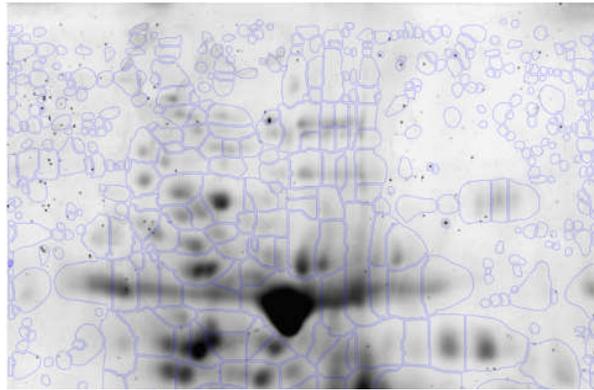


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Investigations into the biological and genetic control of fatty acid levels in perennial ryegrass



Key external stakeholders:

Grass breeders, agronomists, plant science research community, general public

Practical implications for stakeholders:

The outcome of this research informs agronomists and breeders on the effects of management factors influencing fatty acid levels in different perennial ryegrass cultivars.

- Management practices influence the fatty acid composition of perennial ryegrass.
- A perennial ryegrass proteome map has been constructed under contrasting fatty acid levels which can be used for further genetic studies on this topic.

Main results:

The field study component of this project identified the range and extent, along with the seasonality, of variation in fatty acid levels in varieties of perennial ryegrass recommended for use in Irish agriculture. A proteome map of perennial ryegrass under contrasting fatty acid levels was constructed. This work will inform further genetic studies in the fatty acid genetics in perennial ryegrass.

Opportunity / Benefit:

This research is of strategic nature and the results obtained are not currently directly exploitable in a commercial sense. But they will help to inform breeding strategies in the future not only in terms of enhancing fatty acid levels but also in the approach taken to improve other important quality parameters.

Collaborating Institutions:

UCD Dublin

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1. Project background:

Ireland has a grassland based agricultural system with over 80% of the total agricultural area dedicated to grassland. Grass is the cheapest source of feed available to ruminants and so the objective is to optimise grazed grass in the diet. Considerable advances have been made in recent years in breeding more productive and persistent varieties of perennial ryegrass but less emphasis has been placed on the quality of these varieties. Fatty acids in grass are the main precursors for conjugated linoleic acid and polyunsaturated fatty acids in ruminant products. These have well documented associated human health benefits. In order to promote sustainable and profitable agricultural systems by increasing the content of beneficial fatty acids in ruminant products, increasing fatty acid intake from grazed grass is the most appropriate strategy in an Irish context. There is little information on the genetic control of fatty acid levels in grass although a number of management strategies are known to alter grass content. The focus of this study is to profile the variation in fatty acid levels in grass cultivars and determine the biological and genetic control of this variation using new technologies such as real time PCR and 2D protein gels.

Breeding of perennial ryegrass varieties has improved DM yield, persistency, digestibility and water soluble carbohydrate content of the sward but fatty acid levels have received little attention. As grass is the cheapest source of ruminant feed and is maximised in ruminant diets, quality parameters should not be neglected. Grass breeders and producers require information on the genetic control of fatty acid content of perennial ryegrass (PRG) if progress is to be made in breeding varieties with increased fatty acid contents. Grass based diets are known to increase the α -linolenic acid content (18: 3n-3) levels in beef muscle and milk. Therefore there is a need to improve the fatty acid content of PRG and the first step in this is developing an understanding of the genetic control of this important quality trait.

2. Questions addressed by the project:

Internationally there are a small number of groups working on management strategies to augment fatty acid levels in PRG. Shading or extended wilting of fresh grass prior to ensilage will reduce the fatty acid levels in the silage. Fatty acid levels in PRG have been shown to be under genetic control with variation between PRG cultivars in linolenic acid contents identified throughout the growing season and fatty acid profiles were distinctive to species when grasses received the same management. Genetic control of herbage quality traits have been assessed although fatty acid levels were not included in this assessment. Because of the lack of more detailed insights into the genetics of the fatty acid genetics this project was necessary to collect information and data on this subject.

- How large is the variation in fatty acid levels in different perennial ryegrass cultivars under different management regimes grown under field conditions in Ireland?
- Is it possible to correlate fatty acid levels with genetic information gained from candidate genes of the fatty acid pathway?
- Is it possible to construct a 2D proteome map of perennial ryegrass based on a differential response of fatty acid levels?

3. The experimental studies:

Perennial ryegrass varieties used in recommended list testing were grown under different management regimes at Lyons Estate Research Farm and at DAFM Backweston Farm. For a period of three years samples were taken at each harvest and processed for fatty acid quantity and quality testing by gas chromatography.

In the last decade the analysis of RNA expression via Real time PCR has opened up new venues for the accurate determination of the number of transcripts. Furthermore, proteomics has gained new interest with recent advances in protein analysis which allow the generation of complete 2D proteome maps for different cellular compartments. At the time of project application submission very few perennial ryegrass nucleotide sequences had been submitted to public nucleotide databases.

Sufficient genetic variation is the prerequisite to work on the genetics of fatty acid pathways. In perennial ryegrass significant variation has been found for fatty acid contents in different genetic backgrounds applicable to Irish agriculture. For the Poaceae in general a larger number of sequences involved in the fatty

acid pathway have been submitted to genbank. From these a number deemed to be worthwhile testing as candidate genes in expression experiments using Real time PCR were selected. These sequences were from *Lolium rigidum* (a close relative of perennial ryegrass), which were likely to amplify without too much difficulty in perennial ryegrass. For other candidate genes degenerate primers were designed. These genes could prove valuable markers if a tight correlation between the expression of those genes and enhanced fatty acid levels could be found.

4. Main results:

The field study component of this project identified the range and extent along with the seasonality of variation in fatty acid levels in the recommended varieties of perennial ryegrass for use in Irish agriculture. The potential to exploit this natural variation through management practices was then investigated under controlled circumstances on Lyons research farm. This work further identified the extent to which natural variation could be augmented by agronomic practices.

For the Poaceae in general several nucleotide sequences involved in the fatty acid pathway have been submitted to genbank (<http://www.ncbi.nlm.nih.gov/nucleotide>). From these a number have been identified to be worthwhile testing as candidate genes in expression experiments. The strategy of using genomic information from other grass or monocotyledonous species was necessary due to the limited number of publicly available perennial ryegrass sequences. We worked with sequences from closer to more distant related grasses. This approach gave us a chance to amplify those sequences in perennial ryegrass. With the identified sequences multiple sequence alignments were done using the program MEGA (S Kumar, K Tamura, and M Nei (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5:150-163). Areas of conserved sequences were identified which were then used as template regions for primer design. Primers were designed using the program PriFind (<http://cgi-www.daimi.au.dk/cgi-chili/PriFi/main>) which is software specialised to design degenerate primers from sequences of other species. Primers were successfully designed for the following genes lipocalin, lipoxygenase, oleate desaturase, fatty acid hydroxylase and the accase gene. In total we optimised seven PCR assays for fatty acid candidate genes in perennial ryegrass; (1) four independent assays from different sequence gene regions for the key regulatory fatty acid gene accase, (2) two independent assays for the lipocalin gene, and (3) one assay for the oleate desaturase gene. Two working assays for the accase gene, one assay for the oleate desaturase, and one assay for the lipocalin gene are available for real time PCR work in perennial ryegrass. The expression of the genes Accase and lipocalin was quantified using real-time PCR. Quantification was achieved on the LC480 (Roche) using SYBR Green I chemistry. Relative expression was normalised to two housekeeping genes, namely β -actin and GAPDH. Amplification efficiency values (E) were calculated by generating standard curves and incorporated into final calculations. Analysis was performed using the software Qbase (Ghent University, Belgium). Finally correlation analysis between the phenotypic fatty acid data and expression profile data were calculated using the statistical package SAS. The main outcome of this task was the correlations between the levels of expression of the two candidate genes, ACCase and lipocalin, and fatty acid content. A positive correlation was found for the gene expression level of the candidate gene ACCase and C18:0 content ($P < 0.05$) for samples from October 2007, while a negative correlation was found for the expression of the same gene and C18:3 content ($P < 0.05$) for samples from July 2008. While the relationship appears inconsistent it presents evidence of a relationship between the target genes and the fatty acid levels in the varieties tested. This presents strong evidence for future research in this area.

For the perennial ryegrass fatty acid proteome map initial work was focused on the optimisation of protein extraction methods to obtain a high quantity and quality of proteins for 2D SDS-page analysis. The extraction method of Gorg *et al.* (2002) with O'Farrell's lysis buffer was retained as the best working extraction method. For the comparative proteome map two perennial ryegrass varieties, two treatments, two protein ranges, three replications and each gel in technical duplicates were used. The two varieties were picked based on their statistically significant high and low polyunsaturated fatty acid contents. The pH ranges 3-10 and 4-7 were included in the experiments. Gels were stained using Sypro Ruby and digitalized using the Typhoon GE Healthcare scanner. The software package 'Progenesis' (UCD Conway Institute) has been used to compare the 2-D protein gels. A comparative proteome map of differentially expressed proteins for perennial ryegrass with contrasting fatty acid profiles has been built.

5. Opportunity/Benefit:

The outcomes of this research have an impact on the advancement of plant sciences for the forage grass perennial ryegrass. When the grant application for this project was written little research on the molecular

aspects of the fatty acid pathway in perennial ryegrass had been published. This project also increased collaboration between Teagasc and UCD. While this research is at the basic level of the research spectrum and the results obtained are not currently directly exploitable in a commercial sense, they will help to inform breeding strategies in the future not only in terms of enhancing fatty acid levels but also in the approach taken to improve other important quality parameters.

6. Dissemination:

The project resulted in a number of scientific publications and presentations at meetings. The project was also presented to visitor groups and at Open Days in Oak Park.

Main publications:

Cristilli M. (2012) Investigations into the biological and genetic control of fatty acid levels in perennial ryegrass. *PhD. thesis National University of Ireland*, in preparation.

Cristilli M., Lynch M.B., Barth S., McGilloway D., Boland T.M. (2010) Effect of variety, nitrogen fertilization and regrowth interval on fatty acid levels of perennial ryegrass post establishment. *Proceedings of British Society of Animal Science and the Agricultural Research Forum 2010*, p 145 Belfast, Northern Ireland.

Cotecchia L. (2010) Development and testing of assays for the expression of candidate genes involved in the fatty acid pathway in perennial ryegrass. *MSc. thesis National University of Ireland*.

Cotecchia L., Boland T.M., Lynch M.B., McGilloway D., Mullins E., Byrne S. and Barth S. (2009) Investigations into the biological and genetic control of fatty acid levels in perennial ryegrass. *Proceedings of the Irish Plant Scientists Association*, 19th – 20th March 2009 Trinity College Dublin.

Cristilli M, Lynch MB, Barth S, McGilloway D, Kenny DA and Boland TM (2009). Effect of cutting date on fatty acid levels of different perennial ryegrass varieties throughout the growing season. *Proceedings of the Agricultural Research Forum* p. 96.

Cristilli M, Lynch MB, Barth S, McGilloway S, Kenny DA and Boland TM. (2009). Comparison and optimisation of fatty acid extraction methods for high throughput perennial ryegrass sample screening. *Proceedings of the Agricultural Research Forum* p. 104.

Cotecchia L., Boland T, Yang B, McGilloway D., Lynch B. and Barth S. (2008) Investigations into the biological and genetic control of fatty acid levels in perennial ryegrass. *Proceedings of the Irish Plant Scientists Association Meeting*. March 2008

7. Compiled by: Dr. Susanne Barth