around 1.7 fold). As well, we
447 observed a decrease in the amount of C consumed under the 22ºC:4ºC and 10ºC:4ºC
448 temperature regimes compared to 22ºC:18ºC. Finally, we observed that starch was still fully
449 mobilised under cold nights (92%), similarly to Arabidopsis (Pyl et al., 2012), while sucrose
450 and particularly malate, fructose, glucose and fructans percentages of mobilisation during cold
451 nights were strongly reduced (Table 4). Thus a cold inhibition of the enzymes involved in the
452 mobilisation of these compounds can at least partially explain the growth inhibition we
453 observe.

454 Muller et al. (2018) hypothesised that the sensitivity of barley growth to cold nights is
455 mostly due to sucrose mobilisation not being temperature compensated in contrast to starch
456 mobilisation which is under clock control and temperature compensated. Their conclusions
457 were based on data showing that starch was fully consumed at dawn but that large amounts of
458 sucrose remained. We obtained qualitatively the same results at dawn for starch and sucrose
459 levels. Moreover, when we calculated the percentages of consumption of both compounds
460 during the night, we observed a maintenance of the starch mobilisation under the 22 ºC:4 ºC
461 with 92% of the starch mobilised compared to 96% under 22ºC:18ºC, whilst mobilisation of
462 sucrose was strongly depleted, from 96% under 22 ºC:18 ºC to 71% under 22 ºC:4 ºC (Table
463 4). However, if starch was largely consumed, its synthesis was also strongly inhibited and
464 represented only 57% of the starch accumulated under the 22 ºC:18 ºC temperature regime. As
465 a result, when we calculated the respective contribution of these metabolites to the overall C
466 consumed at night, we did not observe any difference between the two growth conditions for
467 sucrose, which contributed for ca 52% of the total carbon consumed at night, whilst starch
468 contribution decreased from 26% to 18% (Table 4). Thus, the turnover of both compounds was

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469 affected by low temperature, which suggests a tight regulation by barley of the night use of
470 these two C stores. We conclude that cold nights affect both starch and sucrose metabolism,
471 with starch synthesis and sucrose mobilisation being both repressed.

472 The third hypothesis to explain the inhibition of growth during cold nights is a
473 temperature driven negative effect on the growth machinery. CBF genes, that are expressed
474 under cold acclimation, control DELLA protein levels and gibberellin (GA) biosynthesis,
475 resulting in a dwarf Arabidopsis phenotype by reducing GA synthesis, the overexpression of
476 GA 2-oxidase and the accumulation of non-active forms of GA (Achard, Gong, Cheminant,
477 Alioua, Hedden et al., 2008). Slender barley with defective DELLA is able to maintain its
478 growth under cold, which is a phenotype that can be also mimicked by application of GA
479 (Schünmann, Harrison & Ougham, 1994). Moreover, if the overexpression of Hv CBF2A
480 reduces the time of cold acclimation required for acquiring freezing tolerance in barley, the
481 transgenic plants were smaller than WT under normal growth conditions (Jeknic, Pillman,
482 Dhillon, Skinner, Veisz et al., 2014). This could be partly circumvented by using stress induced
483 specific promotors to modulate the expression of CBF genes (Yang, Al-Baidhani, Harris,
484 Riboni, Li et al., 2019). Thus, growth under cold can be controlled independently of the
485 availability of carbohydrates. However, how CBF and downstream genes do affect the growth
486 machinery remains elusive. Moreover, how CBF genes could explain that cold nights do not
487 affect growth in Arabidopsis but does in barley remains to be answered. Pyl et al. (2012)
488 showed that in Arabidopsis rosettes, an increase of ribosome loading on mRNA in response to
489 cold nights could provide a mechanism to compensate for the slower translational activity of
490 ribosomes at low temperatures. That can only be possible if there is an excess of ribosomes for
491 growth at warm temperatures. Interestingly, ribosomes can represent up to 30% of the total
492 protein content of actively growing tissues in Arabidopsis, in contrast to ca 4% in a mature
493 tissue (Sulpice, Ishihara, Schlereth, Cawthray, Encke et al., 2014). Strikingly, maize, which

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494 like barley, is sensitive to low temperatures for night growth (Poire et al., 2010), does not show
495 such a gradient of ribosome concentrations between the division and mature zones of an
496 actively growing leaf (Czedik-Eysenberg, 2012), with only 1.6 times more ribosomes in the
497 division zone than in the mature zone of a growing leaf. Therefore, we hypothesise that barley
498 and maize might not have an excess in their growth machinery allowing them to compensate
499 for environmental cues such as a drop in temperature. Sucrose and fructan accumulations in
500 specific tissues are enhanced by cold during the light period, but not by cold nights.

501 Barley grown under the three temperature regimes did not show a major consumption
502 of glucose and fructose in any tissue, with the exception of the youngest leaf sheath for plants
503 grown under the 22 ºC:18 ºC regime (Figure S3 and S4). Rao *et al.* (2011) reported increases
504 in glucose and fructose leaf pools followed by increase in fructans and sucrose when *Poa*
505 *pratensis* was submitted to a cold acclimation treatment. However, after 8 days of acclimation
506 at 5 ºC, there was no further increase in glucose or fructose levels. Our plants were grown in
507 the three temperature regimes from sowing, so no accumulation of glucose or fructose were
508 expected. Sucrose content was among the highest of all metabolites we determined at end of
509 day in blades, which is agreement with previous studies (Gordon *et al.*, 1982, Gordon *et al.*,
510 1977, Gordon *et al.*, 1980a). However, sucrose levels in the sheaths were low for the three
511 temperature regimes and this could be explained by significant high invertase activity in the
512 sheaths (Roth *et al.*, 1997), also explaining the presence of glucose and fructose in the sheath
513 of the young third leaf.

514 Fructan function has been largely associated to cold tolerance in plants of temperate
515 regions (Abeynayake *et al.*, 2015, Morcuende, Kostadinova, Perez & Martinez-Carrasco, 2005,
516 Tamura *et al.*, 2014, Tyrka *et al.*, 2015). In response to cold treatment, the expression of fructan
517 synthesis genes is increased and consequently fructan levels rise (Meguro-Maoka & Yoshida,
518 2015, Morcuende *et al.*, 2005, Rao *et al.*, 2011, Tamura *et al.*, 2014, Yokota, Iehisa, Shimosaka

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519 & Takumi, 2015). Accordingly, we would have expected increased content of fructans in plants
520 grown under the 22 ºC:4 ºC and 10 ºC:4 ºC temperature regimes. However, this was not the
521 case, and fructans were mostly observed in sheaths of youngest leaf 3 for all three temperature
522 regimes, with the highest levels being observed for the 10 ºC:4 ºC temperature regime,
523 representing less than 5% of the C accumulated at ED (Table 4).

524 Fructan synthesis is also stimulated by increases in sucrose content (Apolinario, de
525 Lima Damasceno, de Macedo Beltrao, Pessoa, Converti *et al.*, 2014, Arkel, 2014, Cairns, 2003,
526 Chalmers, Lidgett, Cummings, Cao, Forster *et al.*, 2005, Cimini, Locato, Vergauwen, Paradiso,
527 Cecchini *et al.*, 2015, Xue, Drenth, Glassop, Kooiker & McIntyre, 2013). In agreement,
528 fructans are synthesized particularly at the end of the day, when the diurnal levels of sucrose
529 are highest (Sicher, Kremer & Harris, 1984). This might explain the accumulation of fructans
530 in the oldest blade of the plants grown in 10 ºC:4 ºC temperature regime, as this leaf
531 accumulated the highest sucrose levels at end of the day, but it does not well explain why other
532 blades, which also accumulated high sucrose levels, did not accumulate fructans. It has been
533 proposed that fructan synthesis is induced only after a certain concentration of sucrose is
534 reached in the tissues, and that this level vary according to the species (Cairns, Cookson,
535 Thomas & Turner, 2002, Nagaraj *et al.*, 2004, Obenland, Simmen, Boller & Wiemken, 1991,
536 Suarez-Gonzalez, Lopez, Delano-Frier & Gomez-Leyva, 2014, Wagner & Wiemken, 1987,
537 Wagner, Wiemken & Matile, 1986). Whether sucrose accumulation was not enough to reach
538 the minimal level required to enhance fructan accumulation in other blades, or that fructan
539 accumulation is not naturally directly induced by sucrose due the different
540 compartmentalisation of sucrose and fructans (Cairns, Turner & Gallagher, 2008, Keerberg,
541 Ivanova, Keerberg, Parnik, Talts *et al.*, 2011) remains unclear. It is also possible that young
542 barley redirect sucrose towards growth instead of accumulating fructans even if temperature
543 drastically slows the development.

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544 **4.2 Starch accumulation is highly sensitive to cold nights, but not its mobilisation**

545 Starch has been described as a minor reserve in fructan accumulating plants (Cairns *et*
546 *al.*, 2002, Farrar & Farrar, 1985, Roth *et al.*, 1997, Wang & Tillberg, 1996, Wang, Van den
547 Ende & Tillberg, 2000). However, our data show that starch is still an important transient
548 carbon pool in young barley grown under 22 ºC:18 ºC (Table 4), representing 26% of the total
549 C used during the night (Table 4). Conversely, we were surprised that starch accumulation was
550 impaired (43% decrease) when the plants faced cold only at night (Figure 4c). Considering that
551 the temperature in the light was the same in both conditions, the sucrose content at end of day
552 was similar (Table 4), and that CO_2 assimilation was only slightly decreased (Table 3), we
553 would expect no restrictions on starch accumulation. This result is in stark contrast with Pyl *et*
554 *al.* (2012) who reported no change in starch accumulation and turnover in Arabidopsis plants
555 submitted to cold nights. Starch turnover in Arabidopsis is strongly dependent on circadian
556 clock (Graf *et al.*, 2010, Yazdanbakhsh, Sulpice, Graf, Stitt & Fisahn, 2011), and trehalose 6-
557 phosphate (T6P) levels also regulate rates of starch degradation, allowing Arabidopsis plants
558 to avoid exhaustion of starch prematurely during the night (Dos Anjos, Pandey, Moraes, Feil,
559 Lunn *et al.*, 2018, Figueroa, Feil, Ishihara, Watanabe, Kolling *et al.*, 2016, Martins, Hejazi,
560 Fettke, Steup, Feil *et al.*, 2013).

561 It has been proposed that the growth of both dicots and monocots is regulated by an
562 additive effect of circadian-clock controlled processes and environmental cues such as
563 temperature, with the monocots being more sensitive to environmental changes. We observed
564 that in response to cold, either during 10 ºC:4 ºC or 22 ºC:4 ºC, the turnover of sucrose, glucose,
565 fructose and malate were strongly reduced. But starch was still largely mobilised at night with
566 80-92% of it consumed under 22 ºC:4 ºC and 10 ºC:4 ºC compared to 96% for 22 ºC:18 ºC
567 (Table 4). It appears that starch mobilisation is largely cold compensated in barley, like for
568 Arabidopsis, but its accumulation during the day is impaired by night temperature. Thus, we

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569 hypothesise that cold nights, which induce a strong reduction in growth of barley, inhibit starch
570 accumulation in daytime (Figure 8). The mechanism in not known, but the circadian clock
571 and/or T6P are likely candidates for such regulation and further studies are required. Both the
572 clock and T6P signalling provide mechanisms for adjusting the rates of starch degradation
573 (Graf *et al.*, 2010, Martins *et al.*, 2013), but recently they have been more largely involved in
574 the diurnal control of both carbohydrate, organic acids and nitrogen metabolisms in
575 Arabidopsis (Figueroa *et al.*, 2016, Flis *et al.*, 2019). Gordon, Ryle and Webb (1980b)
576 suggested that starch consumption at night is triggered by a decrease of sucrose below a
577 threshold value, rather than the onset of darkness. We also found a reduced rate of starch
578 degradation in the first hours of the night for the 22 ºC:18 ºC treatment (Figure 3), so starch
579 and sucrose use at night might be partly sequential. However our results show that if there is a
580 threshold value for sucrose triggering starch degradation, then temperature do affect this value.

581 **4.3 Malate plays important role as alternative carbon supply to growth**

582 The levels of malate observed in barley leaves are very high. They are about 10 times
583 more than in Arabidopsis rosettes grown at similar temperature and photoperiod (Medeiros,
584 Barros, Barros, Omena-Garcia, Arrivault *et al.*, 2017). It is partly explained by Arabidopsis
585 accumulating up to 10 µmol.g^{-1} FW of fumarate (Pracharoenwattana, Zhou, Keech, Francisco,
586 Udomchalothorn *et al.*, 2010), in contrast to barley where fumarate was below detection levels
587 (not shown). Malate is an intermediary of the tricarboxylic acid cycle (TCA) and it plays an
588 important role in stomatal function, pH regulation and can refill the TCA cycle to restore NAD$^+$
589 and NADP$^+$ in the cell, besides being a major carbon storage in C_4 and CAM plants (Fernie &
590 Martinoia, 2009). Plants that undergo chilling might increase reactive oxygen species (ROS)
591 due to photo-inhibition (Allen & Ort, 2001, Hurry & Huner, 1992). Although malate could be
592 a reductive equivalent complementing antioxidative mechanisms in presence of oxidative
593 stress, the accumulation of malate in cold-hardened leaves of rye was probably about a storage

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594 of carbon and a vacuolar osmolyte to balance cytosolic accumulation of sugars (Crecelius,
595 Streb & Feierabend, 2003). The increased malate accumulation and significant turnover rates
596 we observed in barley leaves under 22 ºC:4 ºC (Figure 5, Table 4) suggests that the malate pool
597 might be an alternative carbon storage to starch and fructans, especially in young tissues
598 (Figure 6). Malate contributed 15, 28 and 24% of the total carbon used at night for the
599 22ºC:18ºC, 22 ºC:4 ºC and 10 ºC:4 ºC temperature regimes, respectively (Table 4). It makes
600 malate one of the major contributors of C for growth at night in barley. However, its turnover
601 was only partial and high levels remain at dawn, especially under cold treatments, suggesting
602 a role as an osmolyte in addition to a source of C for night use. Interestingly the highest levels
603 of malate were in the youngest leaf, for all temperature regimes. Thus, malate might participate
604 in the growth of young leaves, as well as in their protection, which was unexpected and
605 strengthen the need for future metabolic studies in barley to include the determination of this
606 compound.

607 **4.4 Mobilization of C reserves is controlled by *elf3* but without an effect on growth**
608 **under cold nights**

609 Bowman WT and *elf3* mutants were grown under 22ºC:4ºC. Although there was no
610 visual phenotype and mutants reached the same three leaf stage at same time as WT without
611 differences in photosynthesis and elongation rates, we decided to investigate further if an
612 impairment in *elf3* function caused disturbances in the regulation of C reserves. Glucose and
613 fructose mobilization were affected in both *elf3* introgression lines at night, and importantly
614 only a partial mobilisation of the starch and sucrose at night was observed (Table S2). While
615 first and second blades showed a decrease in the consumption of sucrose at night in *elf3*
616 mutants, the third sheaths of the mutants accumulated less hexoses at night compared to WT.
617 These results suggest that in the *elf3* mutants, even if sucrose is only partially degraded in
618 blades during the night and starch less accumulated during the day, it is partly compensated by

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619 a decrease in the accumulation of hexoses during the night. These results exclude the possibility
620 of growth impairment at night due to cold inhibition of phloem sap flow but supports the
621 hypothesis of growth being sensitive to low temperature, as discussed above.

622 The participation of the crowns in the overall C accumulated at end of day, and the use
623 of these reserves at night was very small compared to the shoots, because the C content
624 accumulated in crowns was very low (Table S3). However, a small consumption for some of
625 the carbohydrates (i.e. sucrose and starch) was observed, thus crowns act as transient reserve
626 tissue. Crowns accumulate more fructans than the shoot parts at end of day, but they are not
627 consumed during the night, instead, we see accumulation at end of night when cold night is
628 imposed (Table S3). Accordingly, winter wheat shows decrease of almost 50% in assimilation
629 rates under cold, but the ability to mobilize sucrose in leaves to fructans and starch in crowns
630 allows higher assimilation rates than spring varieties (Savitch *et al.*, 2000). Interestingly,
631 Vágújfalvi, Kerepesi, Galiba, Tischner and Sutka (1999) reported a significant correlation
632 between accumulation of soluble carbohydrates in wheat varieties and freezing acclimation
633 only after 19 days of treatment. Thus, the accumulation of fructans in spring varieties is limited
634 at early stage, however, small increases of fructans in crown at night may play a role as
635 osmolyte on the protection of young tissues from cold night and being mobilized during the
636 day under warmer temperature.

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637 **5 CONCLUSION**

638 Most carbon reserves used at night were stored in both young and mature blades and
639 not in the sheaths, while crowns had little relevance in the accumulation of reserves for cold
640 acclimation in young spring barley plants. Carbon consumed at night originated primarily from
641 sucrose. However, malate was important, especially under cold treatments, and can be
642 considered a major contributor to night growth in barley. Starch accumulation was strongly
643 inhibited by cold in the daytime, but surprisingly also under warm day and cold night.

644 However, its mobilisation was not affected by cold nights. The clock *elf3* mutants showed
645 changes in glucose, fructose, sucrose and starch levels compared to WT, however, it did not
646 impact on their growth, maybe because these metabolites were not drastically affected
647 quantitatively. Altogether our data suggest that enzymes involved in the mobilisation of sucrose
648 and malate and/or barley growth machinery are sensitive to cold night because C was available
649 for night growth and despite that, the plants were not growing. Thus, breeding for increased
650 photosynthetic performance under cold might not lead to increases in biomass in barley because
651 it is the use of the photosynthates which is limiting. In contrast, desensitising growth inhibition
652 by cold might be an alternative target, through e.g. higher polysome recruitment, manipulation
653 of the CBF pathway and the regulation of DELLA proteins.

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Table 1. Leaf 2 water content and fluorescence parameters of Propino cv. grown under different temperature regimes.

	Water content (%)	F_v/F_m	$Y_{(II)}$	**ETR**
22ºC:18ºC	91.15 ± 1.11 a	0.796 ± 0.003 a	0.745±0.016 a	156.4±3.4 a
22ºC:4ºC	88.97 ± 0.92 b	0.788 ± 0.006 a	0.743±0.009 a	156.1±1.9 a
10ºC:4ºC	84.59 ± 0.96 c	0.77 ± 0.016 b	0.722±0.009 b	151.5±1.9 b

Propino plants were grown until third leaf stage in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. The plants reached third leaf stage under 22°C:18°C day:night at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS. Values represent mean and SD. F_v/Fm: maximum photochemical quantum yield of PS II; $Y_{(II)}$: effective photochemical quantum yield of PS II; ETR: electron transport rate in µmol electron $m^{-2}s^{-1}$. Letters represent significant differences between treatments for Tukey's test P<0.05, n=6.

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Table 2. Elongation rate and height of Bowman WT and *elf3* introgression lines under cold nights.

Elongation rate (mm h^{-1})			
Second leaf	**WT**	**289**	**290**
Day	1.9 ± 0.2 a*	2.1 ± 0.2 a*	2.0 ± 0.2 a*
Night	0.4 ± 0.2 a	0.3 ± 0.1 a	0.3 ± 0.1 a
Third leaf	**WT**	**289**	**290**
Day	1.9 ± 0.1 a*	1.9 ± 0.1 a*	2.0 ± 0.2 a*
Night	0.4 ± 0.1 a	0.4 ± 0.0 a	0.2 ± 0.1 a
Height (cm)			
WT	20.0 ± 2.0 a		
289	20.7 ± 1.3 a		
290	18.7 ± 1.3 a		

Plants were grown in a 12h:12h light:dark photoperiod with 500 μmol photons $m^{-2}s^{-1}$ 22°C:4°C day:night for 20 DAS, until third leaf stage Values represent mean and SD. Letters represent differences between genotypes by Tukey P<0.05; * represents differences between day and night by t-test P<0.05, n=6.

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Table 3. Photosynthesis, stomatal conductance, internal CO_2 concentration, transpiration and water use efficiency of Bowman (WT), *elf3* mutants and Propino, grown under different temperature regimes

		A		g_s		C_i		E		WUE	
22ºC:18ºC	**WT**	17035 ± 1817	B	0.004 ± 0.001	A	22 ± 5	A	0.043 ± 0.011	A	112 ± 30	B
	289	17354 ± 2869	B	0.005 ± 0.001	A	22 ± 6	A	0.056 ± 0.007	A	91 ± 18	B
	290	16516 ± 1373	B	0.004 ± 0.001	A	21 ± 6	A	0.043 ± 0.012	A	111 ± 31	B
	PRO	17567 ± 3149	B	0.004 ± 0.000	A	20 ± 5	A	0.050 ± 0.011	A	110 ± 15	C
22ºC:4ºC	**WT**	15674 ± 2168	B	0.004 ± 0.001	A	20 ± 2	A	0.053 ± 0.011	A	88 ± 16	B
	289	15266 ± 1213	B	0.005 ± 0.001	A	19 ± 2	A	0.055 ± 0.009	A	80 ± 14	B
	290	14781 ± 1514	B	0.004 ± 0.001	A	22 ± 5	A	0.048 ± 0.011	A	92 ± 15	B
	PRO	13150 ± 1290	A	0.004 ± 0.001	A	18 ± 3	A	0.045 ± 0.006	A	87 ± 17	B
10ºC:4ºC	**WT**	11498 ± 1175	A	0.016 ± 0.006	B	22 ± 3	Ab	0.080 ± 0.019	B	18 ± 6	A
	289	10920 ± 1764	A	0.018 ± 0.006	B	21 ± 2	Aab	0.081 ± 0.022	B	16 ± 6	A
	290	11012 ± 1334	A	0.014 ± 0.003	B	23 ± 4	Ab	0.073 ± 0.010	B	18 ± 4	A
	PRO	10601 ± 983	A	0.013 ± 0.004	B	16 ± 3	Aa	0.060 ± 0.015	A	20 ± 4	A

Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night for 15 DAS, 22°C:4°C day:night for 20 DAS and 10°C:4°C day:night for 45 DAS, until third leaf stage. Values represent mean and SD. WT: Bowman WT; 289 and 290: introgression lines for *elf3* in Bowman background; PRO: cv. Propino; A: net photosynthesis, µmol g^{-1} DW day^{-1}; g_s: stomatal conductance, mol H_2O g^{-1} DW day^{-1}; C_i: substomatal concentration of CO_2; E: transpiration, mol.g^{-1}.day^{-1}; WUE: water use efficiency µmol CO_2 mol^{-1} H_2O. Capital

letters represent differences between temperature regime within a genotype; small case letters represent differences between genotypes within temperature regimes by Tukey $P<0.05$, n=6.

Do not distribute

Table 4. Accumulation and mobilization of reserves in barley grown under three temperature regimes.

Total accumulation at end of day (C µmol g-1 FW)			
	22°C:18°C	**22°C:4°C**	**10°C:4°C**
Glucose	11 ± 1 b	13 ± 1 b	8 ± 1 a
Fructose	4 ± 0 b	3 ± 0 a	4 ± 0 b
Sucrose	159 ± 14 a	174 ± 7 a	181 ± 8 a
Starch	82 ± 3 c	46 ± 4 b	27 ± 0 a
Fructans	7 ± 5 a	6 ± 1 a	13 ± 0 a
Malate	52 ± 3 c	127 ± 14 a	89 ± 8 b
Total[1]	316 ± 10 a	370 ± 21 b	321 ± 6 a
Depletion of reserves during the night [%]			
	22°C:18°C	**22°C:4°C**	**10°C:4°C**
Glucose	94	30	46
Fructose	97	-85	-1
Sucrose	96	71	83
Starch	96	92	80
Fructans	93	47	55
Malate	84	53	64
Total[2]	94	64	74
Contribution to C use at night [%]			
	22°C:18°C	**22°C:4°C**	**10°C:4°C**
Glucose	4	2	2
Fructose	1	-1	0
Sucrose	52	52	63
Starch	26	18	9
Fructans	2	1	3
Malate	15	28	24

Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night for 15 DAS, 22°C:4°C day:night for 20 DAS and 10°C:4°C day:night

for 42 DAS, until third leaf stage. Values represent mean and SD. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. n= 3

*Total of reserves accumulated at end of day in the shoot

**Percentage of the total reserves consumed at night in shoots from total reserves accumulated at end of day

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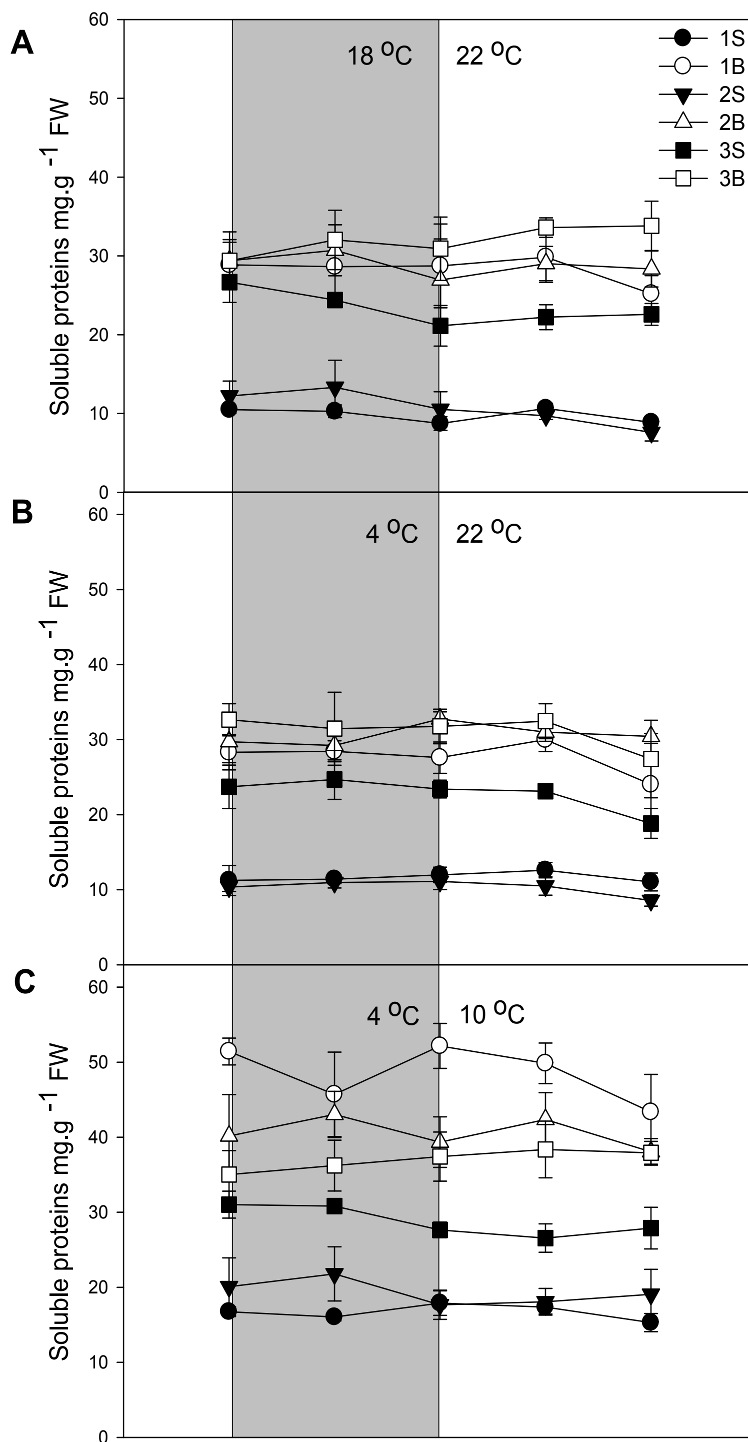

Figure 1 - Diurnal protein levels of barley grown under three temperature regimes. Protein levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

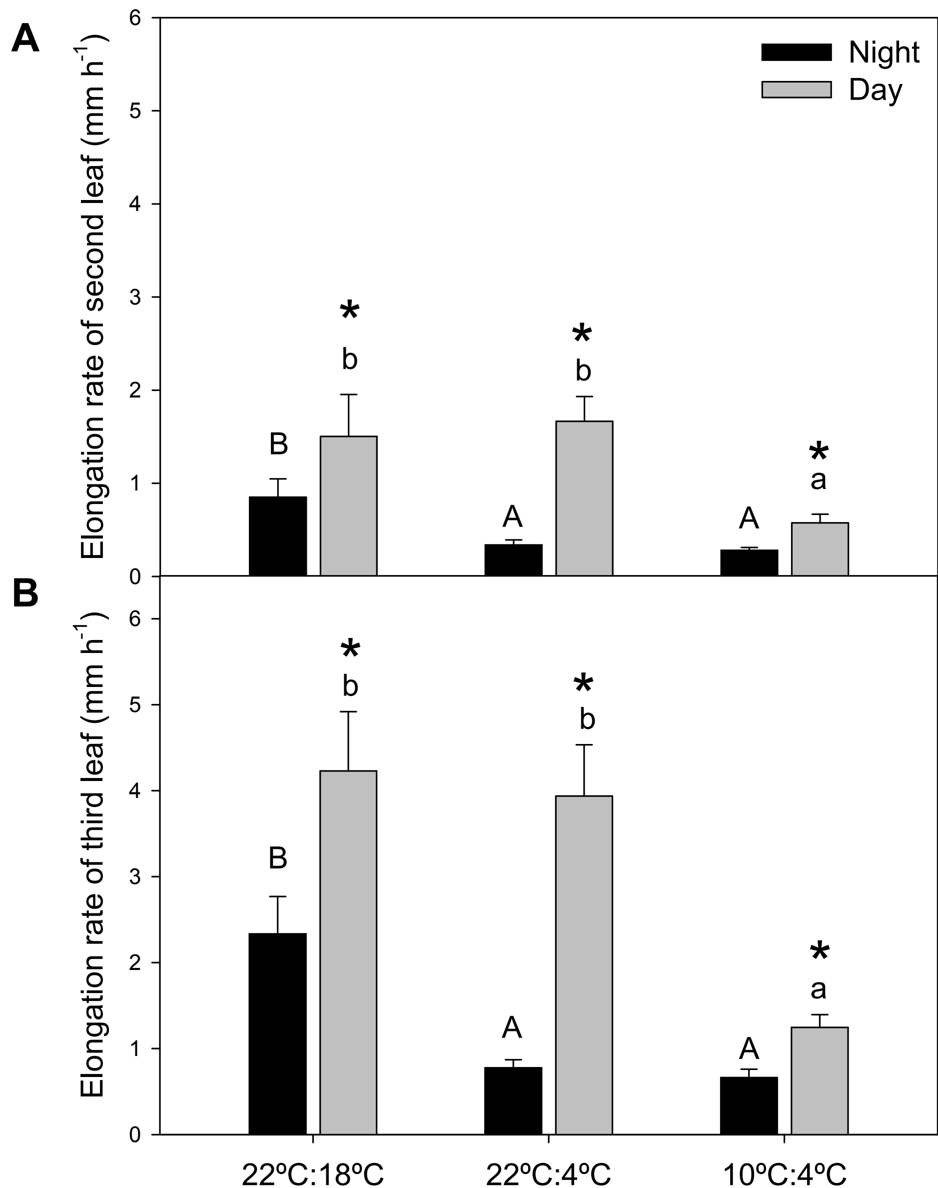

Figure 2 - Elongation rates of barley grown under three temperature regimes. (A): elongation rate of second leaves. (B): elongation rate of third leaves. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night harvested at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS; *: difference between day and night by t-test at P<0.05; lowercase letter: differences between daytime measurements; uppercase letters: differences between night-time measurements; significantly different by Tukey test at P<0.05, error bar represents SD; n=6

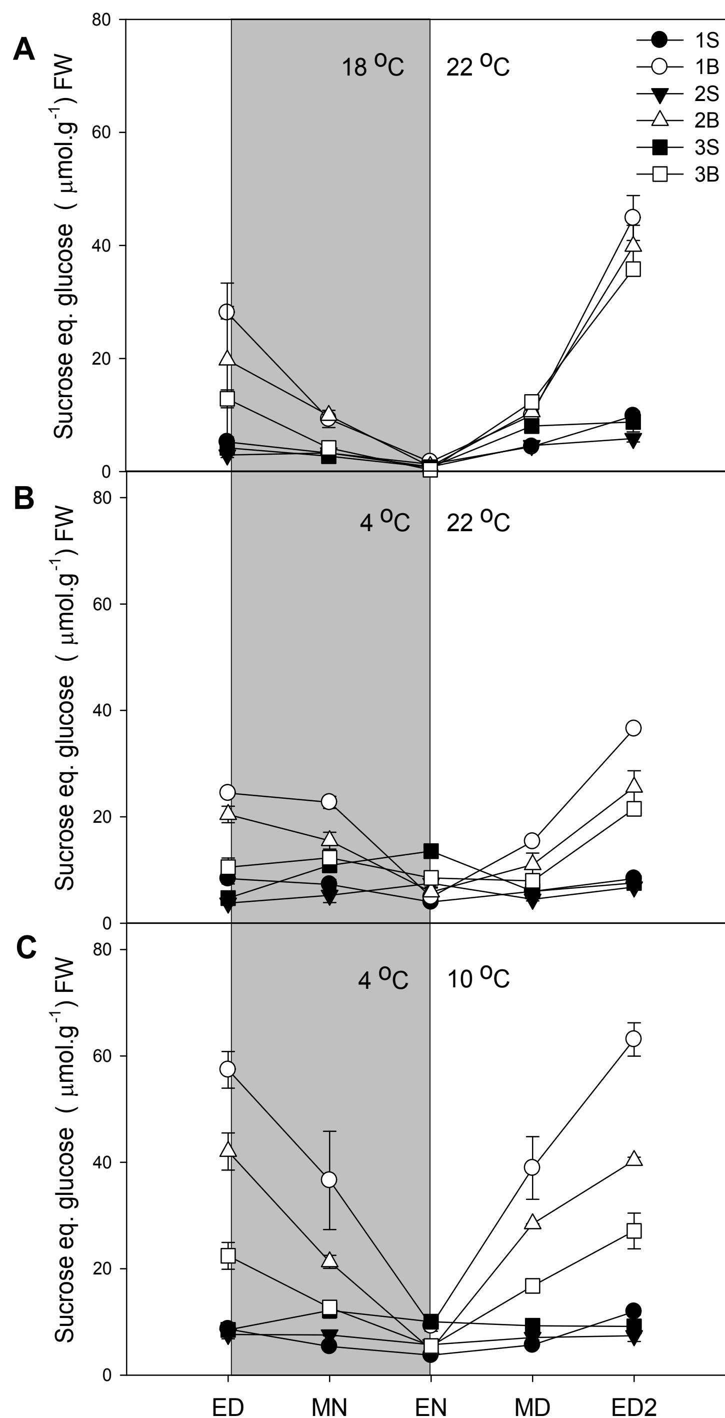

Figure 3 - Diurnal sucrose levels of barley grown under three temperature regimes. Sucrose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

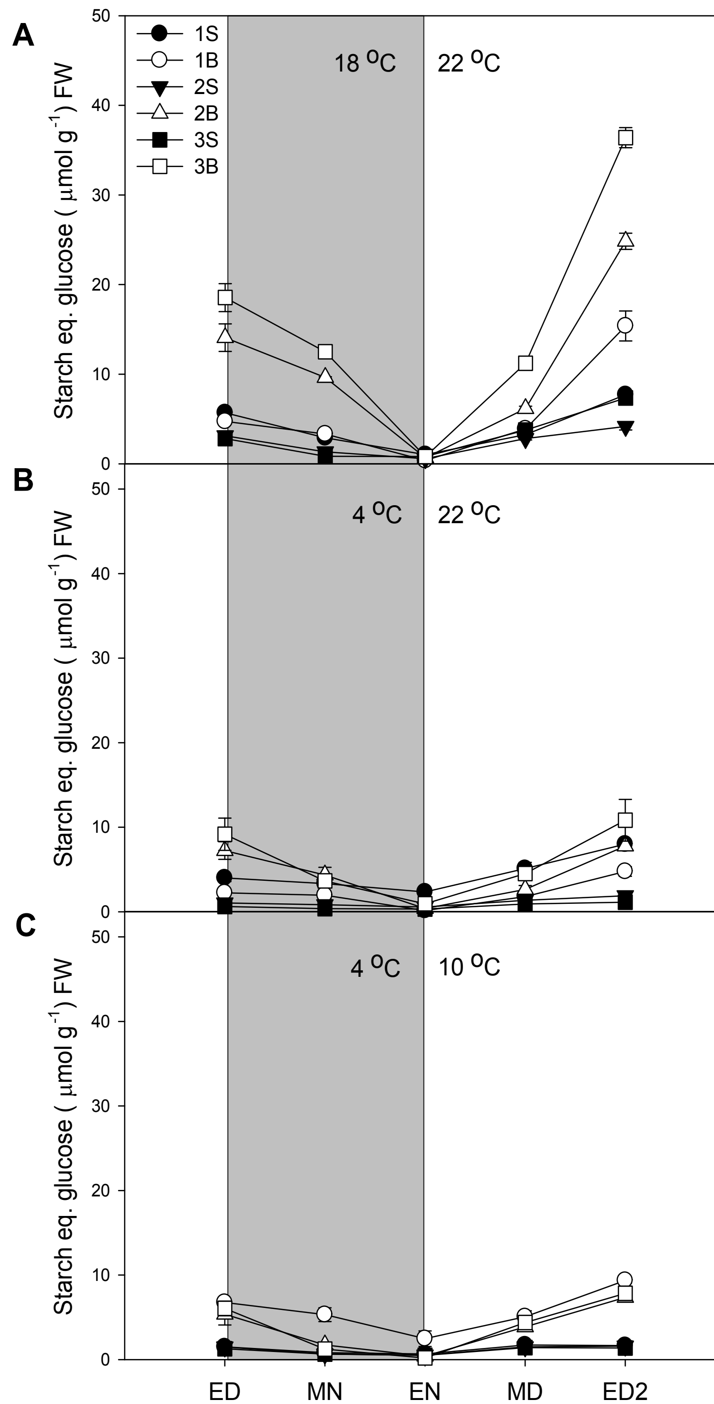

Figure 4 - Diurnal starch levels of barley grown under three temperature regimes. Starch levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

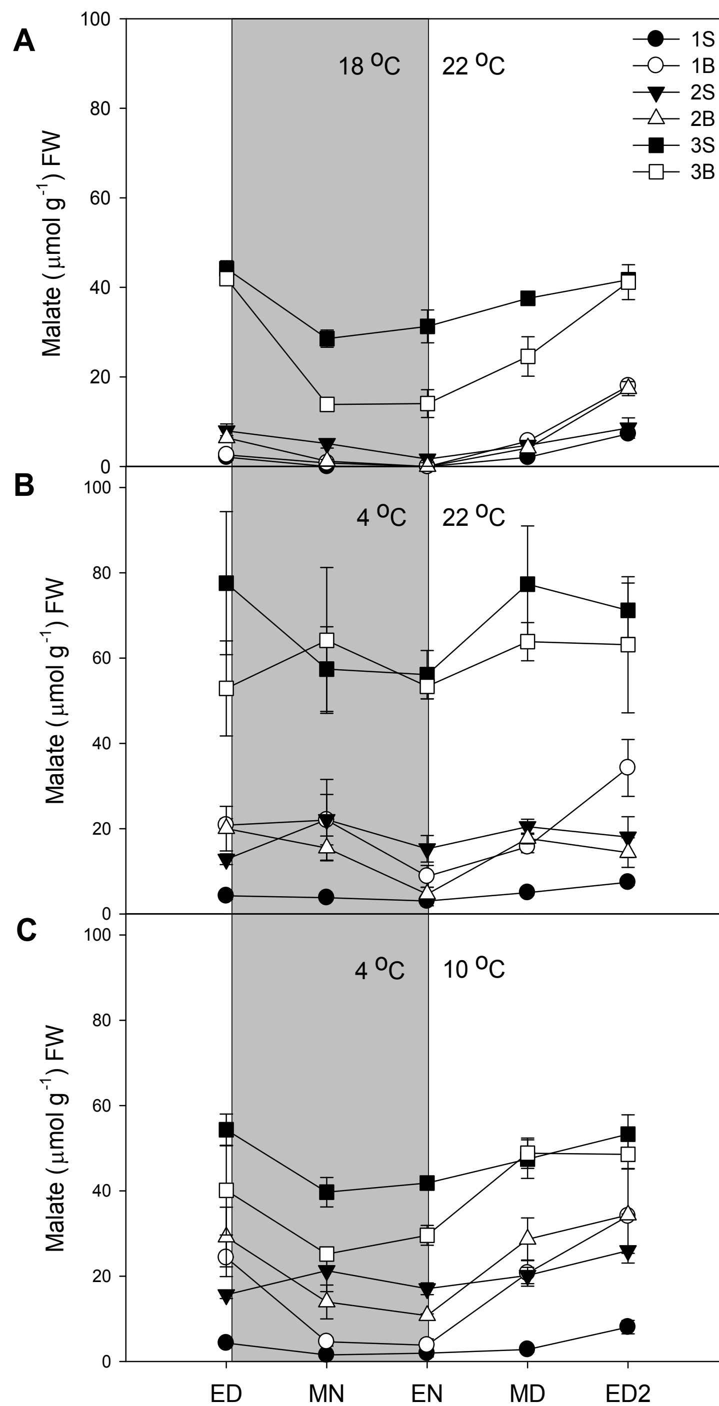

Figure 5 - Diurnal malate levels of barley grown under three temperature regimes. Malate levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

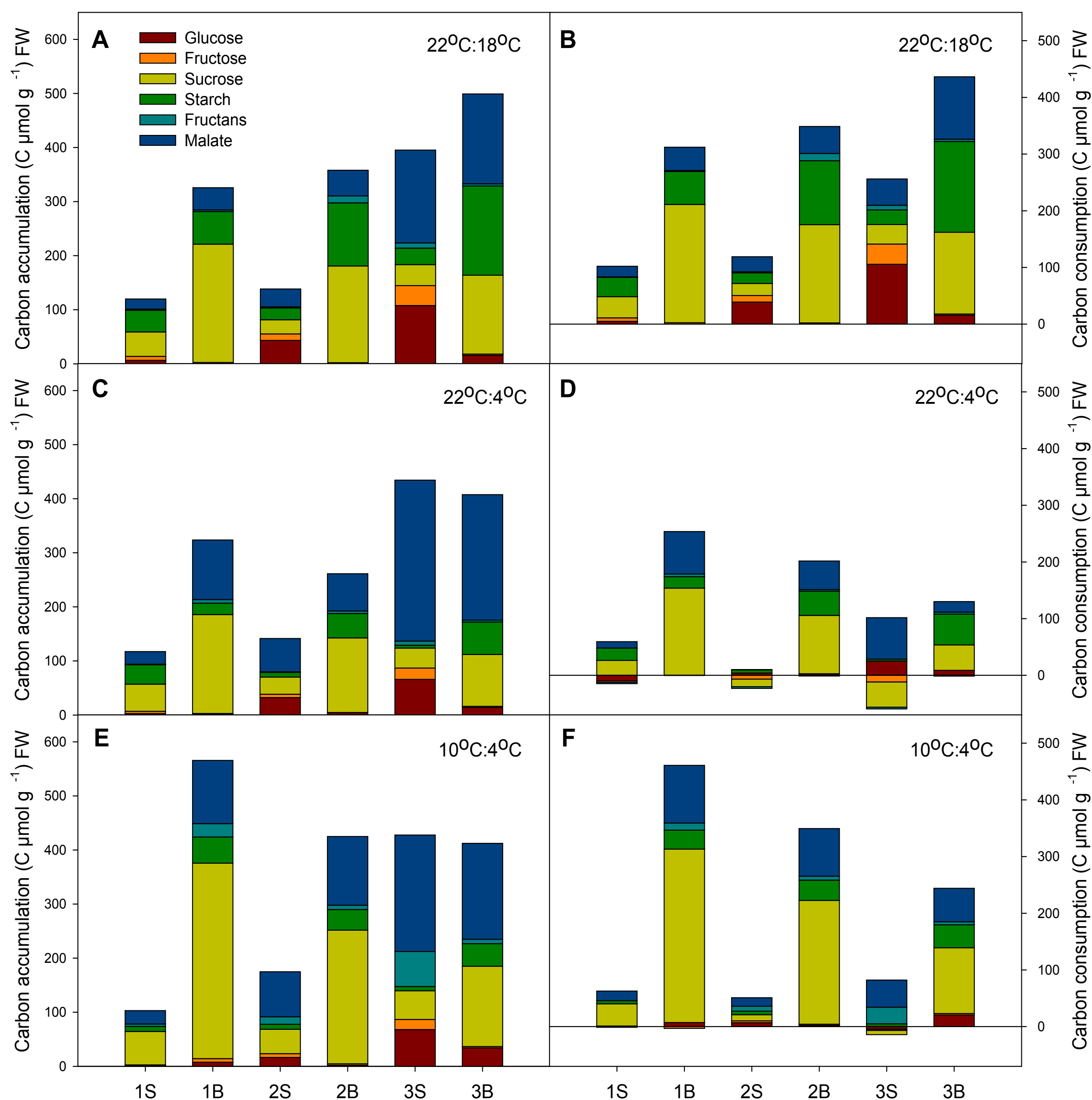

Figure 6- Carbon accumulation at end of day and carbon consumption at night per organ under three temperature regimes. Composition of carbon accumulation (A) and consumption (B) at end of the day of sheaths and blades of barley grown under 22°C:18°C day:night at 15 DAS; (C) and (D) under 22°C:4°C day:night at 20 DAS, and (E) and (F) under 10°C:4°C day:night at 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$, 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; DW: dry weight; n= 3.

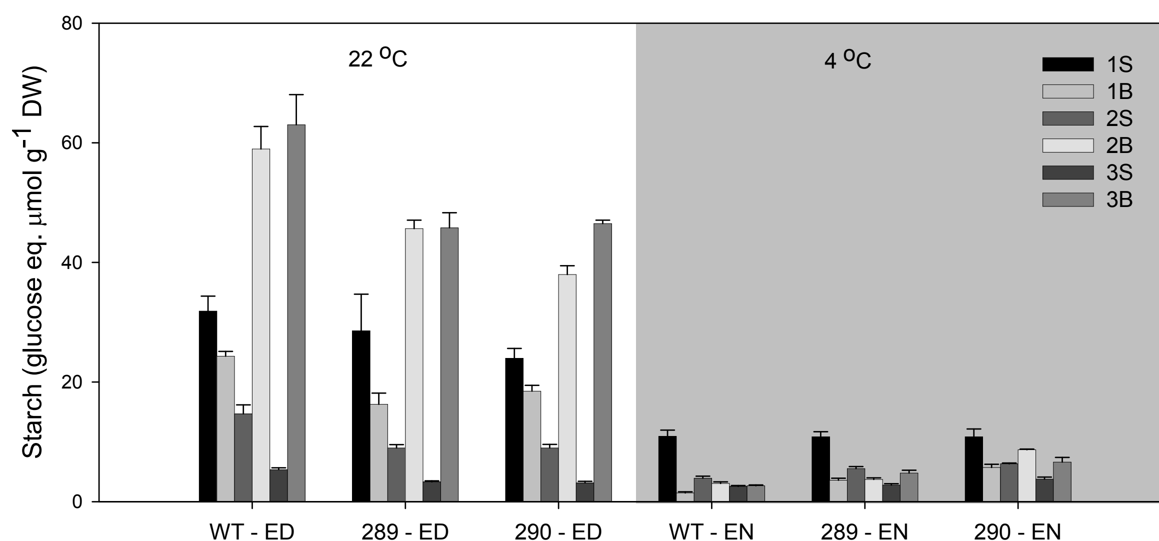

Figure 7- Diurnal starch levels of barley WT and *elf3* mutants grown under cold nights. Plants were grown under 22°C:4°C day:night, 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ for 20 DAS, until third leaf stage. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; EN: end of night; DW: fresh weight; grey panel: night period; error bar represents SD; n= 3

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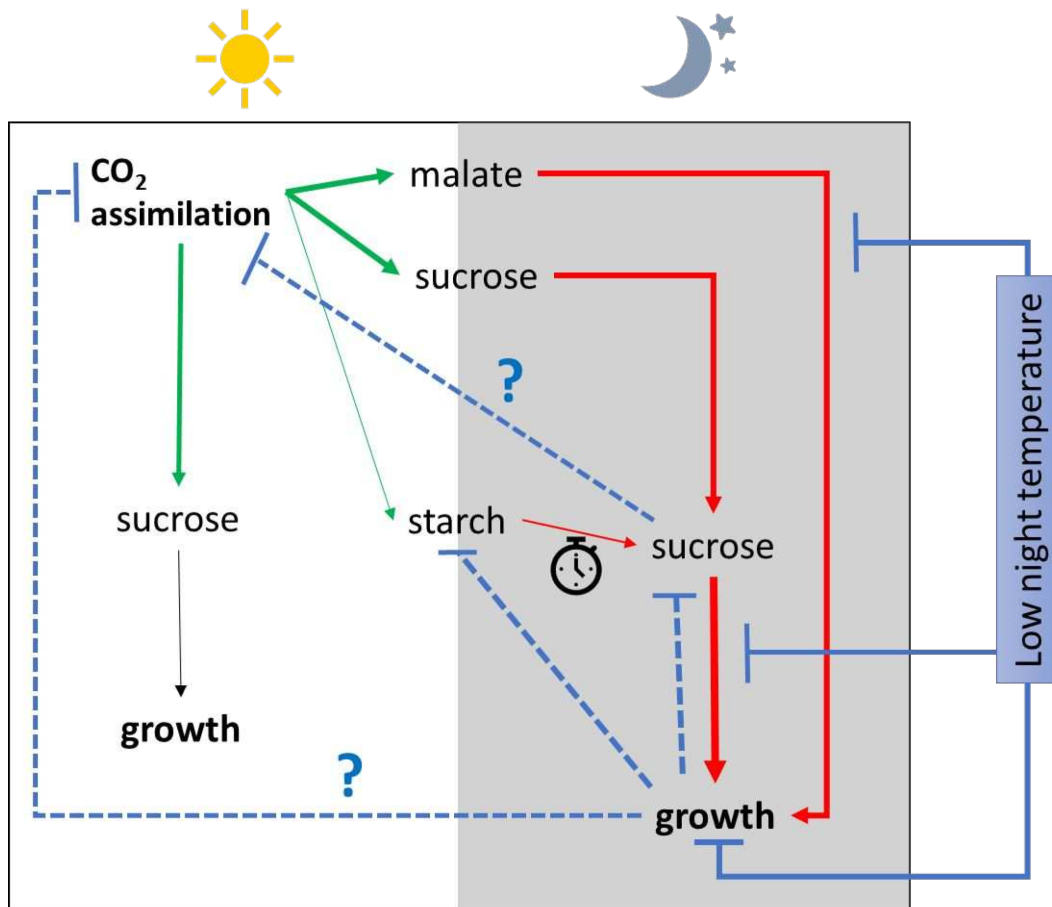

Figure 8– Schematic representation of changes in the metabolism of spring barley caused by low night temperature. During the day a large fraction of the photosynthates are accumulated in the form of sucrose and incorporated in growth. A fraction is as well accumulated, mostly as sucrose, malate and starch. Under warm nights, the growth is fuelled by sucrose, malate and starch. Sucrose and starch are almost fully consumed while malate remains with high basal levels. Under low temperature at night the growth is inhibited. It can be due to an inhibition of the mobilisation of both sucrose and especially malate stores, leading to a lack of C blocks to fuel the growth. A second hypothesis is that cold nights affects directly the growth machinery which then lead to the decrease in the mobilisation of C stores. Low night temperature also affects starch accumulation during warm days, but starch degradation at night is cold compensated, partly via a clock regulation (*elf3*).
Metabolic pathways are represented by solid lines; green: synthesis; red: degradation, thickness of lines represents proportional accumulation or degradation; blue lines: possible effects of low night temperature.

Barley uses almost equally sucrose, starch and malate for night growth under optimal growth temperatures. Under cold, mostly sucrose and malate are used, starch synthesis being strongly repressed, even when cold is only applied at night.

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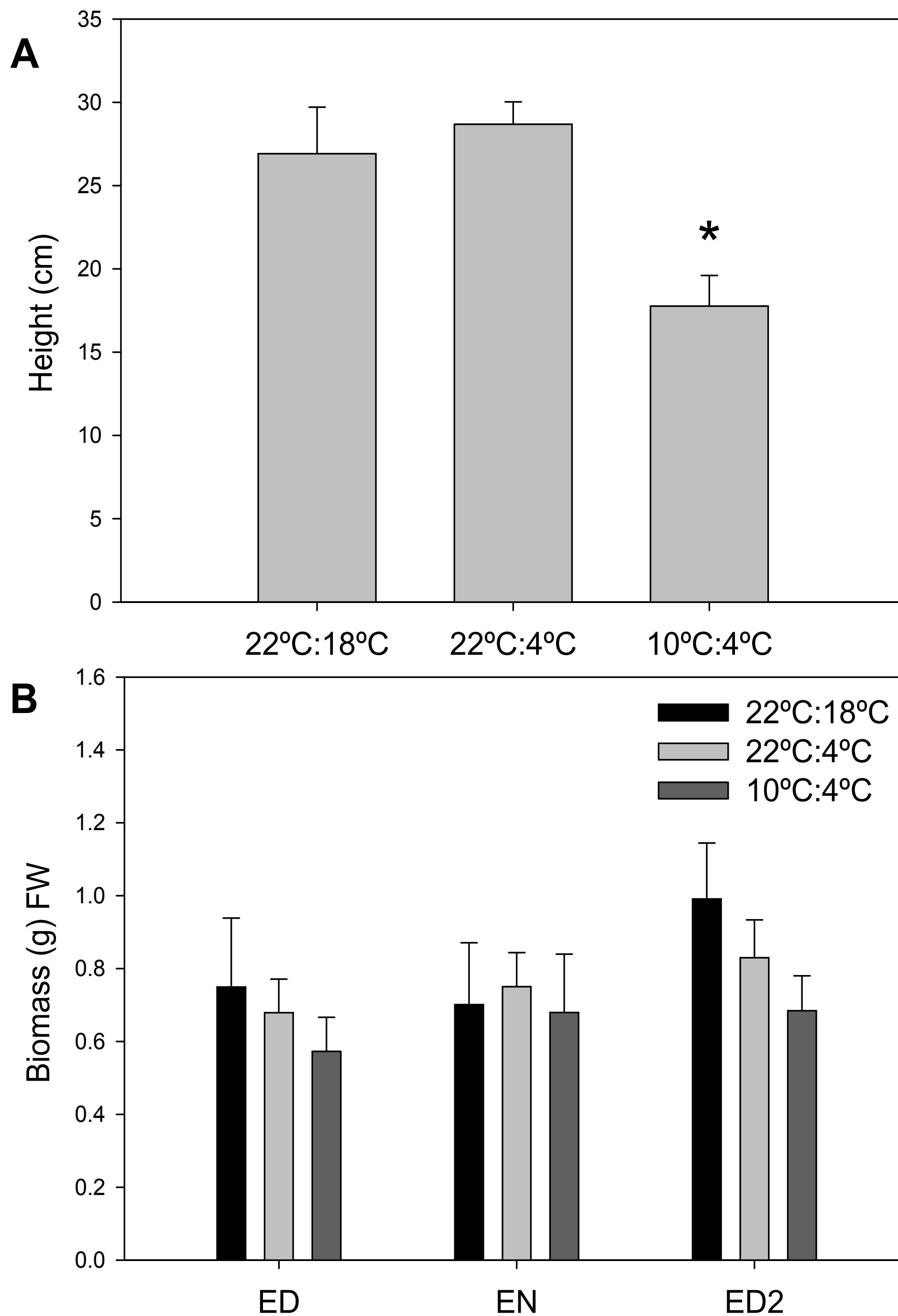
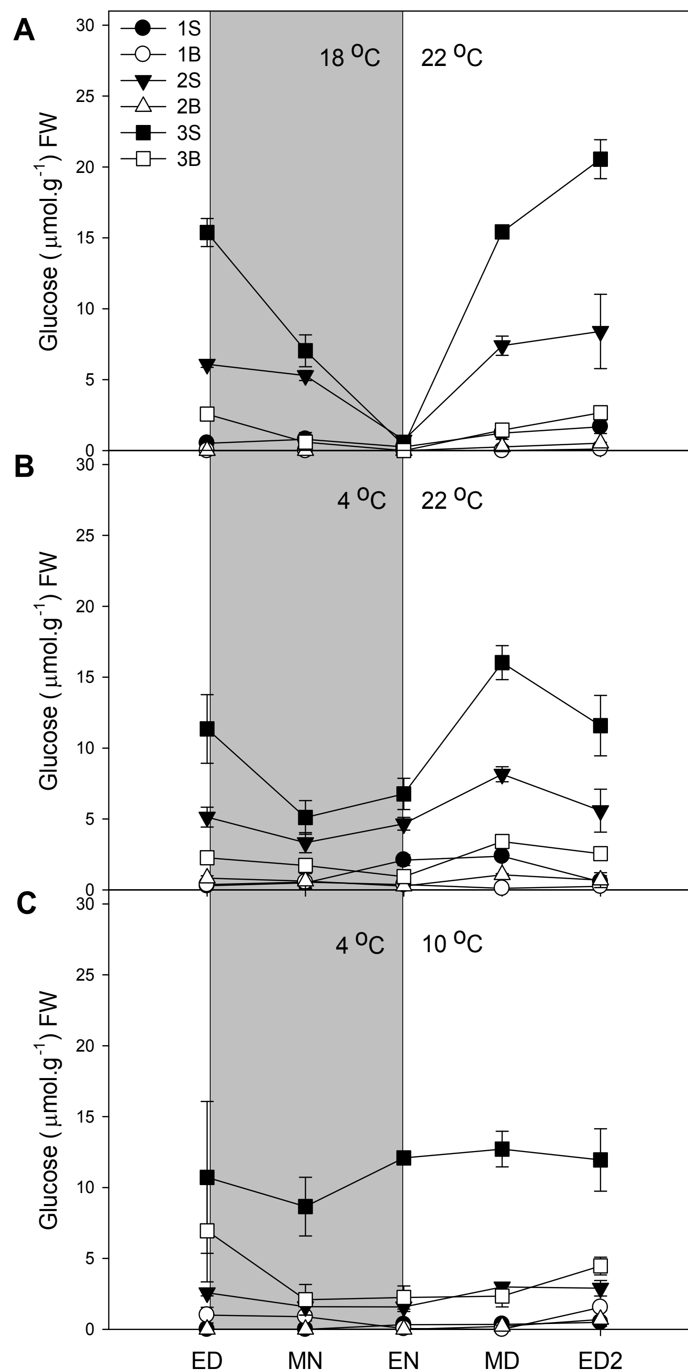
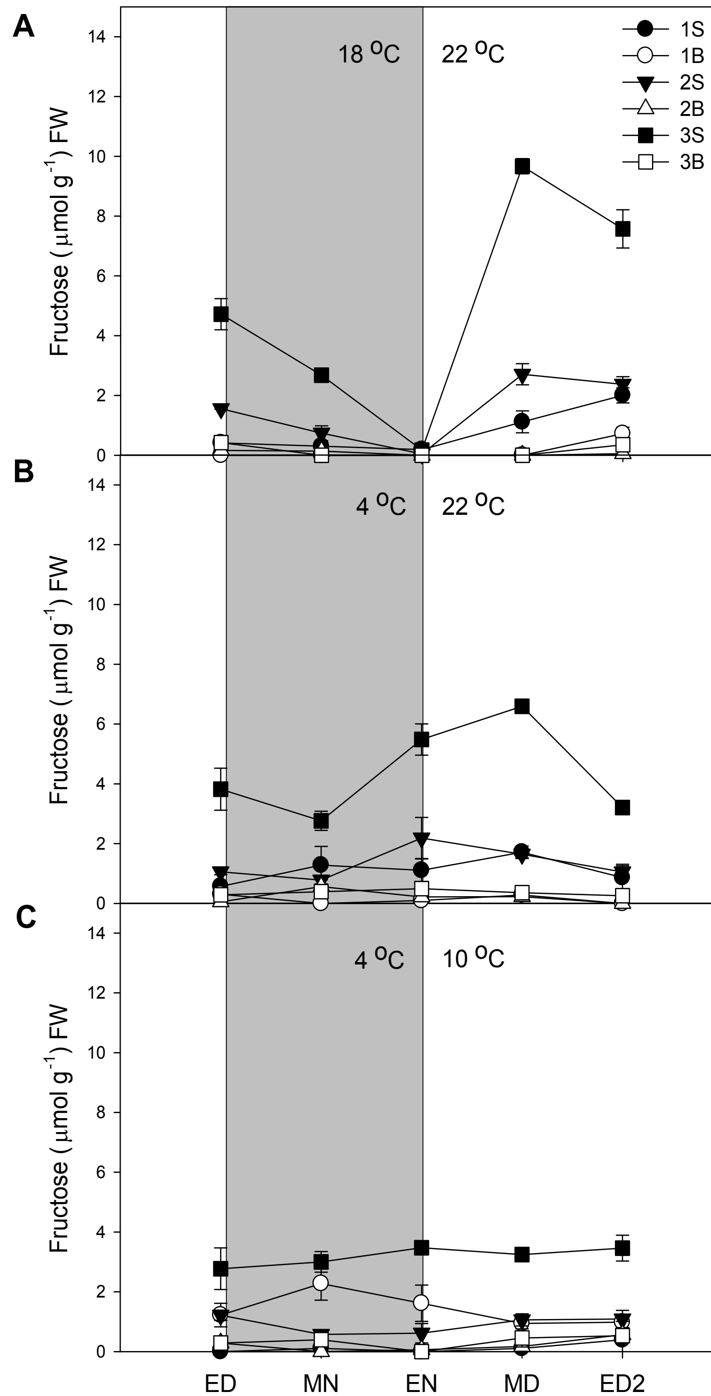


Supplemental figure S1 - Height and biomass of barley grown under three temperature regimes. (A): height at the last timepoint harvested: 22°C:18°C day:night at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS; (B): fresh weight of shoot biomass. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. DAS: days after sowing; error bar represents SD; n=6; ED: end of day; EN: end of night; ED2: end

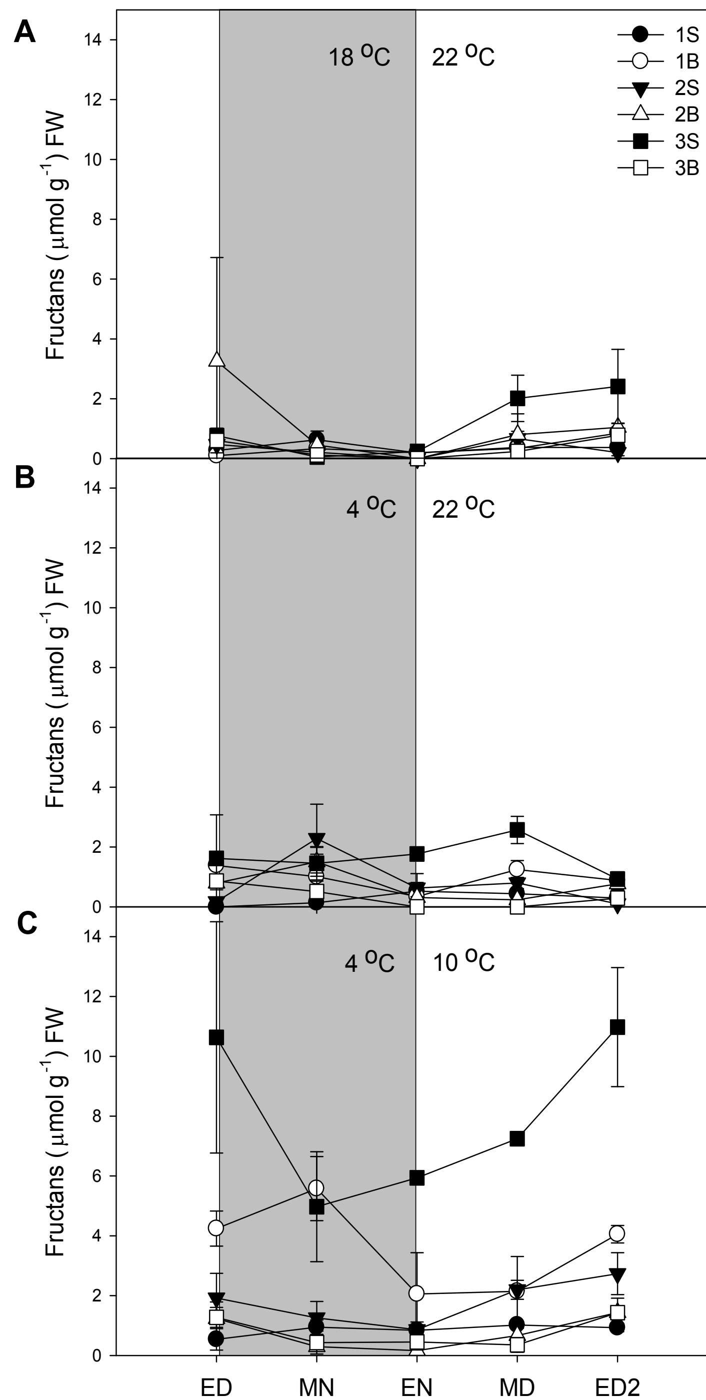
of subsequent day * represents significant difference for Tukey test at P<0.05



Supplemental figure S2- Diurnal glucose levels of plants grown under three temperature regimes. Glucose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.



Supplemental figure S3 Diurnal fructose levels of plants grown under three temperature regimes. Fructose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.



Supplemental figure S4- Diurnal fructan levels of plants grown under three temperature regimes. Fructan levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

WARM DAY AND NIGHT 22ºC:18ºC

A	columns	between timepoints
a	lines	between tissues

GLUCOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.52 ± 0.0	ABa	0.00 ± 0.0	Aa	6.09 ± 0.2	Bb	0.00 ± 0.0	Ac	15.38 ± 1.0	Ca	2.57 ± 0.3	Cd
MN	0.79 ± 0.5	ABa	0.00 ± 0.0	Aa	5.29 ± 0.3	Bb	0.00 ± 0.0	Aa	7.04 ± 1.1	Bc	0.59 ± 0.3	Aa
EN	0.26 ± 0.1	Ab	0.00 ± 0.0	Aa	0.74 ± 0.1	Ac	0.00 ± 0.0	Aa	0.37 ± 0.1	Ab	0.00 ± 0.0	Aa
MD	1.23 ± 0.4	BCbc	0.00 ± 0.0	Aa	7.39 ± 0.7	Bd	0.26 ± 0.1	Bab	15.42 ± 0.2	Ce	1.44 ± 0.3	Bc
ED2	1.67 ± 0.5	Ca	0.11 ± 0.1	Aa	8.40 ± 2.6	Bb	0.52 ± 0.1	Ca	20.55 ± 1.4	Dc	2.67 ± 0.3	Ca

FRUCTOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.41 ± 0.1	Aa	0.00 ± 0.0	Aa	1.56 ± 0.0	Cb	0.16 ± 0.0	Aa	4.72 ± 0.5	Cc	0.42 ± 0.0	Ba
MN	0.30 ± 0.2	Aab	0.00 ± 0.0	Aa	0.74 ± 0.2	Bb	0.14 ± 0.2	Aa	2.68 ± 0.2	Bc	0.00 ± 0.0	Aa
EN	0.19 ± 0.1	Aa	0.00 ± 0.0	Aa	0.04 ± 0.1	Aa	0.00 ± 0.0	Aa	0.17 ± 0.2	Aa	0.00 ± 0.0	Aa
MD	1.12 ± 0.4	Bb	0.00 ± 0.0	Aa	2.71 ± 0.4	Dc	0.00 ± 0.0	Aa	9.67 ± 0.3	Dd	0.00 ± 0.0	Aa
ED2	2.00 ± 0.3	Cb	0.72 ± 0.2	Ba	2.37 ± 0.3	Db	0.05 ± 0.1	Aa	7.57 ± 0.6	Ec	0.35 ± 0.1	Ba

SUCROSE (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	5.23 ± 0.3	Cab	28.11 ± 1.1	Cc	2.92 ± 0.5	Ba	19.73 ± 13.6	Bbc	4.18 ± 0.7	Ba	12.86 ± 1.6	Cabc
MN	3.27 ± 0.0	Ba	9.30 ± 1.5	Bb	3.28 ± 0.6	Ba	9.93 ± 0.2	ABb	2.72 ± 0.0	ABa	4.17 ± 0.1	Ba
EN	1.30 ± 0.2	Ac	1.70 ± 0.1	Ad	0.82 ± 0.2	Ab	0.89 ± 0.1	Ab	0.75 ± 0.1	Ab	0.30 ± 0.1	Aa
MD	4.40 ± 0.2	BCa	9.95 ± 0.5	Bc	4.59 ± 0.3	Cb	10.55 ± 0.3	ABc	8.04 ± 0.7	Cb	12.30 ± 0.4	Cd
ED2	9.83 ± 0.8	Da	44.86 ± 4.0	Dc	5.83 ± 0.6	Da	39.85 ± 3.7	Cbc	8.78 ± 1.8	Ca	35.80 ± 1.0	Db

STARCH (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	5.70 ± 0.2	Cb	4.73 ± 0.3	Bab	3.12 ± 0.5	Bab	14.07 ± 1.5	Dc	2.81 ± 0.3	Ba	18.55 ± 1.6	Cd
MN	2.90 ± 0.2	Bb	3.32 ± 0.3	Bb	1.33 ± 0.1	Aa	9.63 ± 0.1	Cc	0.84 ± 0.0	Aa	12.50 ± 0.3	Bd
EN	1.02 ± 0.1	Ac	0.38 ± 0.1	Aa	0.58 ± 0.1	Aab	0.68 ± 0.2	Ab	0.80 ± 0.1	Abc	0.80 ± 0.0	Cbc
MD	3.25 ± 0.2	Bab	3.89 ± 0.5	Bb	2.81 ± 0.1	Ba	6.16 ± 0.3	Bc	3.76 ± 0.3	Bb	11.21 ± 0.4	Bd
ED2	7.71 ± 0.3	Cb	15.38 ± 1.7	Cc	4.17 ± 0.4	Ca	24.83 ± 0.9	Ed	7.35 ± 0.7	Cb	36.39 ± 1.1	De

FRUCTANS (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.27 ± 0.2	Aa	0.10 ± 0.2	Aa	0.47 ± 0.1	BCa	3.25 ± 3.5	Aa	0.76 ± 0.1	ABCa	0.61 ± 0.4	BCa
MN	0.63 ± 0.3	Ab	0.33 ± 0.1	Aab	0.21 ± 0.2	ABab	0.45 ± 0.1	Aab	0.05 ± 0.1	Aa	0.12 ± 0.1	ABa
EN	0.19 ± 0.2	Aa	0.20 ± 0.2	Aa	0.00 ± 0.0	Aa	0.00 ± 0.0	Aa	0.24 ± 0.2	ABa	0.00 ± 0.0	Ba
MD	0.38 ± 0.2	Aa	0.34 ± 0.3	Aa	0.67 ± 0.2	Ca	0.80 ± 0.7	Aab	2.01 ± 0.8	BCb	0.24 ± 0.1	ABCa
ED2	0.37 ± 0.1	Aa	0.85 ± 0.0	Ba	0.20 ± 0.1	ABa	1.04 ± 0.1	Aab	2.41 ± 1.2	Cb	0.77 ± 0.2	Ca

MALATE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	2.08 ± 0.7	Ba	2.58 ± 1.0	Ba	7.96 ± 1.5	BCb	6.40 ± 0.5	Cb	44.15 ± 1.8	Cc	41.93 ± 1.0	Cc
MN	0.00 ± 0.0	Aa	0.77 ± 0.6	Aa	5.11 ± 1.0	ABCb	1.16 ± 0.4	Ab	28.54 ± 1.9	Ad	13.85 ± 0.9	Ac
EN	0.00 ± 0.0	Aa	0.00 ± 0.0	Aa	1.64 ± 0.8	Aa	0.00 ± 0.0	Aa	31.28 ± 3.7	Ac	14.05 ± 3.1	Ab
MD	2.05 ± 0.5	Ba	5.67 ± 0.7	Ca	4.72 ± 0.6	ABa	4.09 ± 0.6	Ba	37.56 ± 1.4	Bc	24.57 ± 4.4	Bb
ED2	7.29 ± 0.5	Ca	17.95 ± 0.7	Db	8.58 ± 2.3	Ca	17.38 ± 1.6	Db	41.70 ± 1.4	BCc	41.17 ± 3.9	Cc

PROTEINS (mg g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	10.49 ± 0.4	Ca	28.87 ± 2.9	Ab	12.22 ± 1.9	Ba	29.39 ± 3.7	Ab	26.66 ± 2.6	Bb	29.38 ± 2.7	Ab
MN	10.28 ± 0.8	BCa	28.64 ± 3.9	Abc	13.33 ± 3.4	Ba	30.71 ± 3.2	Abc	24.39 ± 0.5	ABb	32.03 ± 3.8	Ac
EN	8.74 ± 0.9	Aa	28.73 ± 5.3	Abc	10.53 ± 2.2	ABa	26.93 ± 5.2	Abc	21.13 ± 2.6	Ab	30.91 ± 4.0	Ac
MD	10.65 ± 0.7	Ca	29.82 ± 3.2	Acd	9.70 ± 0.5	ABa	29.02 ± 2.2	Abc	22.22 ± 1.6	Ab	33.58 ± 1.2	Ad
ED2	8.87 ± 0.4	ABa	25.19 ± 2.3	Abc	7.60 ± 1.1	Aa	28.35 ± 2.3	Ac	22.57 ± 1.4	ABb	33.82 ± 3.1	Ad

Do not distribute

COLD DAY AND COLD NIGHT 22ºC:4ºC

	1S		1B		2S		2B		3S		3B	
GLUCOSE (µmol g-1 FW)												
ED	0.00 ± 0.0	Aa	1.00 ± 0.5	Ba	2.58 ± 0.2	ABa	0.00 ± 0.0	Aa	10.72 ± 5.4	Ab	6.94 ± 3.6	Bab
MN	0.00 ± 0.0	Aa	0.89 ± 0.3	Ba	1.60 ± 0.6	Aa	0.00 ± 0.0	Aa	8.66 ± 2.1	Ab	2.09 ± 1.1	Aa
EN	0.33 ± 0.2	ABa	0.06 ± 0.1	Aa	1.58 ± 0.3	Ab	0.00 ± 0.0	Aa	12.09 ± 0.4	Ac	2.24 ± 0.8	ABb
MD	0.35 ± 0.0	ABa	0.00 ± 0.0	Aa	2.99 ± 0.2	Bb	0.21 ± 0.1	Ba	12.71 ± 1.3	Ac	2.34 ± 0.8	ABb
ED2	0.50 ± 0.3	Ca	1.55 ± 0.2	Ba	2.90 ± 0.5	Bab	0.70 ± 0.1	Ca	11.94 ± 2.2	Ac	4.47 ± 0.6	ABb
FRUCTOSE (µmol g-1 FW)												
ED	0.00 ± 0.0	Aa	1.22 ± 0.4	ABb	1.22 ± 0.2	Bb	0.29 ± 0.1	Aa	2.77 ± 0.7	Ac	0.28 ± 0.1	ABa
MN	0.11 ± 0.1	Aa	2.28 ± 0.6	Bb	0.58 ± 0.1	Aa	0.00 ± 0.0	Aa	3.00 ± 0.3	Ab	0.39 ± 0.0	ABa
EN	0.00 ± 0.0	Aa	1.62 ± 0.6	ABb	0.62 ± 0.3	Aa	0.06 ± 0.1	Aa	3.47 ± 0.2	Ac	0.00 ± 0.0	Aa
MD	0.11 ± 0.1	Aa	0.94 ± 0.3	Abc	1.06 ± 0.2	ABc	0.17 ± 0.0	Aa	3.25 ± 0.1	Ad	0.46 ± 0.3	Bab
ED2	0.40 ± 0.4	Aa	0.98 ± 0.4	Aa	1.09 ± 0.1	ABa	0.56 ± 0.2	Aa	3.46 ± 0.4	Ab	0.53 ± 0.2	Ba
SUCROSE (glucose eq. µmol g-1 FW)												
ED	8.64 ± 0.7	Ca	57.39 ± 3.4	Cd	7.64 ± 0.8	Ba	42.04 ± 3.5	Dc	8.50 ± 0.6	Aa	22.42 ± 2.5	Cb
MN	5.38 ± 0.4	Ba	36.60 ± 9.2	Bc	7.55 ± 0.2	ABa	21.29 ± 1.2	Bb	12.15 ± 1.4	Bab	12.71 ± 1.1	Bab
EN	3.75 ± 0.1	Aa	9.28 ± 1.0	Ac	5.71 ± 0.6	Ab	4.80 ± 0.7	Aab	10.05 ± 0.7	ABc	5.45 ± 0.2	Aab
MD	5.68 ± 0.3	Ba	38.94 ± 5.9	Bd	7.07 ± 0.4	ABa	28.43 ± 0.0	Cc	9.27 ± 0.5	Aa	16.76 ± 0.6	Bb
ED2	11.94 ± 0.6	Da	63.10 ± 3.1	Cd	7.39 ± 1.1	ABa	40.34 ± 0.6	Dc	9.15 ± 0.0	Aa	27.10 ± 3.4	Cb
STARCH (glucose eq. µmol g-1 FW)												
ED	1.50 ± 0.1	Ba	6.75 ± 0.2	Bb	1.48 ± 0.2	Ba	5.36 ± 1.3	Bb	1.28 ± 0.1	Ba	6.05 ± 0.9	Cb
MN	0.81 ± 0.1	Aab	5.31 ± 0.8	Bc	0.66 ± 0.1	Aa	1.73 ± 0.1	Ab	0.65 ± 0.1	Aa	1.23 ± 0.1	Aab
EN	0.67 ± 0.0	Aa	2.47 ± 0.9	Ab	0.52 ± 0.1	Aa	0.42 ± 0.0	Aa	0.50 ± 0.1	Aa	0.19 ± 0.1	Aa
MD	1.72 ± 0.1	Ba	5.06 ± 0.7	Bc	1.50 ± 0.2	Ba	3.87 ± 0.3	Bb	1.41 ± 0.1	Ba	4.38 ± 0.5	Bbc
ED2	1.66 ± 0.1	Ba	9.35 ± 0.3	Cc	1.60 ± 0.2	Ba	7.36 ± 0.3	Cb	1.36 ± 0.1	Ba	7.84 ± 0.8	Db
FRUCTANS (glucose eq. µmol g-1 FW)												
ED	0.54 ± 0.4	Aa	4.24 ± 0.6	ABa	1.92 ± 0.8	ABa	1.23 ± 0.6	Ba	10.63 ± 3.9	ABb	1.27 ± 0.3	ABa
MN	0.94 ± 0.2	Aa	5.58 ± 1.1	Bb	1.26 ± 0.6	ABa	0.29 ± 0.1	Aa	4.97 ± 1.8	Ab	0.43 ± 0.4	Aa
EN	0.84 ± 0.1	Aab	2.05 ± 1.4	Ab	0.86 ± 0.3	Aab	0.16 ± 0.1	Aa	5.94 ± 0.2	ABc	0.45 ± 0.3	Aab
MD	1.02 ± 0.1	Aab	2.14 ± 1.2	Ab	2.20 ± 0.3	ABb	0.67 ± 0.3	ABa	7.24 ± 0.2	ABc	0.35 ± 0.2	Aa
ED2	0.93 ± 0.1	Aa	4.05 ± 0.3	ABb	2.73 ± 0.7	Bab	1.43 ± 0.2	Ba	10.98 ± 2.0	Bc	1.43 ± 0.5	Ba
MALATE (µmol g-1 FW)												
ED	4.31 ± 0.5	Ba	24.34 ± 4.4	Bb	15.64 ± 0.9	Aab	29.17 ± 7.0	Bbc	54.35 ± 3.7	Bd	40.12 ± 10.5	ABcd
MN	1.52 ± 0.0	Aa	4.61 ± 1.0	Aa	21.30 ± 4.9	ABbc	13.95 ± 4.0	Ab	39.69 ± 3.5	Ad	25.19 ± 1.1	Ac
EN	1.95 ± 0.9	ABa	3.80 ± 1.5	Aa	17.04 ± 1.4	Ac	10.75 ± 0.4	Ab	41.83 ± 1.5	Ae	29.58 ± 2.3	ABd
MD	2.79 ± 0.6	Ba	20.72 ± 3.1	Bb	20.16 ± 1.9	ABb	28.64 ± 5.0	Bb	47.45 ± 4.5	ABc	48.82 ± 3.6	Cc
ED2	8.04 ± 1.6	Ca	34.09 ± 11.0	Bb	25.96 ± 0.6	Bb	34.33 ± 0.2	Bb	53.29 ± 4.5	Bc	48.56 ± 3.3	Cc
PROTEINS (mg g-1 FW)												
ED	16.75 ± 0.6	ABa	51.43 ± 1.8	ABd	20.09 ± 3.8	Aa	40.16 ± 5.5	Ac	31.02 ± 1.8	Bb	35.03 ± 3.2	Abc
MN	16.05 ± 0.5	ABa	45.72 ± 5.6	ABd	21.79 ± 3.6	Aa	43.04 ± 3.1	Acd	30.82 ± 1.0	Bb	36.22 ± 3.4	Abc
EN	17.90 ± 1.6	Ba	52.17 ± 3.0	Bd	17.67 ± 1.9	Aa	39.35 ± 3.4	Ac	27.65 ± 1.0	ABb	37.43 ± 3.3	Ac
MD	17.36 ± 0.9	ABa	49.86 ± 2.7	ABd	18.07 ± 1.8	Aa	42.37 ± 3.6	Ac	26.56 ± 1.9	Ab	38.37 ± 3.8	Ac
ED2	15.31 ± 1.2	Aa	43.37 ± 5.0	Ac	19.08 ± 3.3	Aa	38.05 ± 1.8	Ac	27.89 ± 2.8	ABb	37.93 ± 1.5	Ac

A	columns	between timepoints
a	lines	between tissues

WARM DAY AND COLD NIGHT 10ºC:4ºC

	1S		1B		2S		2B		3S		3B	
GLUCOSE (µmol g-1 FW)												
ED	0.30 ± 0.4	Aa	0.38 ± 0.1	Aa	5.13 ± 0.7	Ab	0.83 ± 0.2	Aa	11.35 ± 2.4	Cc	2.26 ± 0.1	Ba
MN	0.49 ± 0.3	ABa	0.55 ± 0.2	Aa	3.33 ± 0.7	Abc	0.62 ± 0.5	Aa	5.11 ± 1.2	Ac	1.72 ± 0.4	ABab
EN	2.10 ± 0.4	BCb	0.37 ± 0.3	Aa	4.66 ± 0.4	Ac	0.27 ± 0.1	Aa	6.77 ± 1.1	ABd	0.94 ± 0.3	Aab
MD	2.38 ± 1.2	Cbc	0.11 ± 0.1	Aa	8.16 ± 0.5	Bd	1.06 ± 0.1	Aab	16.02 ± 1.2	De	3.41 ± 0.3	Cc
ED2	0.57 ± 0.4	ABab	0.25 ± 0.1	Aa	5.59 ± 1.5	Ac	0.69 ± 0.5	Aab	10.61 ± 1.0	BCd	2.55 ± 0.4	BCb
FRUCTOSE (µmol g-1 FW)												
ED	0.57 ± 0.0	Aab	0.31 ± 0.1	Bab	1.06 ± 0.1	Ab	0.05 ± 0.1	Aa	3.82 ± 0.7	Ac	0.28 ± 0.1	Aab
MN	1.28 ± 0.6	ABb	0.00 ± 0.0	Aa	0.78 ± 0.5	Aab	0.56 ± 0.2	Bab	2.76 ± 0.3	Ac	0.39 ± 0.0	Aab
EN	1.11 ± 0.4	ABa	0.10 ± 0.1	Aa	2.19 ± 0.7	Bb	0.22 ± 0.1	ABa	5.48 ± 0.5	Bc	0.49 ± 0.1	Ab
MD	1.71 ± 0.2	Bb	0.28 ± 0.1	Ba	1.65 ± 0.1	ABb	0.23 ± 0.1	ABa	6.60 ± 0.1	Bc	0.36 ± 0.2	Aa
ED2	0.87 ± 0.4	ABbc	0.00 ± 0.0	Aa	1.05 ± 0.2	Ac	0.00 ± 0.0	Aa	3.20 ± 0.2	Ad	0.26 ± 0.2	Aab
SUCROSE (glucose eq. µmol g-1 FW)												
ED	8.38 ± 1.0	Cb	24.47 ± 0.6	Cd	3.79 ± 0.3	Aa	20.44 ± 1.6	CDc	4.71 ± 0.4	Aa	10.52 ± 1.7	ABb
MN	7.29 ± 0.9	BCa	22.73 ± 1.1	Cd	5.24 ± 1.4	ABa	15.51 ± 1.6	BCc	10.87 ± 0.1	Cb	12.30 ± 1.1	Bb
EN	4.00 ± 0.4	Aa	4.92 ± 0.4	Aab	7.50 ± 0.7	Ccd	5.86 ± 0.7	Abc	13.55 ± 1.0	De	8.52 ± 0.4	Ad
MD	5.98 ± 0.2	Bab	15.38 ± 0.4	Bd	4.51 ± 0.3	Aa	11.00 ± 2.2	ABc	5.98 ± 0.6	ABab	7.96 ± 0.8	Ab
ED2	8.38 ± 0.3	Ca	36.55 ± 0.6	Dd	6.80 ± 0.2	BCa	25.57 ± 3.1	Dc	7.57 ± 0.8	Ba	21.48 ± 0.5	Cb
STARCH (glucose eq. µmol g-1 FW)												
ED	4.00 ± 0.4	Bb	2.24 ± 0.2	Bab	1.05 ± 0.1	Ba	7.24 ± 1.0	Cc	0.62 ± 0.1	Ba	9.17 ± 1.9	Bc
MN	3.32 ± 0.1	ABc	1.95 ± 0.4	Bb	0.82 ± 0.0	ABab	4.34 ± 0.9	Bc	0.37 ± 0.0	ABa	3.64 ± 0.5	Ac
EN	2.34 ± 0.1	Ac	0.14 ± 0.0	Aa	0.56 ± 0.2	Aab	0.38 ± 0.1	Aa	0.32 ± 0.0	Aa	0.93 ± 0.3	Ab
MD	5.11 ± 0.7	Cc	1.79 ± 0.2	Bab	1.36 ± 0.1	Ca	2.63 ± 0.5	Bb	0.93 ± 0.1	Ca	4.51 ± 0.5	Ac
ED2	8.00 ± 0.3	Dcd	4.76 ± 0.6	Cb	1.89 ± 0.1	Dab	7.77 ± 0.5	Cc	1.13 ± 0.2	Ca	10.82 ± 2.5	Bd
FRUCTANS (glucose eq. µmol g-1 FW)												
ED	0.00 ± 0.0	Aa	1.38 ± 0.4	Aa	0.17 ± 0.1	Aa	0.80 ± 0.2	ABa	1.62 ± 1.5	Aa	0.86 ± 0.0	Ca
MN	0.14 ± 0.2	Aa	1.01 ± 0.7	Aab	2.28 ± 1.2	Bb	1.50 ± 0.5	Bab	1.46 ± 0.6	Aab	0.51 ± 0.3	BCab
EN	0.52 ± 0.3	Aa	0.34 ± 0.1	Aa	0.63 ± 0.5	Aa	0.31 ± 0.0	Aa	1.77 ± 0.2	Ab	0.00 ± 0.0	Aa
MD	0.44 ± 0.3	Aab	1.25 ± 0.3	Ab	0.80 ± 0.2	ABab	0.23 ± 0.3	Aa	2.57 ± 0.5	Ac	0.00 ± 0.0	Aa
ED2	0.29 ± 0.1	Aa	0.88 ± 0.3	Ab	0.10 ± 0.0	Aa	0.77 ± 0.2	ABb	0.93 ± 0.2	Ab	0.29 ± 0.1	ABa
MALATE (µmol g-1 FW)												
ED	4.24 ± 0.9	Aa	20.83 ± 1.5	ABa	12.83 ± 1.3	Aa	20.02 ± 5.2	Ba	77.58 ± 16.8	Ac	52.89 ± 11.1	Ab
MN	3.79 ± 0.3	Aa	22.00 ± 9.5	ABa	22.11 ± 5.9	Aa	15.44 ± 2.8	Ba	57.41 ± 9.9	Ab	64.15 ± 17.1	Ab
EN	3.03 ± 1.1	Aa	8.82 ± 2.5	Aab	15.27 ± 3.1	Ab	4.65 ± 1.6	Aa	56.10 ± 5.7	Ac	53.36 ± 2.9	Ac
MD	4.99 ± 0.6	Aa	15.74 ± 1.4	Aa	20.45 ± 1.8	Aa	17.70 ± 1.3	Ba	77.34 ± 13.7	Ab	63.86 ± 4.5	Ab
ED2	7.43 ± 0.7	Ba	34.25 ± 6.7	Bb	18.03 ± 4.8	Aab	14.40 ± 3.5	Bab	71.21 ± 6.4	Ac	63.13 ± 16.0	Ac
PROTEINS (mg g-1 FW)												
ED	11.24 ± 2.0	Aa	28.31 ± 2.3	Abc	10.36 ± 0.6	ABa	29.73 ± 2.8	Ac	23.71 ± 2.9	Bb	32.65 ± 2.1	Ac
MN	11.41 ± 0.6	Aa	28.45 ± 1.4	Abc	10.97 ± 0.7	Ba	29.23 ± 2.1	Abc	24.69 ± 2.6	Bb	31.46 ± 4.9	Ac
EN	11.98 ± 1.0	Aa	27.60 ± 2.1	Abc	11.10 ± 1.1	Ba	32.76 ± 0.9	Ad	23.41 ± 1.2	Bb	31.76 ± 2.3	Ad
MD	12.60 ± 1.0	Aa	30.00 ± 1.6	Ac	10.50 ± 1.2	ABa	31.00 ± 1.2	Ac	23.13 ± 0.4	ABb	32.45 ± 2.3	Ac
ED2	11.03 ± 1.2	Aa	24.04 ± 5.5	Abc	8.57 ± 0.7	Aa	30.44 ± 0.4	Ac	18.83 ± 2.0	Ab	27.42 ± 5.2	Ad

A	columns	between timepoints
a	lines	between tissues

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Supplemental table S2- Levels of transient C reserves in shoots of cv. Bowman and *elf3* mutants day:night for 20 DAS, until third leaf stage. Values represent mean and SD. DW: dry weight; 1S: 1 sheath; 3B: 3rd leaf blade; WT: Bowman background; 289 and 290: introgression lines of *elf3*; ED: within a timepoint; small case letters represent differences between tissues within a genotype by Tu P<0.05, n=6.

	1S			1B			2S		
GLUCOSE (µ									
WT ED	21.25	±	4.2 Aab*	2.08	±	1 Aa	86.91	±	8.4 Bc
289 ED	16.94	±	1.7 Ab	5.05	±	0.6 Ba	31.66	±	6.8 Ac
290 ED	29.61	±	9 Ab	6.08	±	1.5 Ba	30.72	±	8.5 Ab
WT EN	11.42	±	3.5 Aa	5.6	±	2.7 Aa	80.98	±	11 ABc
289 EN	14.85	±	1.1 Aab	3.79	±	2.8 Aa	58.91	±	13.2 Ac*
290 EN	26.12	±	6.4 Bb	6.86	±	0.7 Aa	105.32	±	9.3 Bc*
FRUCTOSE (									
WT ED	15.12	±	2.3 Babc*	3.38	±	1.6 Aa	21.68	±	1.7 Bbc
289 ED	9.27	±	2.5 Ab	2.07	±	1.8 Aa	11.65	±	2.3 Ab
290 ED	11.99	±	2.1 ABb	1.67	±	2.5 Aa	12.62	±	3.7 Ab
WT EN	5.64	±	2.8 Aa	5.48	±	1 ABa	46.26	±	5.7 ABb*
289 EN	11.09	±	0.4 ABa	3.93	±	1.2 Aa	30.61	±	2.1 Ab*
290 EN	17.72	±	5.3 Ba	7.87	±	1 Ba*	59.35	±	9.5 Bb*
SUCROSE (eq. glu									
WT ED	39.94	±	5.8 Ab*	262.24	±	26.9 Ad*	22.01	±	6.3 Aab
289 ED	49.53	±	7.2 Ab	229.43	±	8.2 Ad*	34.97	±	4.9 Ab
290 ED	52.07	±	1.4 Ab*	237.43	±	7.6 Ad*	31.04	±	11.5 Aa
WT EN	21.57	±	1.3 Ab	33.49	±	0.5 Ac	61.43	±	5.5 Bd*
289 EN	41.66	±	7.3 Bb	52.86	±	7.8 Bbc	66.21	±	6 Bc*
290 EN	31.93	±	7.8 ABb	87.15	±	5.4 Cd	44.94	±	5.8 Abc
STARCH (eq. gluc									
WT ED	31.86	±	2.5 Ac*	24.32	±	0.8 Bc*	14.67	±	1.5 Bb*
289 ED	28.56	±	6.1 Ac*	16.28	±	1.9 Ab*	8.99	±	0.5 Aab*
290 ED	23.99	±	1.6 Ad*	18.48	±	1 Ac*	8.99	±	0.6 Ab*
WT EN	10.92	±	1 Ad	1.46	±	0.2 Aa	3.93	±	0.3 Ac
289 EN	10.86	±	0.8 Ad	3.58	±	0.3 Bab	5.53	±	0.3 Bc
290 EN	10.85	±	1.3 Ad	5.72	±	0.5 Cb	6.32	±	0.1 Cb
FRUCTANS (eq. glu									
WT ED	5.29	±	2.3 Ba	37.17	±	4 Bb*	10.78	±	4.3 Ba
289 ED	0	±	0 Aa	25.06	±	1.8 Ae*	0.45	±	0.8 Aab
290 ED	4.13	±	3 Ba	34.38	±	6.1 ABc*	6.64	±	1.7 ABa
WT EN	3.71	±	0.6 Aa	6.35	±	1.1 Aab	12.09	±	0.8 Bb
289 EN	0.58	±	1 Aa	13.01	±	2.7 Aab	8.44	±	2.4 ABa*
290 EN	2.46	±	2.6 Aa	9.91	±	7.6 Aa	2.98	±	3.9 Aa

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	MALATE (µ		
	1S	**1B**	**2S**
WT ED	413.37 ± 10 Aa*	637.64 ± 32.8 Bb*	456.56 ± 12.1 Aa
289 ED	449.85 ± 26.4 Aa*	531.53 ± 16.2 Abc	476.68 ± 31.6 Aab
290 ED	526.84 ± 78.7 Aa	550.36 ± 25.4 Aa	459.51 ± 48.4 Aa
WT EN	331.7 ± 14.6 Aa	510.73 ± 13.7 Ac	440.74 ± 29.3 Ab
289 EN	379.83 ± 23.8 Ba	551.82 ± 44.4 Ab	439.83 ± 57 Aa
290 EN	440.15 ± 11.6 Ca	471.67 ± 56.2 Aab	482.47 ± 15 Aab
	PROTEINS (		
	1S	**1B**	**2S**
WT ED	76.48 ± 10.6 Aa	233.28 ± 1.7 Ac	97.81 ± 21.7 Aa
289 ED	66.41 ± 15.7 Aa	243.64 ± 10.7 Acd	129.68 ± 5.6 Ab
290 ED	105.06 ± 26.6 Aa	238.57 ± 8.4 Abc	124.54 ± 14.3 Aa
WT EN	101.28 ± 16.6 Aa	254.37 ± 5.2 Ad*	154.88 ± 16 Ab*
289 EN	98.89 ± 9.2 Aa*	236.75 ± 14.2 Abc	123.77 ± 23 Aa
290 EN	59.19 ± 57.8 Aa	233.53 ± 6.7 Abc	140.69 ± 42.5 Aab

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under 12h:12h light:dark photoperiod with 500 µmol photons m^{-2}s^{-1} under 22°C:4°C
st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf
end of day; EN: end of night. Capital letters represent differences between genotypes
ıkey P<0.05; * represents differences between timepoints within a genotype by t-test

2B	3S	3B
ımol g^{-1} DW)		
2B	**3S**	**3B**
22.76 ± 3.8 Bab	143.57 ± 16.4 Bd	39.26 ± 6.6 Ab
12.08 ± 2.6 Aab	92.33 ± 5.6 Ad	30.93 ± 0.7 ABc
13.5 ± 1.8 Aa	101.99 ± 0.2 Ac	28.22 ± 3.1 Bb
16.01 ± 4.6 Aa	141.22 ± 8.3 Bd	47.27 ± 3.2 Bb
8.99 ± 1.1 ABa	106.93 ± 7.8 Ad	29.96 ± 5.8 Ab
16.22 ± 1.4 Bab	134.47 ± 7.2 Bd*	29.65 ± 5 Ab
µmol g^{-1} DW)		
2B	**3S**	**3B**
11.5 ± 1.5 ABab	47.13 ± 1.4 Bd	28.14 ± 11.9 Ac
8.46 ± 2 Ab	39.86 ± 2.5 Ad	18.31 ± 1.5 Ac
13.42 ± 0.6 Bb	41.2 ± 1.8 Ac	18.41 ± 3.2 Ab
10.21 ± 4.2 Ab	91.97 ± 8.9 Bc*	38.35 ± 5 Bb
10.58 ± 0.3 Aa	66.8 ± 12.9 Ac*	30.8 ± 7.9 ABb
16.5 ± 1.8 Aa*	85.25 ± 4 ABc*	21 ± 1.9 Aa
ıcose µmol g^{-1} DW)		
2B	**3S**	**3B**
160.93 ± 3 Ac*	0 ± 0 Aa	56.53 ± 17.8 Ab*
166.61 ± 13.8 Ac*	11.07 ± 8.1 Aa*	42.68 ± 4.2 Ab*
171.38 ± 4.5 Ac*	12.68 ± 10.9 Aa*	69.4 ± 3.5 Ab*
7.32 ± 3.8 Aa	0 ± 0 Aa	3.19 ± 4.8 Aa
41.27 ± 5.8 Bb	0 ± 0 Aa	9.8 ± 6.1 Aa
55.3 ± 3.1 Cc	0 ± 0 Aa	1.18 ± 2 Aa
cose µmol g^{-1} DW)		
2B	**3S**	**3B**
58.96 ± 3.8 Cd*	5.35 ± 0.3 Ba*	63.02 ± 5 Bd*
45.65 ± 1.4 Bd*	3.32 ± 0.2 Aa*	45.8 ± 2.5 Ad*
37.98 ± 1.5 Ae*	3.15 ± 0.3 Aa	46.48 ± 0.6 Af*
3.05 ± 0.3 Abc	2.56 ± 0.1 Aab	2.67 ± 0.1 Aabc
3.71 ± 0.3 Bab	2.78 ± 0.2 Aa	4.78 ± 0.5 Bbc
8.68 ± 0.1 Cc	3.78 ± 0.3 Ba	6.62 ± 0.8 Cb
ucose µmol g^{-1} DW)		
2B	**3S**	**3B**
15.22 ± 7.6 Aa*	15.82 ± 3.6 Aa	9.8 ± 8.5 Aa
19.22 ± 1.6 Ade*	14.64 ± 6 Acd	7.67 ± 1.8 Abc
15.16 ± 6.6 Aab	19.94 ± 2.7 Ab	5.12 ± 2.1 Aa
0.88 ± 1.5 Aa	26.77 ± 4.9 Ac*	3.35 ± 0.6 Aa
2.1 ± 3.6 ABa	21.94 ± 3.5 Ab	1.84 ± 3.2 Aa
7.62 ± 1.8 Ba	16.93 ± 17 Aa	6.85 ± 4.4 Aa

...mol g^{-1} DW)			
	2B	**3S**	**3B**
	588.94 ± 23.9 Bb*	712.39 ± 29.8 Bc	606.36 ± 17.5 Bb*
	499.6 ± 9.5 Aab	733.27 ± 44.1 Bd	601.21 ± 31.6 Bc
	530.26 ± 17 Aa	573.56 ± 43.4 Aa	475.5 ± 8.3 Aa
	452.02 ± 27.2 Ab	808.02 ± 22.6 Ad*	523.11 ± 3.8 Bc
	564.18 ± 4.1 Bb*	729.28 ± 63.6 Ac	566.8 ± 4.7 Bb
	546.72 ± 26.4 Bb	766.25 ± 20.4 Ac*	404 ± 52.3 Aa
(mg g^{-1} DW)			
	2B	**3S**	**3B**
	230.59 ± 23.9 Ac	171.12 ± 20.5 Ab	258.17 ± 9 Ac
	269.54 ± 8.6 Ad*	213.3 ± 20.7 ABc	240.68 ± 25.8 Acd
	259.39 ± 14 Abc	222.97 ± 7.2 Bb*	272.99 ± 13.1 Ac
	270.75 ± 14.4 Ad	214.09 ± 11.3 Ac*	256.76 ± 4.6 Ad
	235.19 ± 13.9 Abc	191.36 ± 26.9 Ab	264.11 ± 21.5 Ac
	253.3 ± 14.9 Ac	169.16 ± 20.9 Abc	208.57 ± 42.4 Abc

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Supplemental table S3 - Levels of transient C reserves in crown of cv. Bowman, *elf3* mutants and cv. Propino under 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C, 22°C:4°C and 10°C:4°C day:night until third leaf stage. Values represent mean and SD. Glucose, fructose and malate are given in µmol g^{-1} FW, sucrose, starch and fructans are given in µmol g^{-1} FW, proteins are given in mg g^{-1} FW. ED: end of day; EN: end of night; WT: Bowman background; 289 and 290: introgression lines of *elf3*; PRO: cv. Propino; FW: fresh weight.Capital letters represent differences between genotypes within a timepoint by Tukey *P*<0.05; * represents differences between timepoints within a genotype by t-test *P*<0.05, n=6.

22 ºC:18 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	0.70 ± 0.2 B*	0.42 ± 0.1 A	0.31 ± 0.1 A	2.49 ± 0.4 AB*	7.35 ± 1.4 A*	19.74 ± 3.8 C*	18.34 ± 2.3 A
289 ED	0.52 ± 0.2 AB*	0.29 ± 0.1 A	0.34 ± 0.1 AB*	1.89 ± 1.1 A	10.00 ± 2.7 AB*	11.30 ± 3.5 AB	19.44 ± 0.9 A
290 ED	0.25 ± 0.1 A	0.32 ± 0.2 A	0.26 ± 0.1 A	1.40 ± 0.5 A*	9.95 ± 2.1 AB*	9.05 ± 3.5 A	18.49 ± 1.8 A
PRO ED	0.69 ± 0.2 B	0.55 ± 0.2 A	0.49 ± 0.1 B*	3.37 ± 0.6 B	13.20 ± 1.1 B*	17.18 ± 2.7 BC*	20.68 ± 0.5 A*
WT EN	0.20 ± 0.0 A	0.24 ± 0.0 A	0.25 ± 0.1 AB	1.05 ± 0.4 A	4.08 ± 1.4 A	10.84 ± 2.2 AB	17.00 ± 1.9 AB
289 EN	0.20 ± 0.1 A	0.13 ± 0.0 A	0.13 ± 0.0 A	0.87 ± 0.3 A	4.20 ± 1.0 A	8.43 ± 1.5 A	18.67 ± 1.3 AB
290 EN	0.32 ± 0.2 AB	0.30 ± 0.1 AB	0.19 ± 0.0 AB	0.37 ± 0.2 A	2.23 ± 0.8 A	9.13 ± 3.0 A	15.27 ± 3.6 A
PRO EN	0.48 ± 0.2 B	0.46 ± 0.2 B	0.27 ± 0.1 B	2.78 ± 0.5 B	8.80 ± 2.1 B	13.71 ± 1.8 B	19.60 ± 0.9 B

22 ºC:4 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	1.31 ± 0.3 A	0.67 ± 0.2 A	2.36 ± 0.3 A	1.09 ± 0.4 A	10.86 ± 2.2 C	19.96 ± 1.6 A	18.07 ± 4.2 A
289 ED	0.99 ± 0.6 A	0.55 ± 0.3 A	2.07 ± 0.5 A	0.77 ± 0.3 A	5.09 ± 1.8 A	17.83 ± 5.1 A	21.20 ± 3.0 A
290 ED	1.00 ± 0.1 A	0.55 ± 0.2 A	1.92 ± 0.4 A	0.89 ± 0.2 A	7.85 ± 1.1 B	16.61 ± 3.7 A	22.63 ± 1.7 A*
PRO ED	1.54 ± 0.3 A	0.73 ± 0.2 A	2.32 ± 0.7 A	1.95 ± 0.1 A*	10.89 ± 0.7 C	18.91 ± 5.2 A	21.45 ± 2.9 A
WT EN	2.25 ± 0.4 B*	1.09 ± 0.3 A*	4.66 ± 0.9 B*	0.77 ± 0.2 A	22.38 ± 2.9 A*	19.99 ± 2.9 A	19.72 ± 2.4 AB
289 EN	1.61 ± 0.1 A	1.07 ± 0.2 A*	4.87 ± 1.6 B*	0.61 ± 0.1 A	22.66 ± 2.9 A*	21.65 ± 1.7 A	20.59 ± 2.1 B
290 EN	1.73 ± 0.3 A	0.70 ± 0.1 A	2.45 ± 0.3 A	0.68 ± 0.2 A	20.73 ± 1.6 A*	21.75 ± 3.2 A	16.51 ± 1.4 A
PRO EN	2.09 ± 0.2 AB	1.03 ± 0.2 A*	3.17 ± 1.5 AB	1.17 ± 0.1 A	19.67 ± 0.9 A*	24.02 ± 4.9 A	20.71 ± 1.0 B

10 ºC:4 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	1.48 ± 0.2 A	1.23 ± 0.1 A	16.38 ± 1.2 A	3.48 ± 0.8 A	16.88 ± 3.1 A	17.70 ± 1.6 AB	24.08 ± 1.8 A
289 ED	1.11 ± 0.1 A	1.09 ± 0.1 A	16.82 ± 2.1 A	4.04 ± 0.6 A	29.22 ± 2.5 A*	16.81 ± 0.7 A	23.79 ± 2.0 A*
290 ED	1.39 ± 0.3 A	1.29 ± 0.2 A	16.99 ± 1.6 A	3.57 ± 0.6 A	18.85 ± 4.6 A	12.23 ± 1.7 A	22.94 ± 1.3 A
PRO ED	2.00 ± 0.4 B	1.57 ± 0.1 B	19.09 ± 1.1 A	6.40 ± 0.7 A	24.87 ± 1.9 A	23.45 ± 1.6 B*	25.83 ± 2.9 A
WT EN	1.38 ± 0.2 A	1.17 ± 0.1 A	24.40 ± 0.7 A*	2.88 ± 0.6 A	17.79 ± 3.0 A	18.59 ± 0.6 A	21.61 ± 1.9 A
289 EN	1.38 ± 0.2 A*	1.40 ± 0.1 AB*	24.55 ± 2.3 A*	4.02 ± 0.5 AB	18.00 ± 2.5 A	18.24 ± 0.5 A*	21.05 ± 2.2 A
290 EN	2.16 ± 0.4 B*	1.71 ± 0.1 B*	26.85 ± 3.4 A*	3.32 ± 0.6 AB	27.33 ± 7.9 A	15.02 ± 1.9 B*	21.36 ± 1.7 A
PRO EN	2.40 ± 0.3 B	1.74 ± 0.2 AB	24.77 ± 1.8 A*	6.27 ± 0.5 B	23.69 ± 5.1 A	21.04 ± 0.8 B	23.98 ± 2.5 A

A = comparison between genotypes within timepoint
* = comparison between timepoints within genotype