Molecular mechanisms regulating carbohydrate metabolism during *Lolium perenne* regrowth vary in response to nitrogen and gibberellin supply

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5   **Abstract**

7   The promoting effects of both high nitrogen (N) and exogenous gibberellin (GA) supply on regrowth of *Lolium*

8   *perenne* have been widely reported. The mobilisation of carbohydrate reserves in response to N is a critical

9   mechanism for promoting plant regrowth. However, our knowledge about GA regulation of carbohydrate

10   metabolism remains limited. Here, we analysed the effects of both N and exogenous GA on the molecular

11   mechanisms controlling perennial ryegrass regrowth and investigated the similarities and differences. Our

12   analyses show that both high N and exogenous GA supply lead to a decline in the accumulation of carbohydrate

13   reserves, but the regulatory mechanisms responsible for this decline varied between N and GA supply. The effects

14   of elevated N were mainly through declining fructan biosynthesis which results in improving photosynthate use

15   efficiency to promote plant regrowth, whereas the application of exogenous GA resulted in an increase in the

16   hydrolytic activities of fructan exohydrolase and invertases capable of cleaving reserved carbohydrates to release

17   energy sources for plant regrowth.

19   **Key words:** Perennial ryegrass, Regrowth, Fructans, Exogenous gibberellin, Nitrogen, Gene expression

21   **Introduction**

23   Intense pasture-based animal production systems are strongly dependent on nitrogen (N) supply and face

24   mounting pressure to mitigate resource-driven adverse environment impacts, such as nitrous oxide (N_2O)

25   emissions and nitrate (NO_3^-) leaching. In New Zealand the primary source of N_2O emissions, 57% of the total

26   anthropogenic N_2O emissions (Ministry for the Environment 2013), comes directly from soils following N

27   fertilisation and excreta deposition by grazing ruminants. One root cause of this high N_2O emission is the high

28   rate of N supply to the ruminant as part of a pasture-based diet. This leads to excessive N intake per animal that

29   is voided, predominantly in urine, adding to the concentrations of highly labile mineral N in soils already supplied

30   with mineral N fertiliser (Parsons et al. 2013). A decrease in N fertilisation of soil and concurrent increase in

31   carbohydrate content of forages, the latter providing a readily fermentable source of energy in the rumen, have

32   been proposed to improve the N use efficiency of ruminants grazing pasture (Edwards et al. 2007; Parsons et al.

33   2013). Reducing N fertilisation has also been shown to increase carbohydrate content and reduce crude protein

34   concentrations in *Lolium perenne* (Lattanzi et al. 2012). However, lower N fertilisation can reduce plant yield,

35   adversely affecting pastoral production. The challenge therefore is to reduce N fertilisation without negatively

36   impacting forage yield and quality.

38   Gibberellins (GAs) have been known for decades for their strong growth-promoting effects on stems and leaves

39   (see review by Yamaguchi 2008). Applied to pasture, they can stimulate winter and spring pasture production or

40   manipulate seasonality of production through speculative reserve mobilisation, leaf and stem elongation and

41   promotion of flowering (see review by Mathew et al. 2009; Cai et al. 2016; van Rossem et al. 2013). In a

42   previously reported experiment (Parsons et al. 2013), we simply applied an exogenous gibberellin (GA₃,

43   commercially labelled ProGibb-SG*TM*) and demonstrated that growth of winter-derived *L. perenne* plants was

44   promoted, both at low mineral N supply (25% increase in blade biomass) and substantially more (38%) so at high

mineral N supply. These results support many earlier observations on GA promoting responses to pasture growth, e.g. for a sample of 72 responses from experiments reviewed by Mathew et al. (2009), an increase in sward height and yield in the 3 – 4 weeks after GA application was almost universally observed. These studies indicate that there is significant scope to increase growth without the need for substantial increases in N input, offering far greater N use efficiency through the plant, animal and soil, and this has valuable implications and prospects for mitigation of not only nitrous oxide emissions but for significantly improving multiple aspects of the efficiency of use of nitrogen (Parsons et al. 2013). Although these studies have advanced our understanding of the effect of GAs at specific developmental phases, compared to N nutrient which is known to affect carbohydrate metabolism in fundamental ways (Stitt and Krapp 1999; Wang et al. 2000; Wang and Tillberg 1996), they provide limited information concerning the general role of bioactive GAs in the regulation of carbohydrate metabolism under regrowth conditions in *L. perenne*.

Defoliation and subsequent regrowth is a major and essential event with respect to *L. perenne* growth and productivity in pastures. Carbohydrate and N reserves serve a critical role as substrates for refoliation (Schnyder and de Visser 1999). Consequently, the accumulation and mobilisation of storage carbohydrates, predominantly fructans, in the remaining tissues are of great importance in restoring photosynthetic tissues for regrowth. It has been shown that during the first 2 - 3 days following defoliation, shoot growth of *L. perenne* relies predominantly on the mobilisation of carbohydrate reserves from both sheaths and elongating leaf bases (de Visser et al. 1997; Morvan et al. 1997); and the refoliation rate during the first week following defoliation is an important determinant of subsequent regrowth and herbage yield (Schnyder and de Visser 1999). The accumulation and mobilisation of reserve carbohydrates requires a substantial change in the expression of genes coding for enzymes involved in carbohydrate metabolism, such as invertases, fructosyltransferases (FT) and fructan exohydrolases (FEH) (Lee et al. 2011; Rasmussen et al. 2014; van den Ende et al. 2003); and nutrient level is an important factor regulating the above changes (Conaghan et al. 2012).

Nitrogen deficiency is known to decrease sink strength and growth rate (Kavanová et al. 2008), increase carbohydrate accumulation in vegetative tissues (Keating and O'Kiely 2000; Lattanzi et al. 2012; Lehmeier et al. 2010), and mediate the expression of carbohydrate storage-related genes (McIntyre et al. 2011; Ruuska et al. 2008) and their activities (Morcuende et al. 2004; Wang et al. 2000; Wang and Tillberg 1996). From these relationships, one might expect that the growth promoting effects of exogenous GA supply, apart from its stimulating effects such as enhancing cell division and elongation, may also affect carbohydrate metabolism. Initial studies have implicated that gibberellins may be involved in the regulation of carbohydrate metabolism. For example, Cai et al. (2016) showed that exogenous GA_3 stimulates the regrowth of defoliated sheepgrass (*Leymus chinensis*) regrowth by regulating GA and fructan-related genes, and by promoting endogenous GA synthesis, fructan metabolism and signalling. Morvan et al. (1997) showed that the activity of fructan exohydrolase (FEH, an important fructan mobilising enzyme) in *L. perenne* was able to be repressed by a GA inhibitor of uniconazole and this inhibition was reversed by exogenous supply of bioactive GA_3. However, to our knowledge no study has been undertaken to compare or exploit how nitrogen and exogenous GA differently regulate carbohydrate metabolism within the regrowth cycle in perennial ryegrass.

By comparing the application of nitrogen and exogenous GA, here, we analysed (by RT-qPCR) the expression of genes involved in pathways of *L. perenne* carbohydrate metabolism, gibberellin biosynthesis and transduction, and nitrogen assimilation. Our aims were to investigate the molecular mechanisms of the promoting effects of N and exogenous GA supply on perennial ryegrass regrowth, and the possible interaction between carbohydrate metabolism and GA regulation. The present work is designed to complement and enhance our previously published paper where plant growth data were presented (Parsons et al. 2013).

Materials and Methods

Plants and growing conditions

Field grown *Lolium perenne* plants (cultivar 'AberDart') were dug out of an established pasture in the mid-winter season (June 2011). Plants were washed free of soil and clumps of ten tillers per plant were trimmed and transplanted into pots (9 cm x 9 cm x 18 cm) filled with a 2:1 sand: clay-loam mix. The plants were grown in controlled environment chambers (NZCEL, Plant and Food Research, Palmerston North, NZ) under a single day/night temperature regime of 12.5^{o}C and in a short-day environment ('SD' = 8 hours; radiation flux density at 620 μmols m^{-2} s^{-1}) to 'lock in' their field-derived seasonal/developmental state. Plants transferred to growth rooms were clipped to 6 cm above surface level and allowed to establish in their pots for at least two full defoliation and regrowth cycles (8 weeks in total) before the experiment was initiated. Plants were irrigated with tap water every second day and, prior to the initiation of the experiment, all plants received the 'low' N solution (see below) thus minimising any long-term accumulation (or depletion) of nutrient availability.

Nitrogen/ gibberellin treatments and time-course sampling

Starting in August 2011, half the plants in chambers were supplied, every second day from above, with either 'high' or 'low' mineral N (based on half strength Hoagland solution at 9 mM N as nitrate or adjusted to 2.25 mM N, 60 ml per pot) (Rasmussen et al. 2007). Critical time-course measurements were initiated 8 weeks after imposition of nutrient treatments (26 Sep 2011) by defoliating the plants to 6 cm above the soil surface. Seven replicate plants for each treatment were harvested destructively and dissected following time-course schedules of up to 4 weeks (672 h) as described in Table 1. During each day, harvests were started at 10am and the harvesting periods for each single plant minimised to be within 15mins. The '0 h' of the time-course harvested prior to defoliation (or GA supply) represents the control conditions. For the GA-treated (GA+) plants, gibberellin (GA$_3$, ProGibb-SG^{TM}) and surfactant (Contact^{TM}) were applied to groups of plants 4 days after defoliation following the manufacturer's recommendations (Nufarm, NZ). Application rates were equivalent to the recommended 20 g ha^{-1} ProGibb-SG^{TM}, in a fine foliar spray of 100 L ha^{-1} containing 20 ml 100 L^{-1} surfactant. This created 4 treatments of GA × N combination (i.e. High-N/GA-free, High-N/GA+, Low-N/GA-free and Low-N/GA+). In order to assess the immediate effects of defoliation or exogenous GA supply, corresponding plants were also harvested from 1 to 72 h following defoliation (for GA-free treatments) or from 1 to 48 h after GA supply (which equates to 97 – 144 h after defoliation, for GA+ treatments), respectively (Table 1). Notably, the change in the measures for GA-free treatments within the periods of 97 – 144 h after defoliation were not monitored, mainly due to our inability to

125 sample plants from both GA+ and GA-free treatments simultaneously. As a result, minor defoliation effects from

126 the GA effects cannot be excluded for some analyses.

127

128 At each harvest point, the plant shoots were dissected into three categories of tissues: sheath (S); enclosed

129 elongating leaf (EE), and emerged mature leaf (EM). The latter included (initially) all tissue that had emerged

130 beyond the cutting level, or (later) all tissue that had emerged beyond the ligule of the youngest ligulated leaf, if

131 one was present (see Liu et al. 2015). The harvested tissues were immediately frozen and ground in a mortar under

132 liquid nitrogen. A portion of the powdered samples were freeze-dried for carbohydrate analysis, and others were

133 stored at -80˚C for subsequent RNA isolation. In this study, a total of 280 plants were dissected and up to 840

134 samples were collected for subsequent analyses. Plant growth and dry weight were also measured, and the results

135 have been reported previously (Parsons et al. 2013).

136

137 Chemical analysis

138

139 Water soluble carbohydrates (WSCs) were extracted and quantified as described previously (Hunt et al. 2005).

140 Freeze-dried and powdered plant material (25 mg) was extracted with 2 ml 80% ethanol (representing the low

141 molecular weight (LMW) WSC fraction) and subsequently extracted with 2 ml water (representing the high

142 molecular weight (HMW) WSC fraction). The ethanol extracted LMW WSC fraction contains a mixture of sugars

143 (glucose, fructose, sucrose) and low degree of polymerisation (DP) fructans and the water extracted HMW WSC

144 fraction contains mainly high DP fructans. Extracts were briefly centrifuged and WSCs were determined in the

145 supernatants using anthrone as a colorimetric reagent (Jermyn 1956). Concentrations of glucose, fructose and

146 sucrose were determined enzymatically in the LMW WSC extracts (Rasmussen et al. 2014), and the concentration

147 of low DP fructans were calculated by subtracting those sugars from the LMW WSC.

148

149 Profiling gene expression

150

151 Total RNA from plant material was isolated using TRIzol Reagent (Invitrogen NZ Ltd., Auckland, NZ) and treated

152 with RNA-free DNase I (Roche NZ Ltd., Auckland, NZ) to remove residual genomic DNA. DNase treated RNA

153 was subsequently purified using the RNeasy Plant Mini Spin Kit (Qiagen, Biostrategy Ltd., Auckland, NZ) to

154 remove enzymes, salts and degraded DNA fragments. RNA quality was checked by gel electrophoresis, and the

155 absence of genomic DNA was confirmed by PCR prior to reverse transcription. The treated RNA was reverse

156 transcribed and converted into cDNA using the SuperScript®VILO™ cDNA Synthesis Kit (Invitrogen NZ Ltd.,

157 Auckland, NZ) following manufacturer's instructions. The synthesised cDNAs were then diluted 50-fold; and 6

158 µl diluted cDNA was used for subsequent qPCR analysis in a total PCR reaction volume of 15 µl.

159

160 Transcription profiles of 14 *L. perenne* genes (Table 2) were quantified by RT-qPCR amplifying the target genes

161 using the 96-well LightCycler® 480 II system (Roche Diagnostics NZ Ltd., Auckland, NZ). qPCR reactions were

162 assayed using a LightCycler® 480 SYBR Green I Master mix following the manufacturers protocols (Roche

163 Diagnostics NZ Ltd., Auckland, NZ). After 5 min pre-running at 95˚C, a total of 45 cycles of 10 sec at 95˚C; 10

164 sec at 60˚C; and 10 sec at 72 ˚C were performed for PCR amplification. The primers for qPCR were designed

6

165 based either on sequences in the NCBI Genbank (http://www.ncbi.nlm.nih.gov/) or on the isolated genes from our

166 *L. perenne* Rapid Amplification of cDNA Ends (RACE) cDNA libraries (Liu et al. 2015; Table 2). The primer

167 sequences and relevant information are listed in Suppl. Table S1.

168

169 To normalise the transcript data, an mRNA fragment encoding *Epichlöe*-GFP (eGFP) protein was synthesised and

170 1 pg e*GFP* RNA was spiked into 1 µg of sample RNA prior to cDNA synthesis (Liu et al. 2015). The expression

171 profiles of the genes were normalised by comparing with e*GFP* products, and results were presented as the ratio

172 of target gene copies divided by e*GFP* copies.

173

174 Statistical analysis

175

176 All measures (referred to as 'N and GA effects' at later regrowth stages) obtained within regrowth periods between

177 168 and 672 h after defoliation were analysed by 3-way ANOVA (tissue × N and GA supply × regrowth time)

178 using Minitab statistical software version 16.22. A three-way ANOVA (tissue × N level × regrowth time) was

179 also applied to examine the defoliation effects (for data obtained from 0 to 72 h at GA-free treatments; referred to

180 as 'defoliation effects'), and also to the immediate effects of GA supply (data obtained from 96 to 144 h at GA+

181 treatments; referred to as 'GA immediate effects') (Table 1). A two-way ANOVA was conducted to separate the

182 difference between individual tissues when it was required. A Box-Cox transformation was applied to homogenise

183 the error variances and where appropriate a Tukey's pairwise test was used to help interpret significant effects.

184 We report here untransformed means as a measure of data dispersion in response to treatment effects against the

185 regrowth times.

186

187 In the present study, almost all measures were significantly different between the tissues tested (Suppl. Table S2).

188 As a result, data interpretation in the following sections focused on the 'N and GA effects', 'defoliation effects'

189 and 'GA immediate effects'; and less attention was paid to describing the interactive effects between major factors

190 of tissue. The main and interactive effects between major factors have been summarised in Suppl. Table S2. Note

191 that, to make immediate changes in transcript levels and carbohydrate concentrations more visible, the x-axes in

192 line figures at Figure 1 to 3 have been split into two parts representing 0 -150 h and 160 – 700 h post-defoliation,

193 respectively.

194

195 **Results**

196

197 Effects of defoliation, N and exogenous GA supply on fructan concentration and expression of fructan-regulating

198 genes during regrowth

199

200 Fructan concentrations varied between tissues (P < 0.001 for both high and low DP fructans), with higher

201 concentrations (~2 - 4 folds) generally observed in the enclosed elongation (EE) and sheath (S) tissues compared

202 with the emerged mature blades (EM), supporting previous observations (Guerrand et al. 1996; Liu et al. 2015;

203 Morvan et al. 1997). This lower concentration of fructans in the EM blades was associated with the lower

204 expression of fructan-related genes (~10-fold, data not shown), which is consistent with the observation of others

205 (e.g. Guerrand et al. 1996; McIntyre et al. 2011).

206

207 Defoliation resulted in a dramatic decrease in fructan concentrations within 72 h of defoliation irrespective of N

208 levels ($P < 0.001$), as shown in EE tissues (Fig. 1a, c). Compared to the low N treatments, significantly decreased

209 (~2 - 3 folds; $P < 0.001$) concentrations of both high and low DP fructans were seen under high N supply which

210 is consistent with the observations of others (e.g. Keating and O'Kiely, 2000; Lehmeier et al. 2010). For all tissues

211 tested in the high N treatments, both high and low DP fructan concentrations were consistently reduced during

212 the later regrowth stages following the supply of exogenous GA (Fig. 1b, d; $P < 0.001$ for both DP fractions). The

213 effects of GA supply on fructans in the low N treatments varied in the tested tissues and were statistically

214 insignificant.

215

216 In association with the changes in fructans, rapidly decreased expression (>2 folds) of the fructosyltransferases

217 Lp*1-SST* ($P < 0.001$) and Lp*6-SFT* ($P < 0.001$) and increased expression of the fructan exohydrolase Lp*1-FEH* (P

218 < 0.01) occurred following defoliation as shown in EE tissues (Fig. 1e, g, i). A rapid increase in Lp*1-FEH* gene

219 expression was also observed shortly after GA supply in the high-N and GA+ treatments ($P < 0.01$) in the sheaths

220 and emerged mature blades (Suppl. Fig. S1a, b), which supports the observations in *Leymus chinensis* (Cai et al.,

221 2016). There were no significant differences in GA-immediate effects for Lp*1-SST* and Lp*6-SFT* gene expression.

222

223 During the later regrowth periods of 168 – 672 h after defoliation, significantly increased expression of Lp*1-FEH*

224 (~2-fold) following GA supply was observed in the high N treatment, particularly in the EE tissues (Fig. 1j; P <

225 0.001). This increased expression of Lp*1-FEH* by GA supply in high N treatment was associated with the

226 decreased fructans concentration as stated before (Fig.1b). Compared to the low N treatments, significantly

227 decreased expression levels of Lp*1-SST* and Lp*6-SFT* (both $P < 0.001$) were seen under high N supply (Fig. 1f,

228 h), particularly in the EE tissues, which is consistent with previous observations (e.g. Morcuende et al. 2004;

229 Wang et al. 2000; Wang and Tillberg 1996). Increased Lp*6-SFT* expression after GA supply was also occurred

230 under low N conditions during these periods (Fig. 1h; $P < 0.001$). As expected, the expression of Lp*6-SFT* was

231 significantly correlated with fructan concentrations, particularly in the EE tissues (High DP fructans = 28.204 x

232 Lp*6-SFT*$^{0.8499}$; r = 0.841; $P < 0.001$), which is consistent with our previous observations (Liu *et al.* 2015).

233

234 Effects of defoliation, N and exogenous GA supply on sucrose concentration and the expression of invertase genes

235 during regrowth

236

237 Significant changes in sucrose concentrations occurred shortly after defoliation. Generally, the sucrose

238 concentration decreased significantly to its lowest levels from 72 h after defoliation (Fig. 2a, c, e; $P < 0.001$),

239 although sucrose concentrations were highly sensitive to photoperiods within a day (5 h after defoliation strongly

240 elevated sucrose concentration occurred in EE and EM tissues presumably where emerging and residual leaf

241 blades have resulted in a higher rate of photosynthesis). Compared to the low N treatments, high N supply was

242 consistently associated with decreased sucrose concentrations for all tissues tested (Fig. 2a, c, e; $P < 0.001$),

243 indicating that high N supply induced sucrose cleavage to release hexoses capable of supporting refoliation.

Interestingly, for all tissues tested at the later regrowth stages, sucrose concentration was further reduced in the High-N and GA supply treatment compared with High-N and GA-free treatment (Fig. 2g; P < 0.001), suggesting that GA supply stimulated the release of more hexoses from sucrose which could be used for refoliation.

Rapidly increased expression of the vacuolar invertase gene (Lp*VacInv*), mainly in high N treatments (P < 0.001 compared to low N treatments), peaked at 48 h after defoliation for all tissues tested (Fig. 2b, d, f; P < 0.001). Lp*VacInv* gene expression appeared to be sensitive and negatively associate with the sucrose concentration immediately after GA application. At the later stages of regrowth, increased Lp*VacInv* expression was consistently seen under high N supply for all tissues tested (Fig. 2h; P < 0.001), particularly in the GA-free treatments. The effects of GA supply on Lp*VacInv* expression appeared to interact with N levels and tissues (P < 0.05). Compared to GA-free treatments, GA supply appeared to increase Lp*VacInv* gene expression under low N treatments but decreased Lp*VacInv* gene expression under high N treatments (Fig. 2h). In the EE tissues, both high N level and GA supply were associated with significantly increased expression of the cytosolic invertase (Lp*CytInv*) at the later stages of regrowth (Suppl. Fig. S1c; P < 0.01).

Effects of defoliation, N and exogenous GA supply on expression of GA-regulated genes during regrowth

The expression levels of GA-related genes were generally higher in the EE tissues than in the emerged mature blades as seen in previous studies (Jang et al. 2008; Morvan-Bertrand et al. 2001). Increased expression of GA-activated genes (Lp*GA3ox* and Lp*GA20ox*) was induced immediately after defoliation, particularly under high N conditions as shown in EE tissues (Fig. 3a, b; P < 0.01), and then reverted to the original levels. Conversely, the supply of exogenous GA resulted in decreased expression of Lp*GA3ox* and Lp*GA20ox* shortly (24 - 48 h after GA supply) (Fig. 3a, b; P < 0.001). During the later regrowth periods, the supply of exogenous GA did not result in a significant difference in the expression of both Lp*GA3ox* and Lp*GA20ox*. However, as shown in EE tissues, significantly higher expression of Lp*GA20ox* occurred in the high N treatments compared to the low N treatments (Fig. 3b; P < 0.001).

Expression of the GA-inactivating gene Lp*GA2ox* and Lp*DELLA* highly varied in the early stages after defoliation and after GA supply. Within the later stages of regrowth, gene expression of Lp*DELLA* significantly increased with exogenous GA supply, mainly in the low N treatments as shown in EE tissues (Fig. 3c, d; P < 0.001).

In the present study, the expression of genes encoding nitrate reductase (Lp*NR*) appeared to be elevated by high N levels (P < 0.001; data not shown), but there was no significant difference associated with GA supply. The expression of Lp*NT* (coding for a nitrate transporter) was variable, and there was no difference observed under both N and GA treatments (data not shown).

Discussion

Carbohydrate regulation following defoliation and during regrowth

9

284 Defoliation and the subsequent regrowth of the plant is a major and essential event with regards to *L. perenne*
285 growth and productivity in the pasture. Defoliation removes photosynthetic organs and consequently reduces the
286 available carbon (C) and energy (glucose) for regrowth. Following defoliation, reserve carbohydrates, particularly
287 the fructans, in the remaining stubble tissues have to be mobilised in order to restore photosynthetic tissues for
288 the plants regrowth (Morvan et al. 1997). The refoliation rate during the earlier stages of regrowth is an important
289 determinant of plant viability and eventual herbage yield (Schnyder and de Visser, 1999) and it has been suggested
290 that before the third day of regrowth, C supply to meristematic zones for new leaves emerging from the stubble
291 predominantly relies on this mobilisation (de Visser et al. 1997; Morvan et al. 1997).

292

293 In this study, defoliation was followed by a rapid (> 2-fold within 1-3 days after cutting) decrease in concentrations
294 of both fructans and sucrose. In association, a rapid decrease in expression of the fructan synthetic genes Lp*1-SST*
295 and Lp*6-SFT* and an increase in expression of the fructan mobilising genes Lp*1-FEH* and invertase gene Lp*VacInv*
296 occurred within the first 72 h following the defoliation. All these changes indicate that defoliation induced a shift
297 from maintaining reserves to their conversion to soluble sugars in order to supply energy for rapid refoliation and
298 suggested that the mobilisation and utilisation of reserve carbohydrates is one of the mechanisms that facilitates
299 rapid regrowth. Increased expression of genes encoding the GA-activating Lp*GA3ox* and Lp*GA20ox* were also
300 observed after defoliation. These results are consistent with previous observations (de Visser et al. 1997; Hisano
301 et al. 2008; Lidgett et al. 2002; Liu et al. 2015; Morvan-Bertrand et al. 1999; Morvan et al. 1997) and indicate that
302 GA may be involved in the defoliation response.

303

304 Nitrogen and exogenous GA status affects carbohydrate metabolism differently during regrowth

305

306 *Effects on fructans and fructan-regulating genes*
307 The primary storage carbohydrates in *L. perenne* are fructans (Morvan-Bertrand et al. 2001). Fructans are
308 synthesised from sucrose by fructosyltransferases (FTs) and degraded by fructan exohydrolases (FEHs) (Morvan
309 et al. 1997). They predominantly occur in the youngest cells in the cell division and expansion zones (Morvan-
310 Bertrand et al. 2001), and accumulate as storage molecules, stored in vacuoles, in the carbohydrate-sink tissues
311 (Lattanzi et al. 2012; Pollock and Cairns 1991). High fructan content is a valuable resource in perennial ryegrass
312 as it can be readily mobilised to sustain regrowth immediately after defoliation (Morvan et al. 1997) as well as
313 potentially adding to the nutritive value of the feed (Johnson et al. 2003) and they have also been shown to be of
314 benefit in reducing nitrogen excretion from grazing ruminants (Edwards et al. 2007; Parsons et al. 2013).

315

316 Nitrogen nutrition is known to affect carbohydrate metabolism in fundamental ways (Lattanzi et al. 2012; Morvan-
317 Bertrand et al. 1999; Stitt & Krapp 1999; Wang et al. 2000). N deficiency decreases sink strength and growth rate
318 (Kavanová et al. 2008), increases carbohydrate accumulation in vegetative tissue (Lattanzi et al. 2012; Lehmeier
319 et al. 2010; van den Ende et al. 1999), and increases the expression of carbohydrate storage-related genes (Ruuska
320 et al. 2008) and their activities (Morcuende et al. 2004; Wang et al. 2000; Wang and Tillberg 1996). Nitrate (the
321 major N source applied in this study) has been considered a negative signal regulating fructan biosynthesis
322 although it does not induce fructan degradation directly (Morcuende et al. 2004). In the present study, a
323 comparison between the GA-free treatments under different N levels, showed that the high N supply was

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associated with a significant decrease of both high and low DP fructans in all tissues tested. These decreased fructan concentrations under high N treatments were proportionally related to the initial status of the fructans prior to defoliation and correlated negatively with the gene expression of Lp*1*- Lp*6-SFT*, particularly in EE tissues. Results indicate that the reduced fructan synthesis, via decreased activities of fructosyltransferases under high N levels, were the critical molecular mechanism for the high N effects on ryegrass regrowth; and the fructan-synthetic enzyme of Lp6-SFT may play a key role in the regulation of fructan accumulation under high N conditions. These results support previous studies showing that high N is capable of reducing fructan accumulation (Lattanzi et al. 2012; Morvan-Bertrand et al. 1999; Wang and Tillberg 1996) and the enzymatic activities of 1-SST and 6-SFT (de Roover et al. 2000; Morcuende et al. 2004; van den Ende et al. 1999; Wang et al. 2000; Wang and Tillberg 1996).

Exogenously applied bioactive GA has been known to strongly promote the elongation of shoots in many plants (Gocal et al. 1999; MacMillan et al. 2005; Yamaguchi 2008). In this study, exogenous GA supply appeared to reduce fructan accumulation in a similar manner to high N effects. As shown in Fig. 1 (b, d), at the later stages of regrowth GA supply resulted in reduced fructan concentrations under high N conditions for all tissues tested. The effects of GA supply on fructan concentrations were associated with strongly promoted plant regrowth (38%) in blade biomass following GA supply under high N (Parsons et al. 2013), indicating that the effects of GA supply on *L. perenne* regrowth, like the effects of high N, are also through reducing fructan biosynthesis. Interestingly and notably, unlike N effects where high N supply was strongly associated with declined expression of fructan-synthesising genes, the decreased fructan concentrations in association with GA supply did not appear to be due to decreased expression of the two fructan-synthesising genes. Instead, the decreased fructan concentration in association with GA supply appeared to be a result of increased expression of the gene encoding the fructan-degrading Lp*1-FEH*. Under high N conditions, expression of Lp*1-FEH* was significantly elevated in the presence of exogenous GA throughout the later stages of regrowth in the enclosed elongating tissues (Fig. 1i, j). And increased expression of Lp*1-FEH* also occurred in the sheaths and the emerged mature blades immediately after the supply of GA (Suppl. Fig. S1a, b). These results indicate that, unlike high N effects, the effects of exogenous GA supply on fructan synthesis was through increased activity of the fructan-degrading enzyme Lp1-FEH rather than reduced activity of the fructan-synthesising Lp1-SST and Lp6-SFT. This provides evidence that there are obvious differences and complementarities in the interaction of N and GA affecting fructan biosynthesis during *L. perenne* regrowth. The reduced accumulation of fructans, both under high N and exogenous GA supply, is likely due to the need to provide energy sources to accelerate the regrowth of new leaves emerging from elongating zone, and consequently increase the production of photosynthates and plant regrowth, particularly during the earlier regrowth stages.

In *L. perenne*, Lp1-FEH has been implicated in the fast mobilisation of fructans immediately after defoliation in order to deliver carbon to growing leaf cells. Morvan et al. (1997) showed that the increased activity of Lp1-FEH following defoliation was strongly inhibited by an inhibitor of GA biosynthesis and that this inhibition was overcome by GA treatment. However, little is known about the mechanism of GA regulation of FEH activity, and further studies are needed to exploit the potential regulatory mechanisms. In many plants, sucrose has been reported to be capable of repressing 1-FEH activity directly (Marx et al. 1997; Prud'homme et al. 1992; Yamamoto

and Mino 1987). In the present study, the GA associated expression of Lp*1-FEH* under high N conditions was most likely the consequence of a response to dramatically decreased sucrose concentrations under elevated GA. This is further supported by the fluctuation of Lp*1-FEH* expression within the first day after GA supply (i.e. 1 to 5 h after GA supply), where sucrose concentrations elevated rapidly with increasing photoperiods.

Notably, we observed an increased expression of Lp*6-SFT* associated with GA supply under low N level at the later regrowth stages (Fig. 1h). This unexpected increase in Lp*6-SFT* gene expression under high GA and low N conditions might be linked to the availability of newly synthesised photosynthates during the recovery stages as fructan biosynthesis is highly influenced by the photosynthesis rate.

Effects on sugars and invertase genes

Invertases play an important role in the cleavage of sucrose into glucose and fructose which are then able to provide an energy source for plant growth and also increase the osmotic pressure of the cell, suggesting a possible function of invertases in cell elongation and plant growth (Sturm 1999). Different forms of invertase, including vacuolar (VacInv), cytoplasmic (CytInv) and apoplastic/ cell wall (CWInv) invertases, have been suggested to be distributed between the cellular compartments accordingly (Cairns and Gallagher 2004; Kingston-Smith et al. 1999) with vacuolar invertase suggested to be important during carbohydrate metabolism because major reserve carbohydrates are present in vacuoles (Wang et al. 2000).

In this study, high N treatments resulted in significantly decreased sucrose concentrations and the increased gene expression of the invertase Lp*VacInv* in high N treatments was coupled with deceased concentrations of sucrose (Fig. 2). This suggests that cleavage of sucrose in the presence of high N may be an important prerequisite for improved regrowth of *L. perenne*, which is consistent with results in other crops (e.g. Morcuende et al. 2004; van den Ende et al. 1999). The elevation of vacuolar invertase activities under high N has been observed in other plants such as barley (Wang et al. 2000; Wang and Tillberg 1996) and chicory (van den Ende et al. 1999). It has been suggested that the vacuolar invertase possesses both invertase and fructan exohydrolase-like activities capable of breaking sucrose and low DP fructans into glucose and fructose (Johnson et al. 2003).

GA supply resulted in a further decrease in sucrose concentration in the High-N and GA supply treatment during later regrowth stages (Fig. 2g), an observation that is consistent with results in other species, for example, *Zantedeschia* (Kozlowska et al. 2007). The effects of GA supply on Lp*VacInv* expression appeared to interact with N levels and tissues at the later regrowth stages (Fig. 1h). However, Lp*VacInv* gene expression appeared to be sensitive and negatively associate with the sucrose concentration immediately after GA application, indicating that the increased invertase activity may be an initial response to GA supply. Increased activities of invertases in response to exogenous GA have been observed in many elongating plant tissues such as tulip (*Tulipa gesneriana*) internodes (Ranwala and Miller 2008), *Avena* internodes (Kaufman et al. 1973), bean (*Phaseolus vulgaris*) internodes (Morris and Arthur 1985), and dwarf pea (*Pisum sativum* L.) shoots (Wu et al. 1993).

Exogenous GA is capable of mediating endogenous GA biosynthesis and transduction

12

404 Gibberellin acts as a key mediator between environmental cues and plant morphology across a variety of
405 developmental processes, including stem and root elongation, seed germination, floral development, and
406 determination of leaf size and shape (Middleton et al. 2012; Yamaguchi et al. 1998). The GA biosynthesis and
407 signal transduction pathways have been characterised in great detail in model plants (Hong et al. 2012; Middleton
408 et al. 2012; Peng et al. 1997) and the key gene families associated with GA signalling shown to be GA_{20}-oxidase
409 (GA20ox), GA_3-oxidase (GA3ox), GA_2-oxidase (GA2ox), DELLA and gibberellin-insensitive dwarf1 (GID1).

410

411 GA homeostasis in plants is maintained by both the feedback and feed forward regulation of GA metabolism
412 (Hedden and Phillips 2000; Olszewski et al. 2002; Xu et al. 1999). Endogenous expression of genes involved in
413 GA synthesis and transduction have been shown to be affected by the supply of bioactive exogenous GA
414 (Middleton et al. 2012). In the present study, we found that Lp*GA3ox* and Lp*GA20ox* were both down-regulated
415 immediately after exogenous GA application (Fig. 3a, b), which is consistent with the observations of Middleton
416 et al. (2012) in *Arabidopsis thaliana* (*A. thaliana*). These results indicate that substrate-induced feedback
417 regulation occurs in *L. perenne* to maintain GA homeostasis. Reduced expression of *GA3ox* and *GA20ox* in
418 response to exogenous GA supply has also been reported previously in other plant species (Ayele et al. 2006;
419 Martin et al. 1996; Matsushita et al. 2007; Xu et al. 1999; Yamaguchi et al. 1998).

420

421 Endogenous levels of bioactive GAs are often positively associated with the expression of GA biosynthetic genes.
422 However, an increase in bioactive GA can also be brought about by a reduction in GA degradation. In the present
423 study the expression of the endogenous GA-degrading gene Lp*DELLA* appeared to be up-regulated by exogenous
424 GA supply, particularly under high N conditions during later stages of regrowth (Fig. 3c, d), an observation that
425 is consistent with previous studies (Ayele et al. 2006; Middleton et al. 2012; Ogawa et al. 2000; Thomas et al.
426 1999; Yamaguchi 2008).

427

428 Interactive effects of N and exogenous GA on carbohydrate metabolism

429

430 Both carbohydrate and nitrogen status of the plant at the time of defoliation is an important determinant of the
431 subsequent dynamics of reserve mobilisation for plant regrowth. Phytohormones (e.g. GA) can participate in the
432 regulation of carbohydrate metabolism (Cai et al. 2016; Morvan et al. 1997) and an improved understanding of
433 the interactive effects between carbohydrate, nitrogen and GA regulation on plant growth needs special attention
434 as regrowth response to these elements may be associated.

435

436 At the molecular level, cross-talk between signalling pathways for N assimilation and GA regulation has been
437 suggested previously (Davis 2000; Hong et al. 2012) and GA biosynthesis has been shown to be up-regulated in
438 response to nitrogen application (Jang et al. 2008). A strong interaction between N supply and endogenous
439 concentration of GA on growth has also been reported in other plants (Ali et al. 1996; Krauss and Marschner
440 1982). However, the response to GAs supply in relation to nitrogen status has never been investigated during
441 regrowth in grass species. In the present study the expression of the GA-synthesising genes, Lp*GA3ox* and
442 particularly Lp*GA20ox*, appeared to increase under high N supply. Results suggest that N levels interact with the

13

bioactive GA activity which plays a key role in the regulation of plant development, and perhaps carbohydrate metabolism as well.

It appears that the expression of the GA-degrading gene Lp*DELLA* was also up-regulated under high N treatments particularly in enclosed elongating tissues, indicating that N counteracts GA transduction, probably through feedback processes. Studies in *A. thaliana* demonstrated that RGA (a DELLA protein important for GA signal transduction) was capable of complementing a yeast mutant altered in nitrogen metabolism (Bouton et al. 2002; Truong et al. 1997), suggesting that GA could be involved in controlling N assimilation in plants. However, we did not observe significant changes in N content in herbage and the expression of genes encoding a nitrate reductase (Lp*Nir*) and a nitrate transporter (Lp*NT*) between GA-treated and GA-free plants, although the expression of Lp*Nir* was significantly elevated under high N supply (data not shown). Our observations are consistent with a study of Bouton et al. (2002) who found that N content and the expression of genes encoding a nitrate reductase (NR), nitrite reductase (Nir), cytosolic and chloroplastic glutamine synthetases did not vary in *A. thaliana* plants with different GA genetic backgrounds and in the short-term GA treatment of wild-type plants. The above results indicated that DELLA proteins and gibberellins might not act as major factors controlling nitrate assimilation in the vegetative stage, although in some studies GAs were shown to restore NR and Nir activities in aerial parts of rice seedlings 24 h after root removal (e.g. Gandi et al. 1974). Further studies on the role of GA in regulating N assimilation are required in the future. In some source-sink systems, sugars and other metabolites have been implicated in the interaction of N assimilation and hormonal signals and cross-talk between signalling pathways for N uptake and carbon metabolism has been reported previously (Davis 2000). However, any potential interaction between carbohydrate metabolism and GA regulation still remains to be speculated.

Conclusion

Our study demonstrates that the promoting effects of both high N and exogenous GA supply on regrowth of perennial ryegrass were through reducing the accumulation of carbohydrate reserves in plant tissues, particularly in high N conditions. However, the molecular mechanisms regulating carbohydrate metabolism vary between exogenous GA and high N effects. Our analyses indicate that exogenous GA promotes carbohydrate metabolism through increasing the hydrolytic activities of fructan exohydrolases and invertases capable of cleaving reserve carbohydrates to release an energy source for plant regrowth, whereas high N effects were mainly seen through declining fructan biosynthesis and improved photosynthate use efficiency to support plant regrowth. The stimulating effects of the exogenous GA on plant regrowth appears to be mediated by N levels and this has important implications with regard to the optimal combination of N fertilisation and exogenous GA application in terms of pastoral productivity.

Acknowledgments

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Figure legends

665

666 **Fig. 1** Fructan concentrations (mg g^{-1} DW) and relative expression of fructosyltransferases (Lp*1-SST* and Lp*6-*
667 *SFT*), and a fructan exohydrolase (Lp*1-FEH*) in *Lolium perenne* after defoliation and GA supply. Line charts at
668 left represent the mean ± SE (n=7) of enclosed elongating tissue (EE). Dashed lines: GA-free treatments; Solid
669 lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts at the
670 right represent the overall means ± SE (n=84) of the major N and GA treatments for all three tissues of EE, sheath
671 (S) and emerged mature blade (EM) during the later regrowth periods of 168-672 h after defoliation. Different
672 letters denote significant differences between treatments. Gene expression levels were normalised to relative
673 expression against a spiked external reference gene, *eGFP*.

674

675 **Fig. 2** Concentrations of sucrose (mg g^{-1} DW) and relative expression of an invertase gene (Lp*VacInv*) in *Lolium*
676 *perenne* after defoliation and GA supply. Line charts represent the mean ± SE (n=7) of enclosed elongating tissue
677 (EE), sheath tissue (S) and emerged mature blade (EM). Dashed lines: GA-free treatments; Solid lines: GA supply
678 treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts at bottom represent the
679 overall means ± SE (n=84) of the major N and GA treatments for all three tissues tested during the later regrowth

periods of 168-672 h after defoliation. Different letters denote significant differences between treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, *eGFP*.

Fig. 3 Relative expression of GA-synthesising genes (Lp*GA3ox* and Lp*GA20ox*) and a GA-regulated gene (Lp*DELLA*) in *Lolium perenne* after defoliation and GA supply. Line charts represent the mean ± SE (n=7) of enclosed elongating tissue (EE). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts represent the overall means ± SE (n=84) of the major N and GA treatments for all three tissues of EE, sheath (S) and emerged mature blade (EM) during the later regrowth periods of 168-672 h after defoliation. Different letters denote significant differences between treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, e*GFP*.

Table line numbers in left margin: 15–65

Table 1 Schedules of time-course for plant harvesting (marked by '√') in GA-free and GA+ treatments. * Plant defoliated

Regrowth following defoliation (h)	0	1	5	24	48	72	96	97	101	120	144	168	336	504	672
For GA free treatment	√*	√	√	√	√	√						√	√	√	√
For GA+ treatment	√*						√	√	√	√	√	√	√	√	√
After GA supply (h)							0	1	5	24	48	72	240	408	576
Referred treatment	Defoliation effects						GA immediate effects						N/GA effects		

Table 2 Genes analysed in this study

Gene name	Putative function	NCBI Genbank accession No.
Lp*1-SST*	sucrose: sucrose 1-fructosyltransferase	AY245431
Lp*6-SFT*	sucrose: fructan 6-fructosyltransferase	AB186920
Lp*1-FEH*	fructan exohydrolase	DQ018712
Lp*VacInv*	sucrose-degrading vacuolar invertase	AY082350
Lp*CWInv*	sucrose-degrading cell wall invertase	DQ073969
Lp*CytInv*	sucrose-degrading cytosolic invertase	AM489692
Lp*GA3ox*	GA synthetic Lp*GA$_3$-oxidase*	KP954695
Lp*GA20ox*	GA synthetic Lp*GA$_{20}$-oxidase*	DQ071620
Lp*GA2ox*	GA inactivating Lp*GA$_2$-oxidase*	EF687858
Lp*DELLA*	GA regulator	KP954694
Lp*GID*	GA receptor	This study
Lp*GAMyb*	GA transcription factor	This study
Lp*NR*	nitrate reductase	This study
Lp*NT*	nitrate-transporter	This study
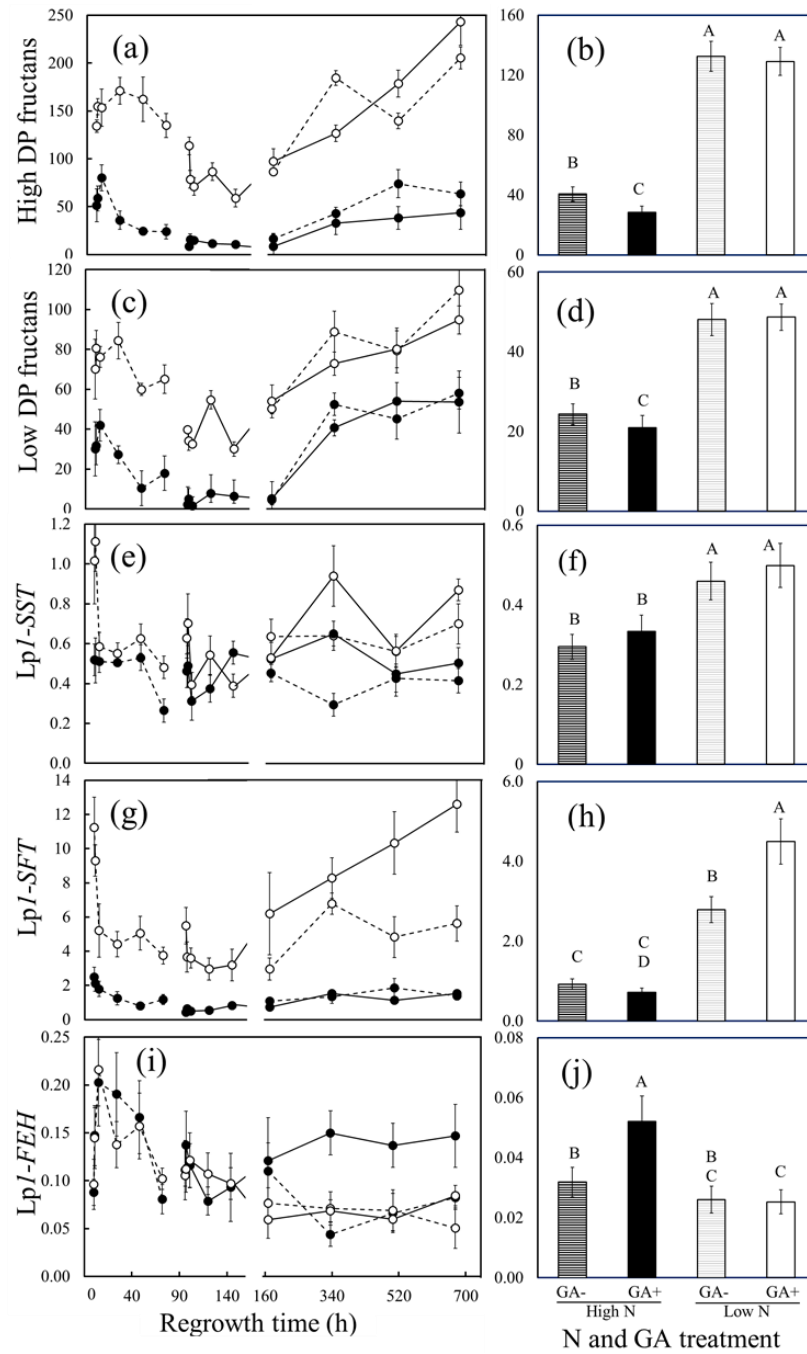
Fig. 1 Fructan concentrations (mg g^{-1} DW) and relative expression of fructosyltransferases (Lp*1-SST* and Lp*6-SFT*), and a fructan exohydrolase (Lp*1-FEH*) in *Lolium perenne* after defoliation and GA supply. Line charts at left represent the mean ± SE (n=7) of enclosed elongating tissue (EE). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts at the right represent the overall means ± SE (n=84) of the major N and GA treatments for all three tissues of EE, sheath (S) and emerged mature blade (EM) during the later regrowth periods of 168-672 h after defoliation. Different letters denote significant differences between treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, *eGFP*.

Fig. 2 Concentrations of sucrose (mg g⁻¹ DW) and relative expression of an invertase gene (Lp*VacInv*) in *Lolium perenne* after defoliation and GA supply. Line charts represent the mean ± SE (n=7) of enclosed elongating tissue (EE), sheath tissue (S) and emerged mature blade (EM). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts at bottom represent the overall means ± SE (n=84) of the major N and GA treatments for all three tissues tested during the later regrowth periods of 168-672 h after defoliation. Different letters denote significant differences between treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, *eGFP*.

Fig. 3 Relative expression of GA-synthesising genes (Lp*GA3ox* and Lp*GA20ox*) and a GA-regulated gene (Lp*DELLA*) in *Lolium perenne* after defoliation and GA supply. Line charts represent the mean ± SE (n=7) of enclosed elongating tissue (EE). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Bar charts represent the overall means ± SE (n=84) of the major N and GA treatments for all three tissues of EE, sheath (S) and emerged mature blade (EM) during the later regrowth periods of 168-672 h after defoliation. Different letters denote significant differences between treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, e*GFP*.
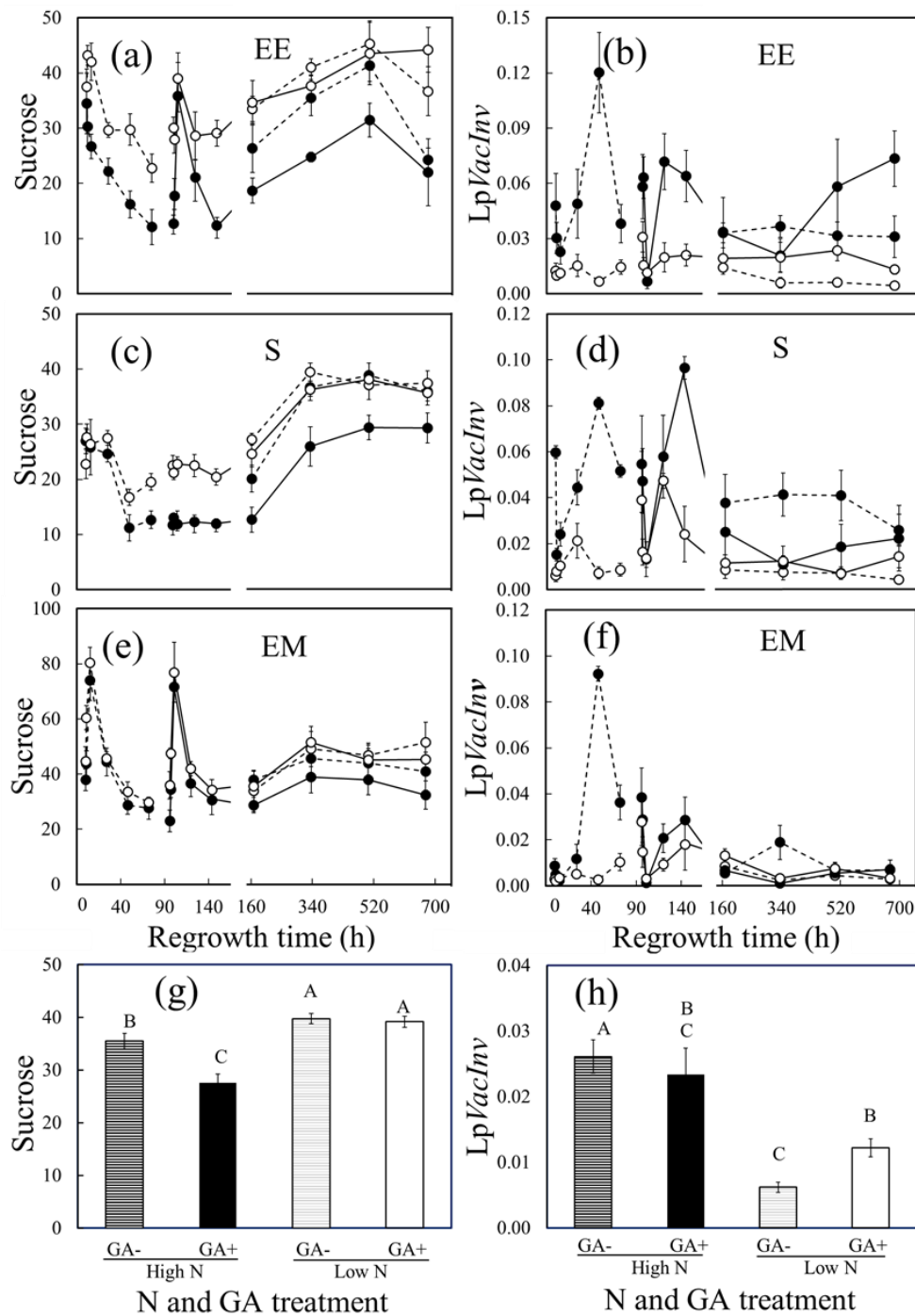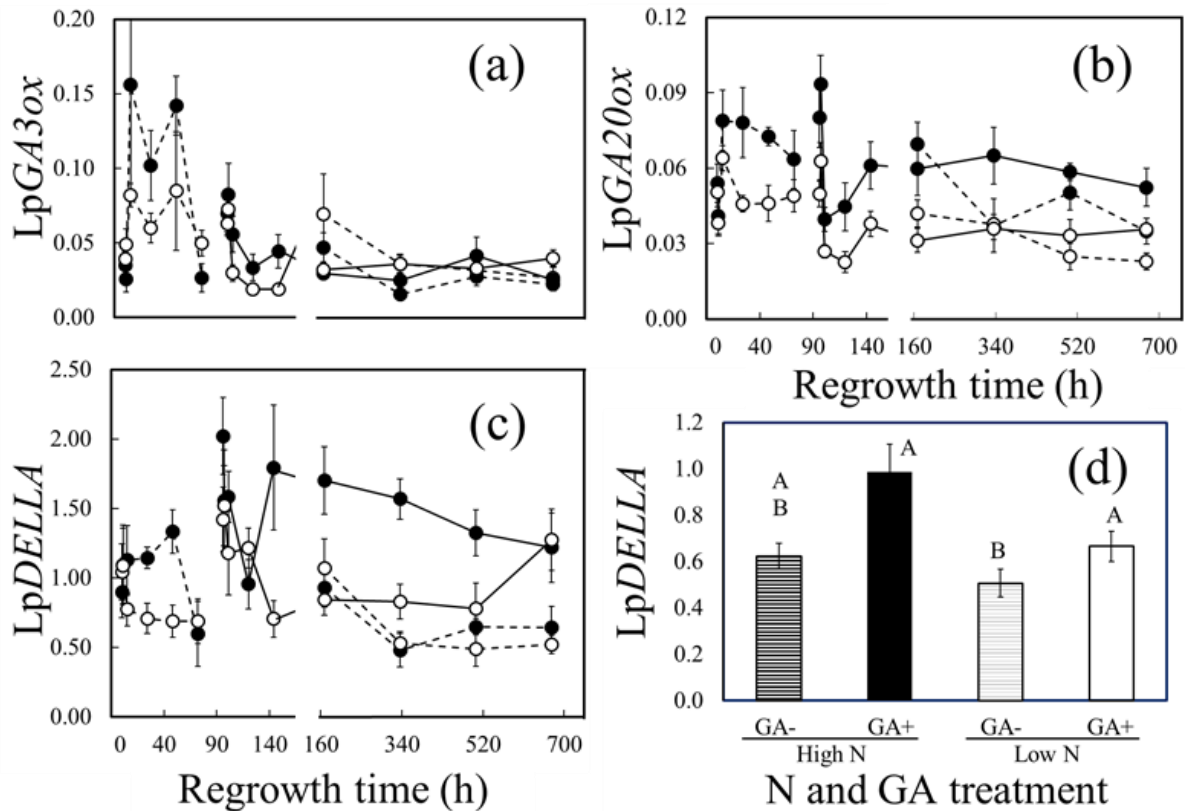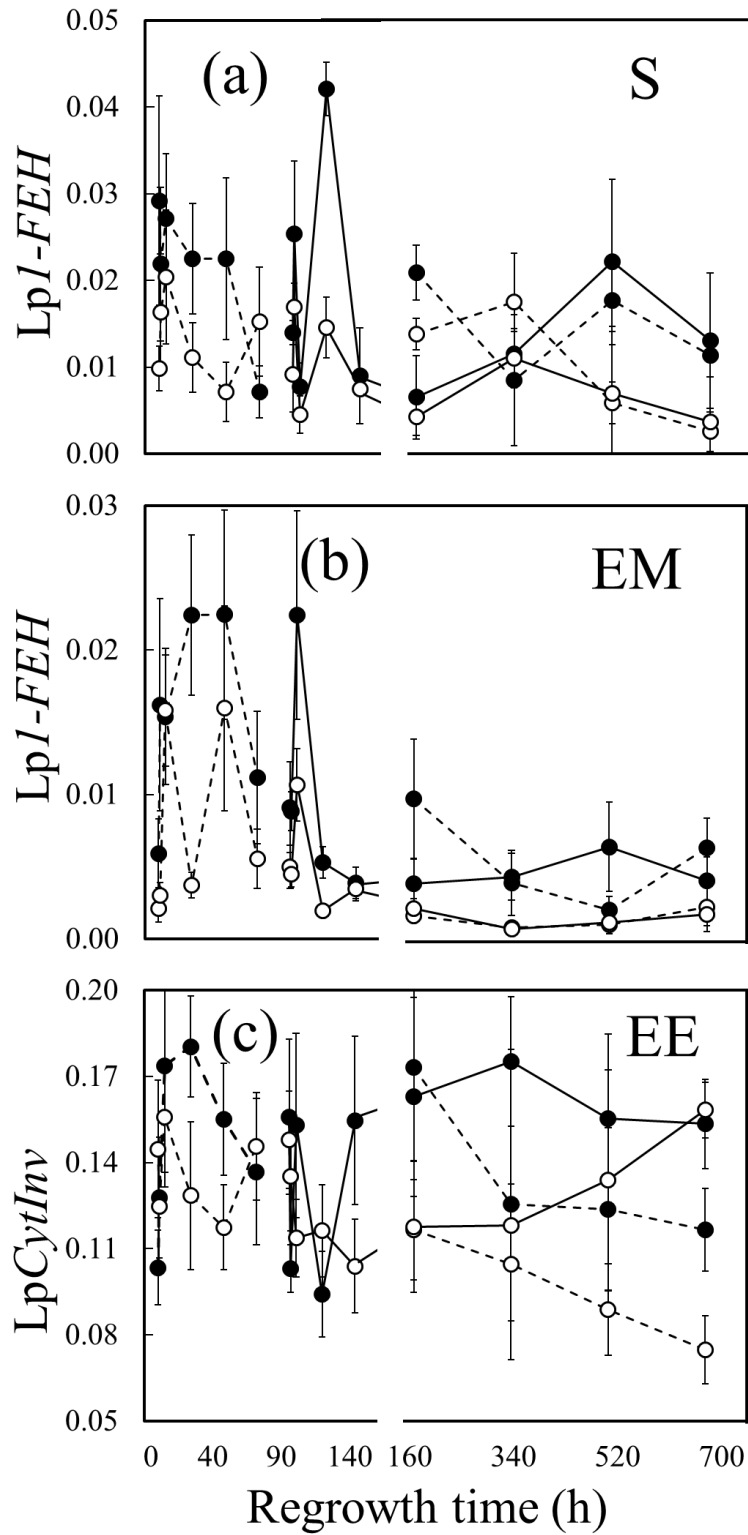
Supplementary materials

Suppl. Table S1 Primer sequences used in this study for RT-qPCR for target genes

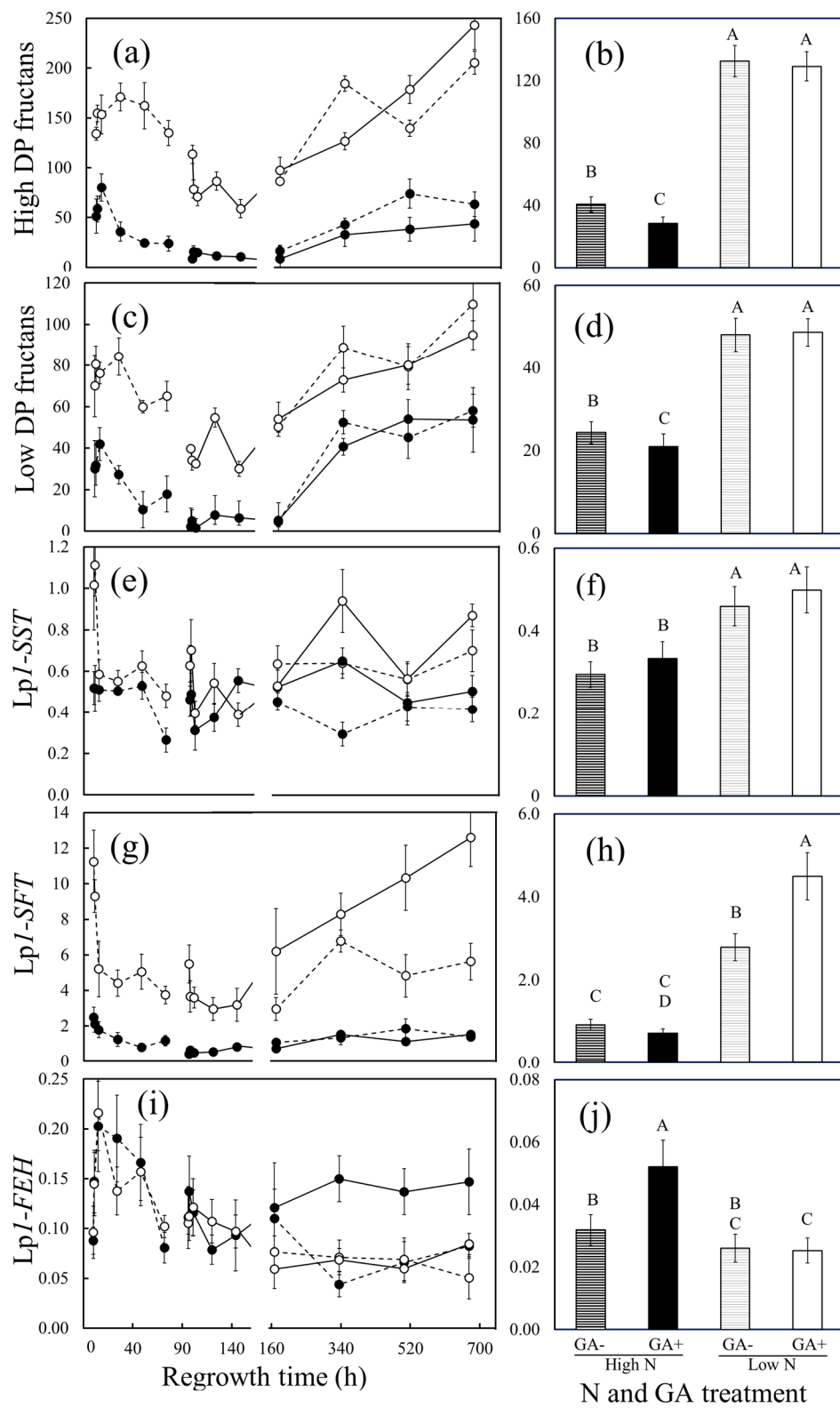| Primer name	Target gene	Sequence (5' to 3'-end)	Size (bp)	Reference
CWInv_for CWInv_rev	qPCR Lp*CWInv*	CTGCCGACCTAGCAGAGAAG GCTGCACATGAGGACGATAG	82	DQ073969
VacInv_for VacInv_rev	qPCR Lp*VacInv*	TCTTCCTAGTTGCTTCCATCG CGCATACGAGTAGGGCATC	92	AY082350
CytInv_for CytInv_rev	qPCR Lp*CytInv*	CTCTGGAACGACGAGGACAC TGCCGAGAACTCACACAATG	102	AM489692
1-SST_for 1-SST_rev	qPCR Lp*1-SST*	AAGTCCTCCGGTGCCTACTC GCGATGTTGCCCCAGCT	196	AY245431
6-SFT_for 6-SFT_rev	qPCR Lp*6-SFT*	GTTCTCGCTCACCCACAG GACACGCTCGAAGCTAAGG	180	AB186920
1-FEH-for 1-FEH-rev	qPCR Lp*1-FEH*	AAGGCGCCAAACATGTCCTC TGCGATGTCATCTGAAGAAC	239	DQ018712
GA3ox_for GA3ox_rev	qPCR Lp*GA3ox*	TGTGAGGTGATGGAGGAGTTC GTACCAGTTGAGGTGCATGG	165	KP954695
GA20ox_for GA20ox_rev	qPCR Lp*GA20ox*	AGGTGTACGCTCGGTACTGC TTGAGCCGCATTATGGATTC	136	DQ071620
GA2ox_for GA2ox_rev	qPCR Lp*GA2ox*	GGTGGATCGAGTACCTCCTG ACGGCAATGTCGAGAACG	81	EF687858
DELLA_for DELLA_rev	qPCR Lp*DELLA*	CATCCTCCTCCTCGTCCTC GAGCGGTATCTGCTTGACG	200	KP954694
Gid_for Gid_rev	qPCR LpGID	AACACCTGGGTGCTCATCTC GGAGTTGTCGATGACGTGGT	168	This study
GAMyb_for GAMyb_rev	qPCR LpGAMyb	GCTCATCATCCAGCTCCAC GCCCTGCTGATCTTCATTTG	187	This study
NT-for NT_rev	qPCR LpNT	GGAGCTGTGTATGGAGTCATC TGCGACGATGTGAAGAAGAG	124	This study
NR_for NR_rev	qPCR LpNR	GACCAACCAAGTACGGCAAG CCAGCAGTTGTTCATCATCC	169	This study
eGFP_for eGFP_rev	qPCR eGFP	CATCGAGCTGAAGGGCATC GACGTTGTGGCTGTTGTAGTTG	84	This study

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Suppl. Table S2 P values of ANOVA for N/GA effects (A), defoliation effects (B) and GA immediate effects (C). Bolded values represent the significant differences
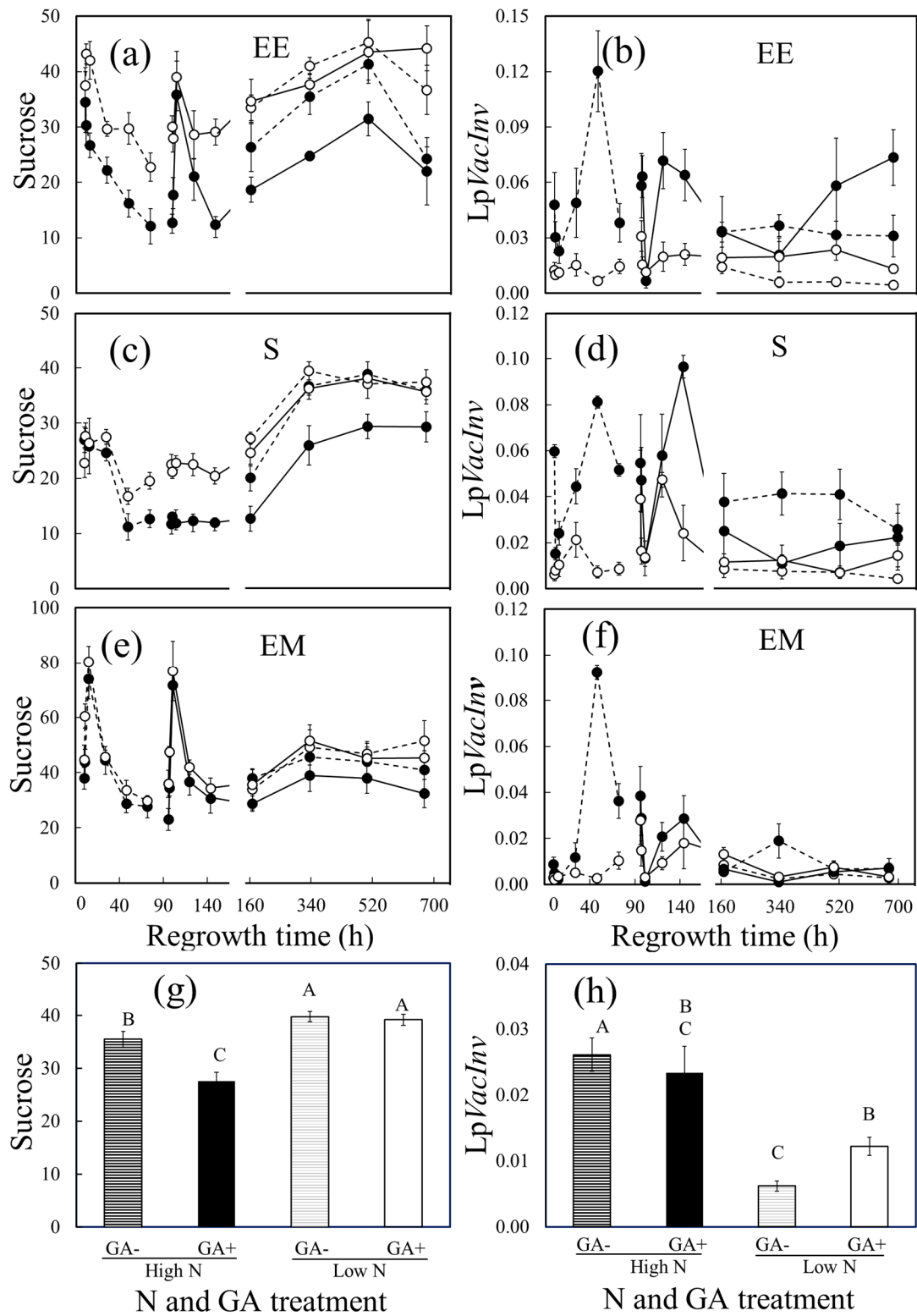
A: P values of N/GA effects. 3-way ANOVA (tissue x N/GA treatment x regrowth time) analysed using data at regrowth periods from 168 to 672h after defoliation

Source	Glu	Fru	Suc	LpCWInv	LpCytInv	LpVacInv	High DP fructans	Low DP fructans	Lp1-SST	Lp6-SFT	Lp1-FEH	LpGA3ox	LpGA20ox	LpGA2ox	LpDELLA	LpGID	LpGAMyb	LpNR	LpNT
Tissues	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
N/GA treatment	**0.000**	**0.000**	**0.000**	0.399	0.231	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.442	**0.000**	0.069	**0.000**	**0.046**	0.897	**0.000**	0.313
Regrowth time	**0.000**	**0.000**	**0.000**	**0.001**	0.126	0.527	**0.000**	**0.000**	**0.005**	**0.000**	0.186	0.209	0.676	0.069	0.469	0.060	0.313	0.298	0.659
Tissues x N/GA treatment	**0.002**	**0.000**	0.386	0.162	**0.029**	**0.016**	**0.008**	0.127	**0.029**	0.220	0.051	**0.014**	**0.001**	0.290	**0.040**	**0.009**	0.062	0.160	0.314
Tissue x Regrowth time	0.485	**0.000**	**0.037**	0.433	0.150	0.793	**0.021**	**0.000**	**0.015**	0.670	0.363	0.882	0.090	0.140	0.240	0.078	**0.000**	0.816	0.372
N/GA treatment x Regrowth time	**0.000**	**0.000**	0.909	0.128	0.338	**0.019**	**0.035**	0.057	**0.034**	0.809	0.002	0.472	**0.007**	0.332	0.114	**0.009**	0.261	0.578	0.648
Tissues x N/GA treatment x Regrowth time	**0.018**	0.051	0.845	0.907	0.217	0.596	0.108	**0.012**	0.519	0.915	0.987	0.863	0.908	0.872	0.971	**0.031**	0.951	0.989	0.934

B: P values of defoliation effects. 3-way ANOVA (tissue x N level x regrowth time) analysed using data at regrowth periods from 0 to 72h after defoliation

Source	Glu	Fru	Suc	LpCWInv	LpCytInv	LpVacInv	High DP fructans	Low DP fructans	Lp1-SST	Lp6-SFT	Lp1-FEH	LpGA3ox	LpGA20ox	LpGA2ox	LpDELLA	LpGID	LpGAMyb	LpNR	LpNT
Tissues	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.127	**0.000**
N level	**0.000**	**0.000**	**0.000**	0.637	0.391	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.015**	**0.000**	**0.050**	0.393	**0.000**	0.548	0.196	**0.000**	0.071
Regrowth time	**0.003**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	**0.008**	**0.004**	**0.000**	**0.007**	**0.000**	0.104	0.052	**0.021**
Tissues x N level	**0.016**	**0.001**	**0.004**	0.828	0.274	0.655	0.411	**0.000**	0.163	**0.045**	0.206	0.081	**0.021**	0.333	0.152	**0.013**	0.531	0.961	0.248
Tissue x Regrowth time	0.357	**0.020**	**0.000**	0.906	0.433	0.286	0.906	0.272	**0.000**	0.077	0.244	**0.046**	0.202	**0.001**	0.699	**0.000**	0.892	0.801	0.160
N level x Regrwoth time	0.341	0.684	0.188	**0.029**	0.071	**0.000**	**0.013**	0.146	0.574	0.580	0.110	**0.013**	0.776	0.713	0.303	0.623	0.219	0.156	0.584
Tissues x N level x Regrowth time	0.640	0.452	0.455	0.854	0.798	0.998	0.293	0.357	0.661	0.870	0.980	0.382	0.854	0.202	0.967	0.876	0.957	0.899	0.979

C: P values of GA-immediate effects. 3-way ANOVA (tissue x N level x regrowth time) analysed using data at regrowth periods from 0 to 48 h after GA supply

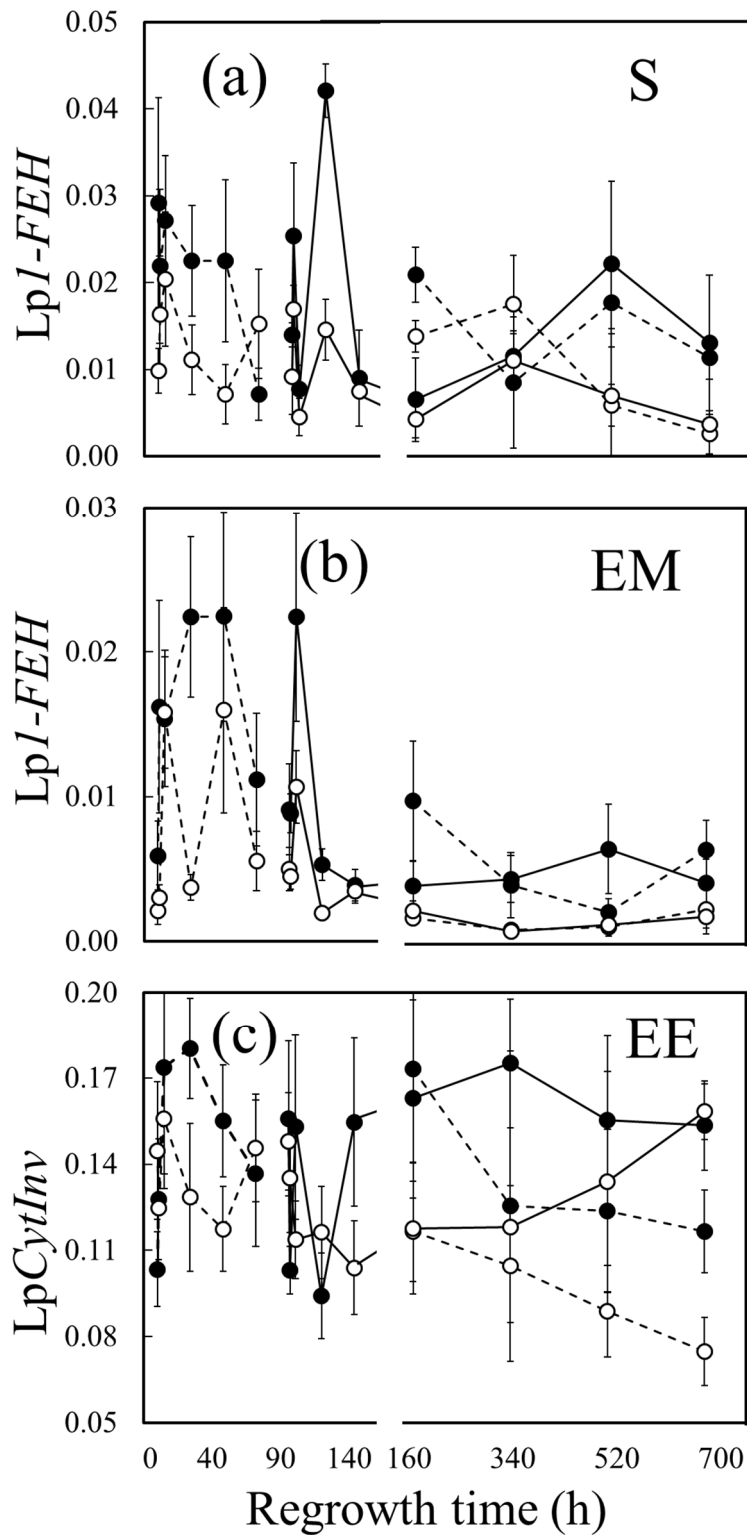| Source	Glu	Fru	Suc	LpCWInv	LpCytInv	LpVacInv	High DP fructans	Low DP fructans	Lp1-SST	Lp6-SFT	Lp1-FEH	LpGA3ox	LpGA20ox	LpGA2ox	LpDELLA	LpGID	LpGAMyb	LpNR	LpNT
Tissues	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.013**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.018**	**0.000**
N level	0.814	**0.000**	**0.000**	0.511	0.225	**0.005**	**0.000**	**0.000**	**0.000**	**0.000**	0.219	**0.000**	**0.001**	**0.000**	0.321	0.261	0.648	**0.000**	**0.018**
Regrowth time	0.074	0.545	**0.000**	0.231	0.111	**0.000**	**0.004**	**0.040**	0.544	0.744	**0.002**	**0.000**	**0.000**	**0.001**	0.719	**0.000**	**0.017**	0.296	0.135
Tissues x N level	0.162	0.432	**0.022**	0.180	0.482	0.574	**0.011**	**0.000**	0.330	0.356	0.125	**0.014**	0.397	**0.023**	0.277	0.266	0.064	0.744	0.113
Tissue x Regrowth time	0.106	0.054	**0.000**	0.881	0.038	0.981	**0.025**	**0.001**	0.334	0.340	**0.003**	0.060	**0.043**	0.246	0.559	**0.000**	0.872	0.965	0.846
N level x Regrwoth time	0.133	0.330	0.143	0.826	0.191	**0.008**	**0.000**	0.064	0.054	0.178	0.149	0.776	0.396	0.362	0.431	0.119	0.489	0.952	0.357
Tissues x N level x Regrowth time	0.394	0.630	0.338	0.501	0.469	0.977	**0.013**	0.105	0.997	0.654	0.983	0.636	0.600	0.566	0.897	0.709	0.670	0.855	1.000

27

Suppl. Fig. 1 Relative expression of a fructan exohydrolase gene (Lp*1-FEH*) in sheath (a) and emerged mature blade (b); and a cytosolic invertase gene (Lp*CytInv*) in enclosed elongating tissue (c) in *Lolium perenne* after defoliation and GA supply (n=7). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, e*GFP*.

28

Figure 1

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Figure 2

Click here to access/download;Figure;Fig-2.pdf ⬇

Figure 2

Figure 3   Click here to access/download;Figure;Fig-3.pdf ±

Suppl. Fig. 1 Relative expression of a fructan exohydrolase gene (Lp*1-FEH*) in sheath (a) and emerged mature blade (b); and a cytosolic invertase gene (Lp*CytInv*) in enclosed elongating tissue (c) in *Lolium perenne* after defoliation and GA supply (n=7). Dashed lines: GA-free treatments; Solid lines: GA supply treatments; Open cycles: low N treatments; Closed cycles: high N treatments. Gene expression levels were normalised to relative expression against a spiked external reference gene, e*GFP*.