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Depletion of carbohydrate reserves limits nitrate uptake during early regrowth in *Lolium perenne* L.

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Abstract

The mechanisms linking C/N balance to N uptake and assimilation are central to plant responses to changing soil nutrient levels. Defoliation and subsequent regrowth of grasses both impact C partitioning, thereby creating a significant point of interaction with soil N availability. Using defoliation as an experimental treatment, we investigated the dynamic relationships between plant carbohydrate status and NO₃⁻-responsive uptake systems, transporter gene expression, and nitrate assimilation in *Lolium perenne* L. High- and low-affinity NO₃⁻ uptake was reduced in an N-dependent manner in response to a rapid and large shift in carbohydrate remobilization triggered by defoliation. This reduction in NO₃⁻ uptake was rescued by an exogenous glucose supplement, confirming the carbohydrate dependence of NO₃⁻ uptake. The regulation of NO₃⁻ uptake in response to the perturbation of the plant C/N ratio was associated with changes in expression of putative high- and low-affinity NO₃⁻ transporters. Furthermore, NO₃⁻ assimilation appears to be regulated by the C–N status of the plant, implying a mechanism that signals the availability of C metabolites for NO₃⁻ uptake and assimilation at the whole-plant level. We also show that cytokinins may be involved in the regulation of N acquisition and assimilation in response to the changing plant C/N ratio.

Key words: Carbohydrate, carbon, cytokinin, fructan, *Lolium perenne*, nitrate transporter (NRT), nitrate uptake, nitrogen, nitrogen use efficiency (NUE), perennial ryegrass.

Introduction

Grasses are well adapted to tolerate and recover from the severe and frequent defoliation associated with grazing (Lestienne *et al.*, 2006). The most obvious developmental aspect of this is the physical protection of the meristem at the crown. Less overt, but no less critical, are the metabolic aspects of this tolerance. For instance, leaf regrowth and the re-establishment of photosynthesis in the leaf prior to its transition from sink to source is dependent upon the remobilization of stored nutrient resources, particularly carbon (C) and nitrogen (N) (Ourry *et al.*, 1989; Lestienne *et al.*, 2006).
In the period immediately following defoliation, photosynthetic capacity is temporarily compromised. This interrupts C assimilation and the capacity to assimilate newly acquired inorganic N into biomass, which is a process requiring reducing equivalents, ATP, and C skeletons generated from respiration of sucrose derived from stored C (Dawar et al., 2010; Nunes-Nesi et al., 2010). Indeed, it has been established that root growth, inorganic N uptake, respiration, and nitrate assimilation decline rapidly after defoliation (Boucaud and Bigot, 1989; Ourry et al., 1989; Richards, 1993; Louahlia et al., 2008).

Some evidence exists to suggest that C and N metabolism is co-ordinated via a sensing of plant C/N balance, and regulated by the expression of genes involved in photosynthesis, metabolic pathways, protein degradation, and N assimilation (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Palenchar et al., 2004; Gutiérrez et al., 2007). Microarray data from Arabidopsis transiently exposed to a matrix of C and N treatments has indicated that a large proportion of genes respond to a C/N interaction, suggesting C and/or N, or a metabolic product of C and N assimilation (e.g. an amino acid), might act as a signal in the regulation of gene expression (Gutiérrez et al., 2007). Despite several studies proposing possible mechanisms underlying C and N signalling in Arabidopsis, a definitive mechanistic understanding remains elusive. The dramatic shift in C/N metabolic partitioning resulting from defoliation and regrowth in forage grasses, such as Lolium perenne, has the potential to reveal insights into the integration of C/N assimilation in plants, something not possible to observe in dicotyledonous species.

Fructans, polymers of fructose that are based on sucrose, are the major storage carbohydrate in temperate forage grasses, such as L. perenne (Turner et al., 2006). We have previously confirmed that the fructan pool in L. perenne is degraded when NO$_3^-$ is supplied to N-deficient plants (Roche et al., 2016), consistent with earlier work by Morvan-Bertrand et al. (1999) and Louahlia et al. (2008), and suggested that both root and shoot fructan pools support nitrate uptake and assimilation (Roche et al., 2016). In response to defoliation, early shoot regrowth is also sustained by remobilization of the C stored as fructans in elongating leaf bases and mature leaf sheathes (Morvan-Bertrand et al., 1999, 2001). Logically, the importance of root-derived C in supporting regrowth must also be considered in terms of the greater root biomass fraction that exists following defoliation. Thus, C and N remobilization under the new metabolic condition induced by defoliation must necessarily be co-ordinated at a whole-plant level.

The cytokinins, in addition to multiple roles in plant growth and development, are well known to play a role in regulating NO$_3^-$ uptake systems in response to fluctuations in N availability (Krouk et al., 2011). The isoprenoid cytokinins are biosynthesized via adenosine phosphate-isopentenyltransferase (IPT). The first formed cytokinins are the nucleotides. The riboside forms are regarded as the main translocated forms, and the free bases the biologically active forms detected by receptors (Sakakibara, 2006; Lomin et al., 2015). The signalling pathway in Arabidopsis involves a multi-step signalling network linking between the receptors and the response regulators (RRs) (Hwang et al., 2012). The cis-forms of the cytokinins derive from the turnover of specific tRNA moities. Cytokinin homeostasis is determined by the rate of cytokinin degradation which is catalysed by cytokinin oxidase/dehydrogenase (CKX) and by conjugation. For a general overview of the cytokinins, see Jameson (2017).

It has been shown that trans-zeatin (iZ)-type cytokinins (which carry a hydroxyl on the isoprenoid side chain) are the major forms in xylem sap, while isopentenyl adenine (iP-type) and cis-zeatin (cZ)-type cytokinins are the predominant forms in phloem sap (Takei et al., 2004; Hirose et al., 2008). Matsumoto-kitano et al. (2008) showed that iP-type cytokinins translocated from the shoot to the root in Arabidopsis were functional. It has been proposed that cytokinins could be involved in local and long-distance signalling to co-ordinate responses at the whole-plant level, and to communicate N status between multiple organs (Takei et al., 2001; Sakakibara et al., 2006; Matsumoto-Kitano et al., 2008; Kudo et al., 2010; Shtratnikova et al., 2015). A split-root experiment in Arabidopsis has demonstrated that NITRATE TRANSPORTER NRT2.1 and NAR2.1 regulation, driven by shoot–root systemic N signalling, is mediated by cytokinin biosynthesis (Ruffel et al., 2011). Further, genome-wide analyses for cytokinin-responsive genes in Arabidopsis have indicated that exogenous cytokinin can down-regulate root-localized transporters including AtNRT1.1, AtNRT2.1, AtNRT2.2, and AtNRT1.5 (Brenner et al., 2005; Li et al., 2007). These gene families have been functionally identified as major components of the NO$_3^-$ uptake and xylem loading system (Liu et al., 1999; Lin et al., 2008). In contrast, cytokinin also up-regulates shoot-localized AtNRT genes under both high and low N conditions (Kiba et al., 2011).

Additionally, global gene profiling coupled with physiological analysis in Arabidopsis has indicated that 74% of cytokinin-regulated genes may be significantly affected by glucose, either agonistically or antagonistically (Kushwah and Laxmi, 2014). Additionally, up-regulation of AtIPT3, and some Type A ARABIDOPSIS RESPONSE REGULATOR (ARR) genes and CYTOKININ RESPONSE FACTOR (CRF) genes by glucose suggests a potential role for glucose in modulating cytokinin signalling (Kushwah and Laxmi, 2014). It is thus plausible that cytokinins may play an active role in balancing the C–N status of the plant (Wang and Ruan, 2016).

Cytokinin deficiency, resulting from overexpression of CKX, in Arabidopsis causes an increase in the root/shoot ratio, with inhibited shoot growth and a larger root system (Werner et al., 2003, 2008). This observation indicates that cytokinin deficiency may trigger a shift in C allocation from shoot to root, enhancing soil ‘foraging’ capacity for nitrate under limited N availability (Ruffel et al., 2011). Once taken up, the increased NO$_3^-$ induces cytokinin biosynthesis in a nitrate-dependent manner (Krouk et al., 2011). Subsequently, the cytokinins translocated to the shoot could regulate C allocation to keep shoot and root development in balance (Wang and Ruan, 2016).

With an ultimate goal of finding ways to better manage N input in grassland-based agricultural systems such as dairy...
farming, we investigated the nitrate uptake response to two independent but coinciding metabolic demands on fructan: defoliation and nitrate addition. Of particular interest was the degree of metabolic constraint, if any, that the defoliation–regrowth cycle and associated fructan depletion had on inorganic N uptake efficiency. Given the C cost associated with inorganic N uptake and assimilation (Roche et al., 2016), we hypothesized that this metabolic demand is not adequately met in the days immediately following defoliation, due to regrowth competing with N uptake and assimilation for the stored C. To test this hypothesis, we carried out a stable isotope N uptake experiment, measured fructan content, and profiled cytokinin metabolites and relevant genes in *L. perenne* subjected to defoliation and glucose supplementation in a hydroponic system.

### Materials and methods

#### Plant growth

Seeds of *L. perenne* L. cv. Grasslands Nui were germinated in Eppendorf tubes filled with perlite (tube tips removed) and the tubes placed in unfertilized soil for 11 weeks at 22°C. The basic N-free Hoagland medium (pH 6.0; Bioworld, USA) was supplemented with 0.05 mM or 5 mM KNO₃ as a sole N source every week. Eight-week-old plants were defoliated at 4 cm above ground level. After 3 weeks regrowth, the plants were transferred to a hydroponic system which contained basic N-free Hoagland medium supplemented with either 0.05 mM or 5 mM KNO₃, consistent with the previous growth conditions. The pH of treatment solutions was maintained at 6.0. Based on preliminary experiments, a 1 week adaptation phase in liquid culture medium was required for plants to retain pre-transfer competence (as assessed by leaf gas exchange and stomatal conductance measurements). After the 1 week adaptation, half the plants were again defoliated. The time zero of the experiments is defined by the second defoliation of plants.

#### K15NO3 uptake measurement and isotope analysis

Root uptake of NO3 was determined by 15N labelling. At 0 h and 48 h after defoliation, plants were gently blotted on tissue paper and then immediately rinsed with 0.1 mM CaSO4 for 1 min to remove any adsorbed compounds on the root surface, followed by 1 h exposure to basic N-free Hoagland medium supplemented with either 0.05 mM or 5 mM 15N-labelled KNO₃ (atom % 15N: 10%). During the uptake experiments, the incubation solutions were aerated by an aquarium pump. At the end of the incubation period, roots were immediately rinsed with 0.1 mM CaSO4 for 1 min. Leaf sheaths and roots were separated, frozen in liquid nitrogen immediately, and stored at −80°C. The incubation timing was based on that used by Brackin et al. (2015). For this study, five plants were pooled as one biological replicate, and each treatment had five independent biological replicates. A 1 g aliquot of fresh samples was ground to a fine powder and freeze-dried for 3 d. Total N and 15N in the samples were determined with an isotope ratio mass spectrometer (Waikato University, New Zealand). A flow diagram of the experimental processes is shown in Supplementary Fig. S1 at JXB online.

#### Glucose treatment

After 42 h of regrowth as described above, 100 plants grown in solutions with 5 mM KNO₃ were then supplemented with either 0.1% or 1% (w/v) glucose (25 plants each) or with 0.1% or 1% (w/v) mannitol (25 plants each) as an osmotic control. After 6 h, K15NO3 uptake measurements were carried out by exposing roots to 5 mM 15N-labelled KNO₃ (atom % 15N: 10%) solutions as described above. A flow diagram of the experimental processes is shown in Supplementary Fig. S2.

#### Target gene sequence determination

Sequences of candidate target gene family members in perennial ryegrass were determined through BLAST searching the NCBI database and an RNA-Seq transcriptome database using refseqBLAST 2.0 software. An Illumina HiSeq2000 genome analyser (Macrogen Ltd, Seoul, Korea) and a pool of combined RNA samples extracted from multiple developmental stages of leaves, flower spikes, and seeds of perennial ryegrass cv. Nui were used to generate the transcriptome database containing 169 862 assembled sequence contigs of 595 bp in average length. All available orthologue sequences of the target gene families in the GenBank database for perennial ryegrass and closely related species were used as local BLAST search query sequences. The putative sequences of interest were verified via BLAST searching the GenBank database and via multiple sequence alignment with representative orthologue sequences in closely related species.

Neighbor–Joining (NJ) phylogenetic trees of the newly identified sequences of *LpNRT1*, *LpNRT2*, *LpNR*, *LpNiR*, *LpCKX*, *LpRR*, and *LpFEH*, and their orthologues were created using Clustal X software with 1000 bootstrap replicates. The phylogenetic trees were visualized with TreeView software (Supplementary Fig. S3). Each tree was rooted with an outgroup orthologue sequence from an unrelated species. The *LpRR* tree was shown in our previous study (Roche et al., 2016). The GenBank accession numbers for the nucleotide sequences determined in this work are listed in Supplementary Table S1.

#### RNA isolation and quantitative RT-PCR

Total RNA was extracted from up to 100 mg of frozen samples using an RNase Plant Mini Kit (Qiagen, Germany), following the manufacturer’s protocols. Three independent tissue samples (each comprising tissues from five plants) for each treatment were used as biological replicates. Up to 1 μg of extracted RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit according to the manufacturer’s instructions (Qiagen).

The relative expression levels were determined using reverse transcription quantitative PCR (RT-qPCR). Specific PCR primers were designed for each family member of the target genes, using Primer Premier 6.2.0. Primer sequences for reference genes and target genes can be found in Supplementary Table S1. A volume of 1 μl was used for all qPCRs containing 1 μl of 10-fold diluted cDNA, the relevant primers, and home-made SYBR Green master mix (Song et al., 2012). PCR products were Sanger-sequenced to confirm homology to genes which were already identified in NCBI gene databases. For RT-qPCR, three technical replicates for each of three biological replicates were carried out for each sample set. The relative expression (fold change) of each target gene was corrected using the geometric means of the two reference genes, *LpELONGATION FACTOR (eEF-1α)* and *LpGAPDH*, and calculated using the 2^ΔΔCT method as described in previous studies (Schmittgen and Livak, 2008; Song et al., 2015).

#### Fructan analysis

Perennial ryegrass samples were harvested and immediately flash-frozen in liquid nitrogen. Ground and freeze-dried plant materials (25 mg) were weighed and placed into 2 ml Eppendorf tubes for fructan extraction. After addition of 750 μl of boiling milliQ water, samples were vortexed for 5 s, placed on a heating block at 90°C for 15 min, and then cooled down to room temperature and centrifuged at 20 000 g for 10 min. The supernatants were transferred to filter tubes, centrifuged at 20 000 g for 2 min, and transferred to LC vials. Extracts were analysed using an Agilent 1290 Infinity LC System (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent
Endogenous cytokinin quantification

Extraction and qualification of cytokinins from ~3 mg DW samples were performed as described previously (Dobrev and Kammek, 2002; Antoniadi et al., 2015) using the LC-MS/MS system consisting of an ACQUITY UPLC® System (Waters) and a Xevo® TQ-S (Waters) triple quadrupole mass spectrometer (Svačinová et al., 2012).

Results

Regrowth 48 h after defoliation

N content was measured in roots and leaf sheaths of 12-week-old L. perenne plants 8 d after they were transferred from soil to a hydroponic solution and then defoliated to 40 mm above the crown. For the purpose of this study, the leaf sheath is defined as tissue from crown to 40 mm above, including elongating leaf bases and mature leaf sheaths, and this same part was harvested for the analysis in the intact plants. As expected, leaf sheath and root N content, regrowth rate, and biomass were significantly greater in plants grown under high NO$_3^-$ (HN, 5 mM) conditions than in plants grown under low NO$_3^-$ (LN, 0.05 mM) (Fig. 1).

Water-soluble carbohydrates and fructan exohydrolase gene transcript levels

Water-soluble carbohydrates (WSCs) were profiled in order to assess the dynamics of C metabolism during the first 48 h of regrowth. WSCs with a degree of polymerization (DP) of 3–8 are referred to here as low molecular weight (LMW) WSCs, and those with DP9 to DP20 are referred to as high molecular weight (HMW) WSCs. With the exception of disaccharides, which showed similar relative abundance in LN and HN plants (Supplementary Fig. S4), LMW and HMW WSCs were found in greater abundance in LN roots and leaf sheaths compared with HN plants (Fig. 2). The detailed changes in the abundance of oligosaccharides with increasing DP are shown in Supplementary Fig. S4. During the first hour after defoliation, the remobilization of fructans occurred only in roots of HN plants, while no differences were observed in either roots or leaf sheaths of LN plants relative to intact plants (Fig. 2A, B; Supplementary Fig. S4). In comparison with the intact plants, the total concentration of LMW and HMW WSCs was significantly reduced 48 h after defoliation in both roots and leaf sheaths of HN plants, whereas in LN plants depletion of the fructan pool was only observed in roots, implying that the nature of defoliation-induced remobilization of the fructan pool after 48 h was tissue specific (Fig. 2C, D). In roots, the extent of exhaustion of the fructan pool of HN plants was more severe compared with that in LN plants (Fig. 2B, D; Supplementary Fig. S4).

Lp1-FEH and Lp6-FEH have been functionally characterized as fructan exohydrolases in L. perenne (Lothier et al., 2007, 2014). Within 48 h of defoliation, Lp6-FEH expression was induced in leaf sheath and root of HN plants, and also in LN roots (Supplementary Fig. S5). Similarly, Lp1-FEH transcription in response to defoliation increased significantly in LN roots and leaf sheaths after 48 h, where the fructan content was highest (Fig. 2D; Supplementary Fig. S5).

Nitrate uptake rate and N allocation

To better understand changes in NO$_3^-$ uptake in response to defoliation, plants were exposed to 0.05 mM and 5 mM NO$_3^-$ to stimulate either the nitrate high-affinity transport system
Carbohydrate storage remobilization impacts NO$_3^-$ uptake

(HATS) or the low-affinity transport system (LATS), respectively. The concentrations chosen were well below or above the point at which HATS or LATS would be saturated (Siddiqi et al., 1990; Garnett et al., 2013). During the first 1 h after defoliation, we observed no difference in NO$_3^-$ uptake rate via HATS or LATS in LN plants compared with intact plants, whereas NO$_3^-$ uptake via both HATS and LATS in defoliated HN plants was significantly greater relative to intact plants (Fig. 3A). In intact plants, HATS uptake in LN plants was greater than that in HN plants, while a much greater LATS uptake was induced in HN plants relative to LN plants (Fig. 3A). At 48 h after defoliation, the uptake rate of both HATS and LATS in LN plants dropped by 93% and 79% relative to intact plants, respectively (Fig. 3B). The HN plants, which had a relatively severe depletion of the fructan pool, showed markedly greater reduction in both HATS and LATS NO$_3^-$ uptake than that of LN plants 48 h after defoliation, implying a dependence on C for the uptake of nitrate.

To investigate the impact of defoliation on N distribution, $^{15}$N allocation to leaf sheaths following the incubations in $^{15}$N-labelled NO$_3^-$ was measured at 1 h and 48 h after defoliation. Relative allocation was determined as the proportion of labelled $^{15}$N in leaf sheaths to that in the total plant (roots and leaf sheaths). During the first hour after defoliation, relatively less $^{15}$N was allocated to leaf sheaths in defoliated LN and HN plants than in the controls (Supplementary Fig. S8). In contrast, despite the decline in NO$_3^-$ uptake rate 48 h after defoliation (Fig. 3B), the $^{15}$N allocation to leaf sheaths was relatively greater in both HN and LN defoliated plants than in intact plants (Supplementary Fig. S8).

NRT, NR, and NiR transcript levels

In our hydroponic system, we expected that HATS and LATS transcripts would be relatively abundant in LN and HN plants, respectively. Indeed, as expected, $NRT2$ and $NAR$ transcript levels in LN roots were generally greater than those in HN roots (Fig. 4A–E). This was particularly evident for the putative HAT candidate genes $LpNRT2.1a$ and $LpNRT2.1b$, where transcript expression in roots of LN plants was 20- to 50-fold greater relative to HN plants (Fig. 4A, B). No significant difference in transcript abundance of $LpNRT2.1a$ and $LpNRT2.1b$ was observed between defoliated and intact LN plants during the first hour or 48 h after defoliation. Although transcript levels were relatively low in HN roots, a reduction in $LpNRT2.1b$ transcript abundance was apparent.
48 h after defoliation (Fig. 4B). Notably, within 1 h of defoliation, LpNRT2.1b expression was slightly but not significantly induced in HN roots (P=0.07; Fig. 4B). However, there was no significant difference in LpNRT2.1a transcription in HN roots following 48 h of defoliation (Fig. 4A). Despite their relatively lower presence, LpNRT2.5 and LpNRT2.7 showed greater expression in LN roots than in HN roots, suggesting that these two putative transporters may play a role in the HATS (Fig. 4C, D). Similarly to LpNRT2.1a and LpNRT2.1b, LpNAR displayed greater expression in LN roots relative to HN roots, confirming an important role for LpNAR in the function of NRT2.1 in NO3 uptake (Fig. 4E).

Although the transcript levels of LpNRT1.1 were relatively low, the expression pattern contrasts with those of the two putative HATS genes (LpNRT2.1a and LpNRT2.1b), with LpNRT1.1 being generally higher in HN conditions than in LN conditions, suggesting that the putative NRT1.1 may play an important role in the LATS (Fig. 4F). However, in LN roots, the transcript level of LpNRT2.1 was reduced 48 h after defoliation, showing a similar pattern to that of LpNRT2.1b (Fig. 4F). Unlike LpNRT1.1, other LpNRT1 genes, LpNRT1.2, 1.3, 1.4, and 1.5, showed extremely low, but constitutive expression patterns regardless of treatment, indicating that these putative NRT1 genes might be less sensitive to changing N conditions (Fig. 4G–J).

In order to determine whether N assimilation is tightly coordinated, we assessed the influence of the C:N balance on the expression of nitrate reductase (NR) and nitrite reductase (NiR) genes. Similar to nitrate transporter gene expression, transcript levels of genes involved in nitrate assimilation (LpNR1, LpNRb, and LpNiR) were more abundant in plants grown under LN conditions relative to those under HN conditions (Fig. 5). With exposure to HN conditions, expression of LpNR1 and LpNRb in sheaths was significantly induced 48 h after defoliation, whereas LpNR expression in roots showed no significant difference 48 h after defoliation (Fig. 5A–D). Notably, in HN roots, expression of LpNR1 and LpNRb was induced during the first hour after defoliation (Fig. 5B, D), which is consistent with the stimulation of NO3 uptake in HN plants following 1 h defoliation (Fig. 3A). The transcripts of LpNiR showed a similar pattern to that of LpNR1 and LpNRb. In LN conditions, expression of LpNR1, LpNRb, and LpNiR was induced in roots 48 h after defoliation, whereas there was no change in leaf sheaths (Fig. 5B, D, F).

**Glucose supplement**

To distinguish further the relationship between carbon change dynamics following defoliation and N uptake responses, a subset of the HN plants were subjected to exogenous glucose treatment 42 h after defoliation. After 6 h of treatment with glucose, fructan abundance in roots increased compared with the mannitol control (Fig. 6B), whereas there was no significant difference in fructan abundance in the leaf sheaths (Fig. 6A). The detailed changes in the abundance of oligosaccharides with increasing DP are shown in Supplementary Fig. S6. Glucose supplementation led to repression of Lp1-FEH and Lp6-FEH expression in the roots but not in the leaf sheaths (Fig. 6D; Supplementary Fig. S7).

After 6 h of glucose treatment, a subset of HN defoliated plants was moved to 5 mM 15N-isotopically labelled NO3 for NO3 uptake measurement. NO3 uptake increased significantly in the plants exposed to 1% glucose compared with those in the mannitol treatment (Fig. 6C). The 1% glucose supplement induced a significant decline in the 15N allocation to leaf sheaths (Supplementary Fig. S8).

Expression of both LpNRT2.1b and LpNRT1.1 was significantly enhanced by exposure to glucose (Fig. 6E, F). There was no significant change in the transcript abundance of LpNRT2.1a following glucose treatment (Supplementary Fig. S9).

**Changes in endogenous cytokinin content following defoliation**

To better understand the role of cytokinin in communicating the root–shoot C:N balance at the whole-plant level, cytokinins were quantified in L. perenne plants. Overall, the total cytokinin complement was greater in the leaf sheaths and roots of HN plants compared with LN plants, irrespective of defoliation. Under HN, both tZ and tZ riboside (tZR) had decreased in the leaf sheaths 48 h after defoliation, whereas both iP and iP riboside (iPR) decreased in the roots 48 h after defoliation, relative to intact controls (Fig. 7). In the LN plants, with the exception of an accumulation of iPR in leaf sheaths 48 h after defoliation, there was little change in other cytokinins (Supplementary Fig. S10). In addition, iP and iPR...
in leaf sheaths was greater in HN defoliated plants following the 6 h 1% glucose treatment relative to HN defoliated plants without glucose treatment. Although there was no significant difference in the iPR content between the glucose and mannitol treatments, the iPR content was greater in HN roots with 1% glucose treatment relative to HN defoliated roots without glucose or mannitol addition (Fig. 7). Substantial amounts of cZ-type cytokinins were detected in HN plants. Among them, cZ accumulated in leaf sheaths in the 48 h following defoliation of HN plants, compared with the leaf sheaths of intact plants (Fig. 7). Further, cZ content decreased in leaf sheaths and roots of plants treated with glucose relative to HN defoliated plants without glucose or mannitol treatment. However, this decrease was also observed in the 1% mannitol treatment (Fig. 7).

Expression of cytokinin oxidase/dehydrogenase and cytokinin response regulator gene family members.

In roots, significant LpCKX4 induction was observed in LN and HN plants 48 h after defoliation (Fig. 8A), and in HN leaf sheaths 48 h after defoliation, relative to intact plants (Fig. 8A). No significant difference was observed during the first hour after defoliation, when compared with intact plants (Supplementary Fig. S11).
In comparison with intact plants, increases in expression of the four Type-B RR genes (LpRR2, LpRR10, LpRR12a, and LpRR12b) and two Type-A RR genes (LpRR3 and LpRR6) occurred in leaf sheaths of HN plants 48 h after defoliation, whereas no significant differences were displayed in leaf sheaths of LN plants (Fig. 8C–H). In roots, transcript levels of LpRR genes were greater in both LN and HN conditions 48 h after defoliation (Fig. 8C–H). Expression of LpCKX4, LpCKX6, and LpRR genes was strongly reduced in roots following the 1% glucose treatment of HN defoliated plants (Fig. 9A–F, H). Under 0.1% glucose treatment, there was no significant difference in expression of these gene families (Supplementary Fig. S12).

**Discussion**

An understanding of the mechanisms linking C/N balance to N uptake and assimilation is vital for optimizing plant growth in pasture-based production systems where defoliation by grazing is followed by N fertilizer addition. As shown previously, the fructan carbohydrate pool in elongating leaf bases and sheaths may be severely depleted in order to sustain regrowth following the removal of photosynthetically active tissues in grasses such as L. perenne (Morvan-Bertrand et al., 1999, 2001). Here, by experimentally defoliating plants to interfere with the C/N balance and by mitigating this with exogenous glucose supplementation, we have uncovered the dynamic relationships between the carbohydrate status of the plant, the nitrate-responsive uptake system, transporter gene expression, and assimilation. We confirm and extend previous studies (Louahlia et al., 1999) by showing that NO₃⁻ HATS and LATS uptake changes in response to the rapid and large shift in C status induced by defoliation. We also explored the involvement of cytokinins in the regulation of the C/N balance in response to defoliation. The results are discussed in relation to the internal C/N balance under defoliation.

**Nitrate uptake and assimilation rely on labile carbohydrate availability**

Consistent with previous studies (Louahlia et al., 2008; Roche et al., 2016), long-term N limitation led to significant accumulation of fructans in both roots and shoots relative to N-sufficient plants, which is important in the context of this study (Fig. 2). Two days after defoliation, severe fructan depletion occurred in both roots and leaf sheaths of HN plants. However, in LN plants, this depletion was only significant in roots, and was incomplete. We suggest that sufficient N in the HN plants was a stimulating factor in remobilization of the available fructan pool in leaf sheaths, which provided the required energy and C skeletons for regrowth. However, efficient regrowth following defoliation requires balanced pools of stored C for energy, cell biosynthesis, and C skeletons for
Carbohydrate storage remobilization impacts NO\textsubscript{3} uptake

 LN plants clearly had insufficient N to utilize the stored C efficiently. These observations indicate that carbohydrate remobilization to support regrowth occurs in an N-dependent manner.

The regulation of root nitrate uptake by C metabolites from photosynthesis has previously been demonstrated through studying the diurnal stimulation of NO\textsubscript{3} uptake. Sugars transported from shoots to roots played an important role in this diurnal stimulatory effect (Rideout and Raper, 1994; Delhon \textit{et al.}, 1996; Lejay \textit{et al.}, 1999). Using defoliation as a treatment in the present study allowed us to reveal the response of root NO\textsubscript{3} uptake to changes in endogenous C. During the first hour following defoliation, we observed the immediate depletion of the fructan pool in roots of LN plants (Fig. 2B). This immediate remobilization of the fructan pool in LN roots was associated with a rapid and significant stimulation of root NO\textsubscript{3} uptake rate (Figs 2B, 3A). Given that there is a substantial amount of sucrose in phloem immediately after defoliation (Morvan-Bertrand \textit{et al.}, 2001), this stimulation of NO\textsubscript{3} uptake by the root may be attributed to the initial increase in sucrose followed by fructan remobilization in roots, suggesting an instantaneous response of root NO\textsubscript{3} uptake to C availability. In contrast, 48 h after defoliation, low- and high-affinity NO\textsubscript{3} uptake decreased following depletion of the fructan pool in the roots, irrespective of the N status of the plants (Figs 2, 3). Interestingly, a relatively greater fructan depletion in LN plants than in LN plants was associated with a much stronger repression of nitrate uptake rate in LN plants than in LN plants, indicating that NO\textsubscript{3} uptake is a C-dependent process. These observations strongly suggest that root NO\textsubscript{3} uptake is modulated by C metabolites at the whole-plant level, which is consistent with the suggestion that photosynthetic supply is closely balanced with mineral uptake and assimilation (Lejay \textit{et al.}, 1999; Coruzzi and Zhou, 2001; Nunes-Nesi \textit{et al.}, 2010).
NO$_3^-$ assimilation is regulated by the C–N status of the plant

According to Scheurwater et al. (2002), the intact shoot of the eight grass species that they tested was the predominant site for nitrate reduction. This can be explained by the fact that nitrate assimilation is more efficient in the shoot when leaves are photosynthesizing in fully saturating light conditions. Under these conditions, nitrate assimilation in roots is inefficient because sucrose has to be synthesized, transported to roots, and respired in order to generate ATP and NAD(P)H for N assimilation, rather than being directly utilized in the shoot (Nunes-Nesi et al., 2010). Our results also support tissue-specific N assimilation being regulated by local C availability. With relative high C availability in HN roots 1 h after defoliation, NO$_3^-$ uptake was induced instantaneously compared with intact plants (Fig. 3A). This induction of nitrate uptake in HN roots was concomitant with stimulation of LpNRI and LpNRb expression in HN roots (Fig. 5B, D). Given that light was non-saturating for expanding leaf tissue immediately following defoliation, because the leaf tissue was shaded by the sheath, we suggest that a relatively greater proportion of NO$_3^-$ was assimilated locally in HN roots during the first hour after defoliation. However, in the days (48 h) after defoliation, when root C was reduced in HN plants and the young leaves were emerging, relatively more N was allocated to shoots compared with intact plants, thereby stimulating shoot growth (Fig. 2D; Supplementary Fig. S8). These observations imply a mechanism that signals the availability of C metabolites for NO$_3^-$ uptake and assimilation at the whole-plant level. Within tissues, where stored C is available, it is apparent that NO$_3^-$ is assimilated locally.

HATS and LATS regulation at the transcriptional level in response to a changing C/N ratio

Four NRT families have been characterized as being involved in nitrate uptake in Arabidopsis (Tsay et al., 2007). Among them, NRT1 and NRT2 are regarded as the main components controlling the HATS and LATS that function at low (<250 µM) or high (>1 mM) nitrate levels, respectively.
Carbohydrate storage remobilization impacts NO$_3^-$ uptake

Siddiqi et al., 1990; Tsay et al., 1993; Huang et al., 1999; Y.Y. Wang et al., 2012). In our study, as expected, the expression of LpNRT1.1 and LpNRT2.1b was significantly repressed in HN plants 48 h after defoliation, coinciding with reduced NO$_3^-$ uptake activity (Figs 3B, 4B, F). Interestingly, within 1 h of defoliation, the increased expression of LpNRT2.1b in HN roots ($P=0.07$) also coincided with the rapid induction of NO$_3^-$ uptake activity (Figs 3A, 4B). This is consistent with observations that regulation of root NO$_3^-$ uptake is correlated with changes in transcript levels of NRT2.1 in response to N and C treatment (Zhuo et al., 1999; Lejay et al., 2003). Conversely, but as expected, this was not the case in LN status plants which showed lower reductions in NO$_3^-$ uptake rate following defoliation relative to HN plants, and only small increases in both LpNRT1.1 and LpNRT2.1 expression (Figs 3B, 4B, F). Indeed, the regulation of high-affinity nitrate uptake is complicated in low nitrate conditions. This result is consistent with previous studies showing that large and rapid increases in HATS uptake in low NO$_3^-$ conditions are not accompanied by increased AtNRT2.1 expression (De Jong et al., 2014). One possible interpretation for the discrepancy between NRT2.1 transcript and nitrate uptake involves the potential for post-transcriptional regulation of existing transporter levels and activity, for example by phosphorylation (Laugier et al., 2012; X. Wang et al., 2012).

**Exogenous glucose mitigates reduced nitrate uptake rate in defoliated plants**

Exogenous glucose supplied for 6 h to defoliated HN plants largely restored LMW WSC levels in roots, which appears to have relieved the C limitation on NO$_3^-$ uptake and assimilation (Fig. 6B). The NO$_3^-$ uptake rate in defoliated plants supplemented with glucose attained a level greater than that in plants maintained in 5 mM NO$_3^-$ but without glucose supply. In contrast, application of 1% mannitol had no impact on the

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**Fig. 8.** The impact of 48 h defoliation on the putative LpCKX and LpRR gene expression. Plants were grown at either 0.05 mM (LN) or 5 mM (HN) NO$_3^-$, and then defoliated (or left intact). Each data point is normalized against the reference genes eEF-1α and GAPDH. Values are means ± SE ($n=3$ pools of five plants each). Means were tested for significance using a two-tailed $t$-test. Asterisks denote significantly different means between intact plants (grey bars) and defoliated plants (black bars) (*$P<0.05$, **$P<0.01$).
NO₃⁻ uptake rate compared with plants kept in 5 mM NO₃⁻, confirming the carbohydrate dependence of nitrate uptake as described above (Figs 3B, 6C). Interestingly, increasing C availability in roots was correlated with a decline in N allocation to leaf sheaths, which supports our observations of C and N interactions in nitrate uptake and metabolism (Fig. 6B; Supplementary Fig. S8).

The NO₃⁻ uptake rescue by glucose was positively correlated with the transcript levels of LpNRT1.1 and LpNRT2.1b (Fig. 6C, E, F), genes that are regarded as the major players in the LATS and HATS for nitrate, respectively (Y.Y. Wang et al., 2012). Glucose and sucrose have been found to regulate the expression of genes involved in metabolism and nutrient uptake (Li et al., 2006; Lejay et al., 2008). However, up-regulation of AtNRT2.1 by sugars is not attributed to well-established specific sucrose or glucose sensing, although, as shown by De Jong et al. (2014), glucose-regulated expression of AtNRT2.1, independent of nitrate-mediated mechanisms, can operate through HEXOKINASE1-mediated oxidative pentose phosphate pathway (OPPP) metabolism.

Cytokinins have been proposed as candidate signalling molecules relaying N status between the root and shoot
(Kudo et al., 2010; Ruffel et al., 2014). We show here that the cytokinin content of intact HN status plants is greater than that in plants of LN status (Fig. 7; Supplementary Fig. 10). This is consistent with the observations that N supply leads to cytokinin accumulation in roots, xylem sap, and shoots in maize (Takei et al., 2001), suggesting that cytokinin serves to signal N availability from the root to shoot (Sakakibara et al., 2006). After defoliation under HN, a decrease was observed in the iZ-type cytokinin in the leaf sheaths and the iP-type cytokinin in the roots which was associated with increased CKX expression (Figs 7, 8A, B). Interestingly, 1% glucose addition to defoliated HN plants induced iP and iPR accumulation in leaf sheaths and roots, and was associated with a significant decline in expression of LpCKX4 and LpCKX6 in roots, implying that iP-type cytokinins might be an integrating signal of the relative availability of C and N metabolites in plants (Figs 7, 9A, B).

In contrast, iZ-type cytokinins increased in the HN leaf sheaths, as did expression of the LpRR genes, 48 h after defoliation (Figs 7, 8). iZ-type cytokinins are recognized by receptors and activate response regulators in rice and maize (Veach et al., 2003; Yonekura-Sakakibara et al., 2004; Lomin et al., 2011; Roche et al., 2016). In this context, it is possible that the LpRR genes could be responding to the cis-cytokinins with the downstream activation of N assimilation in the leaf sheaths. Consistently, while iZ-type cytokinin content declined, LpRR expression was reduced in roots following 6 h of 1% glucose addition (Figs 7, 9). However, this decline of iZ also occurred in plants with 1% mannitol treatment (Fig. 7). The decreased iZ content under 1% mannitol treatment was not consistent with salt or osmotic stress which is suggested to induce iZ (Havlová et al., 2008; Vyrobalová et al., 2009; Macková et al., 2013). The iZ response to mannitol reflects the less well understood biology of the cis-cytokinin forms. As the LpRR expression decreased under glucose treatment but not under mannitol treatment, a cytokinin response to mannitol is not supported. Taken together with the increased NO3− uptake rate, fructan levels, and RR gene profiles under glucose treatment, the glucose supplement may have caused a cytokinin response via its impact on C metabolism.

Conclusions

This study provides clear evidence that the rapid and large shifts in C storage triggered by defoliation have significant impact on nitrate uptake in an N-status-dependent manner. Our results for the perennial grass, L. perenne, are consistent with those in Arabidopsis, where cytokinins act as a systemic N signal regulating N uptake and assimilation (Kiba et al., 2011; Ruffel et al., 2011; Shtratnikova et al., 2015). By perturbing C assimilation and metabolism with a defoliation–regrowth treatment, we propose that cytokinin-mediated NO3− uptake and assimilation may also act to balance C and N resources at the whole-plant level. Building on our results and those of previous workers, we present a model (Fig. 10) whereby cytokinins serve as a signal to integrate C and N metabolism via tissue-specific function: cytokinin-mediated reduction in NO3− uptake in roots and an increase in NO3− assimilation in re-growing shoots under low C/high N status (low N demand); and a concomitant shift of C allocation from shoots to roots which serves to stimulate NO3− assimilation in roots under high C/low N status (high N demand) (Fig. 10). As we develop our understanding of the interaction between defoliation and N uptake, this knowledge may be applied to pasture production for grazing in order to increase N uptake and utilization efficiency.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Sequences of qPCR primers used in this work. Figs S1 and S2. Flow diagrams of the experimental processes

Fig. S3. Phylogenetic trees of LpNRT1, LpNRT2, LpNR, and LpNiR gene families.

Fig. S4. Relative abundance in carbon (C) units of water-soluble carbohydrates (WSCs) in RN and LN plants after defoliation.

Fig. S5. Expression of putative Lp6-FEH and Lp1-FEH in plants grown under either 0.05 mM (LN) or 5 mM NO3− (HN) supply, 1 h and 48 h after defoliation.

Fig. S6. The impact of glucose on relative abundance in carbon (C) units of water-soluble carbohydrates (WSCs).

Fig. S7. Lp6-FEH and Lp1-FEH expression in leaf sheaths with 6 h of supplemental glucose.

Fig. S8. The impact of defoliation or glucose addition on 15N translocation to shoots.

Fig. S9. Effects of glucose addition on putative LpNRT2.1a gene expression in roots grown in 5 mM NO3−, 48 h after defoliation.

Fig. S10. Cytokinin concentrations in LN roots and leaf sheaths 48 h after defoliation.

Fig. S11. The impact of 1 h defoliation or putative LpCKX and LpRR gene expression.

Fig. S12. Effects of 0.1% glucose addition on putative LpCKX and LpRR gene expression in plants grown in 5 mM NO3−, 48 h after defoliation.

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References


