Using molecular biology techniques to improve our ability to manage pasture

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ABSTRACT

Over the past century, substantial research has been devoted to understanding how different environmental conditions and management practices affect plants. These studies have greatly increased our understanding of factors affecting plant growth and physiology. Modern molecular technologies enable even greater insight into the complex biochemical processes underpinning those responses. In a recent study during winter in Hamilton, New Zealand, gene expression profiling was used to understand the way in which perennial ryegrass (*Lolium perenne* L.) responded to different defoliation management practices. Plots in perennial ryegrass-dominant pastures were defoliated to 2, 4 or 6 cm residual height and regrown until the 3-leaf stage before determination of pasture yield. Water-soluble carbohydrate (WSC) energy reserves and the expression of photosynthesis genes were monitored throughout the regrowth cycle. Pasture regrowth was reduced by lax defoliation ($P < 0.001$; 2270, 2250 and 1910 kg DM/ha for residual heights of 2, 4 and 6 cm, respectively). Gene expression profiles indicate that plants defoliated to 2 cm increased photosynthesis in stubble tissue at the 1-leaf stage of regrowth, thereby compensating, at least in part, for the reduced leaf area and WSC reserves. This compensatory mechanism is one possible reason why regrowth of pastures defoliated to 2 cm was not compromised. These data confirm the results of previous studies and, for the first time, provide a physiological basis to explain how ryegrass adapts to different grazing management regimes. Use of such confirmatory techniques should give farmers greater confidence in the extended research results and, depending on the technique used, can provide a mechanism for gene discovery.

Keywords: molecular biology; gene expression; grazing severity; grazing height; perennial ryegrass; pasture**.**

INTRODUCTION

The New Zealand and Australian dairy industries remain largely pastoral-based, contributing to low milk production costs compared with countries that use more supplementary feed (Dillon *et al.*, 2005; Hemme, 2004). To retain these low production costs, growth and utilisation of large amounts of highly nutritious pasture is important. As a result, considerable research has been devoted to understanding how environmental and management factors impact pasture growth and quality.

Some of the factors affecting plant growth and quality are outside farmers' control (e.g. the weather); however, one of the most influential factors that can be controlled is the height to which pastures are grazed, or grazing severity. While considerable research has been devoted to this, results are inconsistent. Some studies demonstrate greater pasture production following severe grazing (< 1500 kg DM/ha; < 4 cm residual height; McKenzie *et al.*, 2004; Reid, 1959; 1962), while others recommend lax grazing (>2000 kg DM/ha; >8 cm residual height) for increased pasture growth (Brougham, 1956; Volesky & Anderson, 2007; Xia *et al.*, 1994).

One of the factors that may have confounded results is the interaction between grazing severity and frequency. Parsons *et al.* (1988) reported that severe grazing (to a leaf area index of 0.5 to 1.1) reduced both rates of photosynthesis and leaf death, resulting in a considerable delay before maximum growth rates were regained. In spite of this however, greater rates of average pasture growth were achieved, compared with lax defoliation. This indicated that severe grazing must be coupled with grazing intervals long enough to obtain maximum growth rates without any deterioration in sward structure caused by increasing proportions of stem or dead material (Parsons & Penning, 1988), i.e. before the 3- to 3.5-leaf stage of regrowth (Fulkerson & Donaghy, 2001).

Physiological measurements have been included in some studies to see how different biological processes were affected by grazing severity. Fulkerson and Slack (1995) and Lee *et al.* (2008) demonstrated that a greater post-grazing height left plants with more water-soluble carbohydrate (WSC) energy reserves, which was generally associated with greater regrowth. In addition, laxly grazed plants had greater residual leaf area remaining, presumably increasing capacity for photosynthesis and, therefore, regrowth (Booysen & Nelson, 1975; Grant *et al.*, 1981).

Conversely, some studies suggest that leaves remaining after grazing tend to be older and less photosynthetically active than younger leaves and, therefore, are unlikely to contribute substantially to subsequent regrowth (Gay & Thomas, 1995; Woledge, 1977). This suggests that there may be no pasture production benefit to leaving more residual leaf after grazing providing the plant has adequate WSC reserves. New and developing technologies, such as gene expression profiling, enable greater insight into how complex biological processes are affected by defoliation management, and to what extent; this may help resolve or better understand the conflicting results.

What is gene expression?

Inside plant cells, numerous genes are encoded in long DNA strands. Gene expression is the process by which the information encoded in a particular segment of DNA (gene) is converted into a functional product, such as a protein. The first step in this process is transcription, in which the DNA sequence is copied by the enzyme RNA polymerase to produce a complementary messenger RNA (mRNA).

There are numerous ways of measuring gene expression; however, the most common method used is quantitative reverse transcription polymerase chain reaction (qRT-PCR; Bustin, 2000). Quantitative RT-PCR enables the number of copies (abundance) of a particular mRNA (the gene transcript) in a sample to be calculated, thereby indicating whether a certain process is occurring in the plant, and at what rate.

Some physiological measurements suggest that photosynthesis may be affected by grazing severity, although the manner in which it is affected is not fully understood. This can be tested using qRT-PCR, by selecting one or more genes that are important to the photosynthetic pathway. The objective of the current study was, therefore, to determine the effect of defoliation severity during winter on pasture growth, WSC energy reserves and the expression of key photosynthesis genes.

MATERIALS AND METHODS

The study was conducted at DairyNZ's Lye Farm in Hamilton (37°47'S 175°19'E; elevation 40 m). In May 2007, 54 plots (each 2 x 3 m) were laid out in a randomised block design in newly-mown perennial ryegrass (*Lolium perenne* L., cv. Bronsyn) dominant pasture. This paper presents a subset of data yield and WSC previously published by Lee *et al.*(2009). After a covariate harvest in late June, plots were defoliated to 2, 4 or 6 cm residual height (1300, 1600 or 1800 kg DM/ha, respectively) using a rotary lawnmower each time three new leaves had emerged on average across all plots. This resulted in two treatment defoliations (late August and early October).

Pasture DM yield

During the early October harvest, each plot was mown to the treatment residual height using a rotary lawnmower. The fresh weight of this sample was recorded on a hanging scale suspended from a tripod in the field. Representative sub-samples (~200 g fresh weight) were oven-dried in duplicate at 95°C for 48 hours to estimate DM content. Pasture DM yield was calculated by multiplying the sample fresh weight by the average DM content of each plot.

Stubble WSC content

Following the late August defoliation, 60 mature perennial ryegrass tillers (including roots) were harvested at random from each plot immediately before defoliation and following the emergence of each new successive leaf (i.e. 1-, 2- and 3-leaf stages of regrowth). Harvests were consistently performed three hours after sunrise to limit diurnal variation in WSC. After harvesting, samples were stored on ice until roots were removed from the base of the tiller and tillers cut to the treatment residual height (2, 4 or 6 cm). Samples were then frozen at -20°C before freeze-drying. Once dried, samples were weighed, ground to pass through a 1 mm sieve and analysed for WSC (Technicon Industrial Method number 302-73A; derived from the method outlined by Smith, 1969). Total stubble WSC content per tiller was calculated by multiplying WSC concentration by the average tiller dry weight of each stubble sample. The amount of WSC per tiller is considered to be a better indicator of regrowth potential (Donaghy & Fulkerson, 1998) because tiller size/weight is taken into account.

Gene expression

At the 1-leaf stage of regrowth, individual perennial ryegrass tillers were harvested to ground level from different plants within each plot at midday. Tissues were frozen immediately in liquid nitrogen, transported in dry ice and stored at –80°C. Leaf (>4 cm from ground level) and stubble (<4 cm, leaf sheaths plus enclosed elongating leaf bases) tissues were separated and ground in liquid nitrogen. DNase I-treated total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and mRNA was isolated from the total RNA using Dynabeads® Oligo $(dT)_{25}$ (Invitrogen Dynal AS, Oslo, Norway). The mRNA (10 ng) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) with anchored-oligo $(dT)_{18}$ primers in total reaction volumes of 20 µl.

Within the photosynthesis pathway, Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)

is arguably the most important enzyme in the photosynthesis pathway; it regulates the conversion of carbon dioxide into 3-phosphoglycerate, the first product in the synthesis of carbohydrates. The Rubisco enzyme consists of two types of protein subunits assembled together; the large subunit and the small subunit. Therefore, the two key photosynthesis-related genes chosen for this study were *rbcS*, which encodes for the small subunit of the Rubisco enzyme, and *rca*, which encodes for Rubisco activase, an enzyme responsible for activating Rubisco. Primer pairs for these two genes were designed using Primer3 (http://primer3.sourceforge.net/; Rozen & Skaletsky, 2000) and supplied by Invitrogen (Auckland, New Zealand). Forward and reverse primer pairs were ATATCACCTGGGTCGAGGAA and CCAACGGCGAATAAAGAAAC for *rbcS*, and CCAAGAACTTCGACCCAACTG and AACTTTCATGCCCAGCCATC for *rca*, respectively.

The qRT-PCR analyses were performed in 384-well plates with a LightCycler® 480 real-time PCR instrument (Roche Diagnostics) using the LightCycler® 480 SYBR Green I Master kit. Each reaction contained 5 µl of 2x SYBR Green I Master mix, 2 µl PCR-grade water, 2 µl of 100-fold diluted cDNA, and 0.5 µl of each of the 10 µM forward and reverse gene-specific primers in a final volume of 10 µl. All reaction set-ups were performed using the epMotion® 5075LH automated liquid handling system (Eppendorf). Reactions were incubated at 95°C for 5 minutes to activate the FastStart Taq DNA polymerase, followed by 45 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 8 seconds. The specificity of the PCR reaction was confirmed with a heat dissociation protocol (from 60°C to 95°C) following the final PCR cycle.

Relative abundance of each photosynthesis-related gene was calculated by:

$$\text{Relative gene abundance} = \frac{(\text{T/R1} \times \text{T/R2})^{1/2}}{(\text{C/R1} \times \text{C/R2})^{1/2}}$$

This formula provides an efficiency-corrected relative quantification, normalised to a calibrator sample (either leaf or stubble at the 3-leaf stage), where T is the concentration of the target photosynthesis gene in a particular sample; R1 and R2 are the concentrations of the first and second validated reference genes (eukaryotic elongation factor 1α and YT521-B-like protein family protein, respectively; Lee *et al.*, 2010) in either a particular sample, or the calibrator sample; and C is the concentration of the target photosynthesis gene in the calibrator sample.

Statistical analyses

All data were analysed in GenStat (VSN International Ltd., 2008) using ANOVA with defoliation height as a fixed effect. The relative abundance of each photosynthesis gene was $\log_{10}$-transformed before analysis. Following statistical analyses, averages for each treatment were back-transformed.

RESULTS AND DISCUSSION

There was no effect (P >0.1) of defoliation height on leaf appearance rates, therefore, all 1-, 2- and 3-leaf stage sampling procedures and 3-leaf stage harvests were carried out at the same time for all defoliation treatments.

Pasture yield at the 3-leaf stage of regrowth was reduced following defoliation to 6 cm residual height (P <0.001; 2270, 2250 and 1910 kg DM/ha for pastures defoliated to 2, 4 and 6 cm, respectively). This is consistent with previous research by Brougham (1960), who investigated the effect of defoliation height on a seasonal basis and demonstrated that severe winter grazing (2.5 cm) encouraged growth of all sown pasture species. Modelling work by Parsons *et al.* (1988) also emphasised that severe defoliation followed by 'relatively short' durations of regrowth was the best method for effective grazing management. 'Relatively short' was defined as long enough to achieve the maximum average growth rate without the deterioration of sward structure caused by substantial increases in stem (Parsons & Penning, 1988), a definition which aligns with the 2- to 3-leaf stage of regrowth as the recommended time for optimal defoliation (Fulkerson & Donaghy, 2001).

The WSC reserves stored in the stubble increased as the defoliation height of the pasture increased (Figure 1). Stored WSC are important to plants as they provide energy for growth when energy supply via photosynthesis is insufficient, such as immediately after defoliation (White, 1973). Plants containing greater WSC, therefore, may achieve greater growth (Davies, 1965; Fulkerson & Slack, 1994; Gonzalez *et al.*, 1989). In the current study, however, pasture yield was not correlated with stubble WSC content (P >0.1), with greater pasture yield achieved in pastures containing less WSC. During winter, plants contain more WSC reserves than other seasons (between 44 and 105% more WSC compared to between spring and autumn; Lee *et al.*, 2008), due to slower growth rates and cooler temperatures reducing respiration. Therefore, WSC may not have been a limiting growth factor during this study, even in pastures defoliated to 2 cm.

Gene expression analyses resolved, at least in part, why perennial ryegrass plants defoliated to 2 cm were able to regrow more than plants defoliated to 6 cm, despite the latter containing more WSC. At the 1-leaf stage of regrowth, expression of both

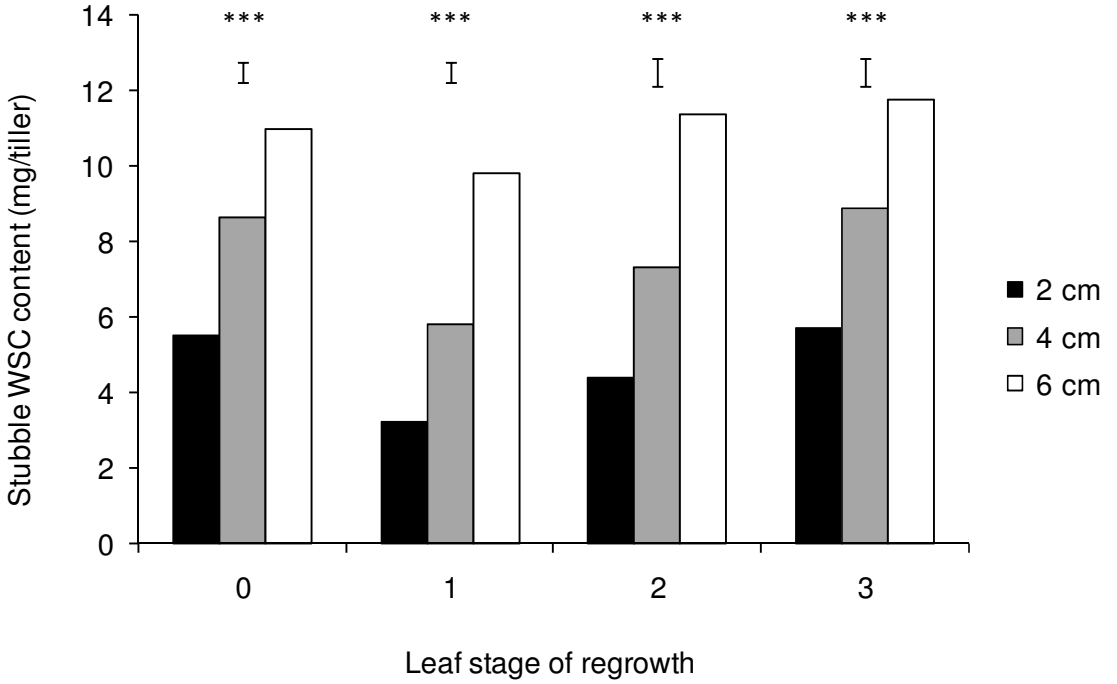

FIGURE 1: Water-soluble carbohydrate (WSC) content stored in the stubble of perennial ryegrass tillers that had been defoliated to 2, 4 or 6 cm residual height (0-leaf stage) and regrown to the 3-leaf stage. Errors bars indicate the standard error of the difference for each leaf stage (where: *** = P< 0.001).

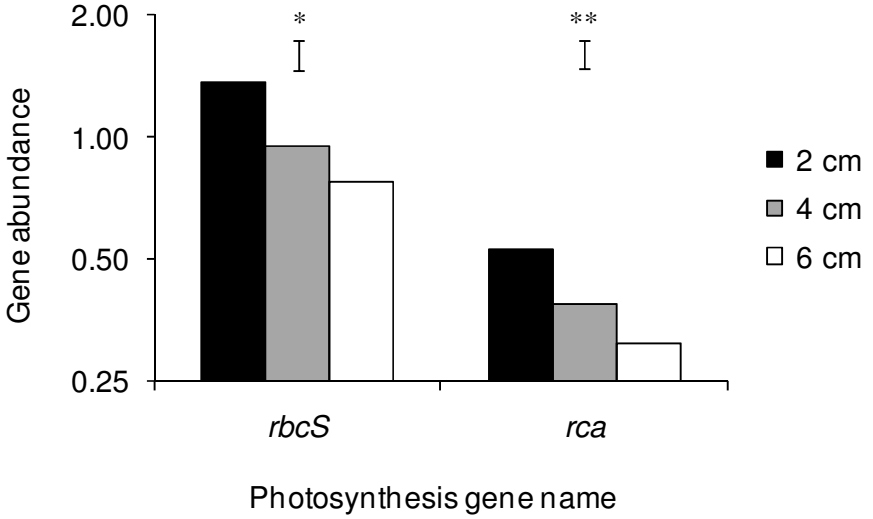

FIGURE 2: Abundance of two photosynthesis genes (the small subunit of Rubisco, *rbcS*; Rubisco activase, *rca*) in stubble tissue of perennial ryegrass plants at the 1-leaf stage of regrowth. Plants had been previously defoliated to 2, 4 or 6 cm residual height. Errors bars indicate the standard error of the difference for each photosynthesis gene (where: * = P <0.05; ** = P <0.01).

photosynthesis genes was greater in the stubble tissue of plants defoliated to 2 cm compared to those defoliated to 6 cm (Figure 2). This indicates that plants severely defoliated to 2 cm were able to increase the photosynthesis of the outer leaf sheaths to compensate for less residual leaf area (thus lower initial photosynthetic capacity) and reduced WSC reserves. Although leaf sheaths are not normally considered to be a primary site of photosynthesis, previous studies have noted that they do possess the

capability (Leafe & Parsons, 1983). This compensatory effect is one possible reason why regrowth was not compromised in pastures defoliated to 2 cm in the current study.

Although defoliation to 2 cm did not compromise pasture yield, regrowth was only measured following a single severe defoliation at a time when WSC content is at its greatest. A negative effect of severe defoliation (2 cm) may have become evident if severe grazing had occurred over several rotations, as was demonstrated between spring and autumn (Lee *et al.*, 2008). Therefore, grazing as low as 2 cm (1300 kg DM/ha) is not advised between spring and autumn, otherwise reductions in pasture production will occur, even following a single rotation (Lee *et al.*, 2008). In addition, pastures should only be severely grazed during winter when soil and environmental conditions are suitable to avoid pugging and soil damage.

In conclusion, during winter grazing pastures to between 2 and 4 cm residual height (1300 to 1600 kg DM/ha, respectively) increased pasture yield. Plants defoliated to 2 cm upregulated expression of photosynthesis genes in the stubble, thereby compensating for less residual leaf area and reduced WSC reserves. These data provide a physiological basis to explain how perennial ryegrass adapts to different grazing management regimes. In the future, the ability to breed plants will be improved by a better understanding of gene expression and regulation; therefore, continued progress in this area is important.

ACKNOWLEDGEMENTS

We thank Barbara Dow for her statistical expertise and Pastoral Genomics, ViaLactia Biosciences and Roche Diagnostics for providing equipment and facilities. This study was funded by DairyNZ Inc., the Foundation for Research, Science and Technology, the T.R. Ellett Agricultural Research Trust and a Tasmanian Graduate Research Scholarship from the University of Tasmania.

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