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1. Abstract

The research describes whole or part genome incorporation of the fescue species *Festuca glaucescens* into Italian ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*). As hybrids the ryegrass and fescue genome combinations provide opportunities to harness complementary adaptive, agronomic, and ecosystem service traits.

Ruminant protein use is inefficient and leads to losses of N and harmful greenhouse gas emissions. Previous research indicated this was in part due to plant-mediated proteolysis, particularly by *Lolium* species, a consequence of the stresses encountered within the rumen. *F. glaucescens* is more resilient to abiotic stress and has genome potential to aid protein protection.

Under simulated rumen conditions the protein stability of alternative *L. multiflorum, L. perenne,* and *F. glaucescens* genotypes, and progeny of contrasting genome dosage, was compared. Amongst the combinations assessed, the amphiploid breeding approach (two equivalent fescue and ryegrass genome complements) provided balanced F_1 hybrid genome combinations and achieved the greatest protein stability. Interactions between the *Lolium/Festuca* genomes in the F_1 provided enhanced protein retention compared to one and often both parental genotypes. Both *Lolium* and *Festuca* genotypes showed large intraspecific variation in protein stability but this *per se* was no indicator for protein stability found in their progeny. Backcross breeding programmes led to unbalanced *Lolium/Festuca* genome combinations and negated their positive F_1 hybrid interactions. The initial lower protein content of the F_1 compared to their parental genotypes may have contributed to their superior protein stability. Novel polypeptides were induced in the F_1 by rumen-simulated stress but were not evident in *Lolium*.

A detailed introgression breeding programme (targeted transfers of a limited number of fescue genes) was undertaken to assess the efficacy for *F. glaucescens* gene transfers and trait expression in *L. perenne.* Both *Festuca*-specific morphological and genetic markers were identified that were later transmitted and expressed in high numbers in backcross derived progeny. Their high transmission confirms the efficacy of an introgression-breeding approach for transfers of valuable *F. glaucescens* traits into *L. perenne*.

2. Introduction

Strategies in crop design and management are required that offer a balance to safeguard food security, efficient use of the earth's scarce resources, and a reduced environmental "footprint". Success will depend on a co-ordinated strategy that enjoys the full engagement and support from scientists, industry, policy makers, and the public.

Changes to ruminant farming practices will play an important part in providing future food security whilst protecting the environment because ruminants are an important part of the human food chain (Grigg, 1995). Ruminant digestion converts fibrous biomass such as grass, which is in abundance around the world often in areas deemed unsuitable for arable crops, and produces via livestock intervention high quality protein sources fit for human consumption. However, the advantages drawn from livestock agriculture must be weighed against the environmentally damaging concomitant methane production (Moss *et al.*, 2000). In the UK, 65% of agricultural land is grassland, much of it permanent grasslands unsuitable for cereal crop cultivation but highly amenable to livestock agriculture where it sustains a £6bn industry (Humphreys *et al.*, 2006). However, the ruminants on which UK livestock agriculture depend are poor converters of the nitrogen they ingest. This results in the generation of the greenhouse gases nitrous oxide (N_2O) and ammonia (NH_3) in addition to methane (CH_4) and in combination contribute significantly to the emissions responsible for environmental pollution and global warming (Kingston-Smith *et al.*, 2010).

Ruminant livestock agriculture is responsible for around 28% of anthropomorphic emissions which is around 80 million tonnes of methane globally every year (Moorby *et al.*, 2008). In the EU in the 1990's methane produced by ruminants (enteric fermentation 67%; livestock manure 33%) accounted for an estimated 10.2 million tonnes per year and represents the greatest source of methane produced (Moss *et al.*, 2000). Methane (CH_4), and carbon dioxide, (CO_2) contribute 20% of global warming and in the UK, livestock agriculture, a large proportion of which is dairy farming, constitutes 43% of CH_4 emissions (Smith *et al.*, 2008). Under the Kyoto Protocol a target was set to reduce greenhouse gas (GHG) emissions by 12.5% of 1990 levels by the year 2008-2012 which is under review. Mitigation of GHG emissions, especially CH_4 (which has 23 times the warming potential of CO_2), is now a priority and ways in which this can be achieved have been suggested with the proviso that they are effective over broad spatial scales and under future scenarios (Smith *et al.*, 2008).

Although it is important for ruminants to be able to absorb large amounts of the necessary proteins in their diet, poor protein capture from the large amounts of plant biomass has serious implications for livestock agriculture sustainability because there is a poor return with only 30% of nitrogen ingested being converted into animal protein products milk and meat (Dewhirst, 1996; Firkins, 1996). Plant biomass eaten as forage is exposed in the rumen to hostile conditions including darkness, no oxygen,

high temperatures (39°C) and microbes that in unison cause the plant cells to initiate an early regulated cell death (senescence). This results in poor utilization of nutrients and creates excess waste N of up to 70% as urea and ammonia (Kingston-Smith *et al.*, 2008). GHG's and waste N may also be caused by the imbalance of substrates supplied to the ruminal microbial populations that relates to methane produced by the methanogenic *Archaea* at the final stage of fermentation (Kingston-Smith *et al.*, 2010). The main concerns for livestock agriculture are to provide feed that will increase microbial protein supply, reduce production costs and decrease losses of N to the environment causing pollution. Grassland accounts for over 70% of crude protein (CP) consumed by ruminants in the UK with other sources of CP including cereals (14%), oilseeds (10%) and legumes (peas and beans < 5%) (Wilkins & Jones, 2000).

The problems associated with the inefficiency of the rumen's microbial population to capture non-protein N (NPN) i.e. NH_3 also include the poor conversion of dietary N to N products (meat and milk). The cause of poor utilization of dietary protein and loss of N from the rumen has been attributed to the 'Asynchrony Hypothesis' (Kingston-Smith & Theodorou, 2000) with regard to the rumen microbial populations' protein requirements. Asynchrony occurs because it has been shown that ruminants grazing on fresh forages supply the rumen microbial populations with excess RDP (Rumen Degradable Protein) relative to fermentable energy; this is because of the rapid availability of plant proteins due to their degradation by plant and microbial enzymes (Nolan *et al.*, 2005; Moorby *et al.*, 2008). Loss of NPN limits microbial growth because of reduced amino acid production for the host animal's tissue production and is then absorbed from the rumen when it is excreted in urine and faeces. In order to solve the problem of asynchrony, Sinclair *et al.*, (1993) suggested (in experiments done on sheep) that efficient use of dietary crude protein (CP) would be at its highest when fermentable carbohydrates are in an appropriate ratio to RDP in the diet alongside the energy substrate being fermented being in synchrony with release of RDP sources (Fig. 1.3). Nocek & Russell (1988), also observed that if water-soluble carbohydrate (WSC) is relatively low in the diets of lactating cows, rumen microorganisms will use structural components of the plant (cellulose and hemicellulose) as fermentable energy and, as a result, both the balance and the temporal release of N and energy yielding components can be out of phase.

Nitrogen and energy-use-efficiency in ruminant digestion to reduce greenhouse gas emissions caused by livestock agriculture and improve animal nutrition has been addressed using two approaches involving plant breeding techniques; (1) Matching the availability of energy to protein intake and (2) Reduction of protein degradability to synchronize with energy availability (Moorby *et al.*, 2008). This project focused on the second technique.

Greater understanding of the digestion of plant protein by ruminants has become an extremely important environmental objective in order to reduce the release of damaging N by-products by livestock agriculture. Kingston-Smith &Theodorou (2000), addressed the traditional views of the time and also provided further insight and suggested hypotheses of other factors likely to affect protein conversion by ingested forage and the outcomes on the environment. The authors explained that breakdown of ingested forage in the rumen was traditionally thought to be a process mediated by the large and diverse rumen micro-organism community involved in pre-gastric fermentation. It was also known that fresh living forage grasses ingested by grazing ruminant animals are mostly still intact when entering into the rumen, but the idea of plant-mediated proteolysis was over-looked because research into livestock animal feed was mainly carried out with conserved forages such as ground feed, hay and silage as opposed to fresh forage grasses with live cells intact (Kingston-Smith &Theodorou, 2000).

In response to the hostile rumen environment of high temperatures and anaerobic conditions (39°C, no O_2), it has been shown that living cells in freshly ingested forage release enzymes which could be part of a defence mechanism that may be considered as a programmed cell death (PCD). PCD is fundamental for the processes of seed germination and senescence (Pennell & Lamb, 1996). PCD also coincides with colonisation by the rumen micro-organisms (Kingston-Smith *et al.*, 2005), which can degrade ingested forage plant protein but their access is restricted by the structural compartmentation of plant cells by cellulose-layered plant cell walls which limits and delays activity by the microbial-generated proteases (Fig.1.4) (Kingston-Smith &Theodorou, 2000).

The forage grass species of choice for the UK and temperate grasslands where the climate permits are *Lolium perenne* (perennial ryegrass) and *Lolium multiflorum* (Italian ryegrass), the former used extensively in the UK, with *L. multiflorum* grown more widely in short-term leys in Continental Europe (Humphreys *et al.*, 2006). The ryegrasses are selected for their high yields and nutritious forage and used especially in intensive agricultural systems (although perennial ryegrass is also used extensively in improved permanent grassland pastures) subjected to temperate weather climatic conditions. However, they are not very well adapted to more environmentally extreme conditions. Their close relatives, the fescues (*Festuca spp.*), which in total number more than 500 species have evolved collectively with adaptations to diverse and extreme conditions and as a consequence have colonised widely throughout Europe and beyond (Humphreys *et al.*, 1997). Fescue species, although of lower forage quality than ryegrass, are also of agronomic importance in areas deemed unsuitable for ryegrass. The fescue species of major agricultural importance are meadow fescue (*Festuca pratensis*) chosen for its winter hardiness and grown primarily in northern Europe and tall fescue (*Festuca arundinacea*) grown more extensively in southern Europe or the USA for example, in areas

prone to heat and drought stress (Yamada *et al*., 2005; Ghesquière *et al.* 2010). In Europe the *Lolium/Festuca* complex comprises 175 species, only 8 of which are *Lolium* (Thomas *et al.*, 2003).
The genomes of *Lolium* and *Festuca* species are closely related and readily interchangeable. In combination, their genomes provide great genotypic and phenotypic diversity providing a wide range of trait expression. They are obligate and highly heterogeneous out-breeders and their potential for successful interspecific hybridization and gene exchange has led to avoidance of the genetic bottlenecks seen so often in domesticated crops that have been developed from a very narrow genetic base and have become highly inbred (Humphreys, 2005).
The increasing likelihood that UK grasslands will face more irregular and severe weather patterns and more persistent stress conditions has made improved resilience of *Lolium* a priority and provided fresh opportunities for the development and marketing of *Festulolium.* Care should be taken that the attributes of high production and highly digestible forage characteristic to *Lolium* should not be compromised when combined with *Festuca* species.

Forage grasses for the future need to combine good quality nutrition for the livestock agriculture industry and capabilities to withstand the effects of global warming as well as reducing environmental pollution caused by livestock agriculture (Humphreys *et al.*, 2006). One grass species that in combination with *Lolium* spp. has potential to help fulfil all these requirements is the species *Festuca arundincea var glaucescens* (Mediterranean fescue), a progenitor species of the well-known forage grass species, tall fescue (*Festuca arundinacea*) (Humphreys *et al.*, 1995). The adaptive ability of *F. glaucescens* to withstand extreme heat stress conditions in the field has been suggested to provide co-adaptive strategies for ingested living cells in the hostile conditions of the rumens of grazing animals and thereby reduce the rates of plant-mediated proteolysis (Shaw, 2006). This is because the temperature of the rumen is 39^0C which would be similar to the high temperatures encountered by *F. glaucescens* in its natural environment within Mediterranean grassland regions. Hybrids between *F. glaucescens* and *Lolium spp.* have the potential to match the availability of energy (as in the HSG's) to protein intake as well as reducing the rate of protein degradation to synchronize with energy availability in the rumen.

The overall aim of this project was to assess the potential to combine the forage production and quality of *Lolium spp.* with *F. glaucescens* traits especially those stress tolerance traits able to provide improved protein stability in ingested fodder within the rumen to improve livestock nutrient-use-efficiency and to subsequently lower their greenhouse gas emissions. The hypothesis that natural adaptations in *F. glaucescens* that protect plant proteins from high temperature stress may also protect protein from heat and other stresses in the rumen was tested. Shaw (2006), in her PhD thesis, tested the ability of *F. glaucescens* to withstand simulated rumen conditions alongside other *Lolium* and *Festuca* grass species) and showed that it took up to four times longer for the protein of *F.*

glaucescens to be broken down when compared with *L. perenne* when subjected to simulated and equivalent rumen conditions. This suggests a role for the incorporation of relevant genes from *F. glaucescens* in *Lolium* that could benefit the nutrition of livestock and lead to more efficient N conversion with increased livestock gains and reduced releases of harmful gaseous wastes. However, in the preliminary studies undertaken by Shaw (2006), when a limited number of targeted *F.glaucescens* genes were introgressed into *L. multiflorum* chromosomes, the *F. glaucescens* attributes in regard to protein stability decreased significantly questioning the efficacy of an introgression-breeding approach.

This prompted the current project which aimed to discover the optimal *Lolium/Festuca* genome dosage required to combine the forage quality attributes of *Lolium* with the protein protection mechanism present in *F. glaucescens.* Alternative genome numbers of both *L. multiflorum and L. perenne* were assessed in turn in combination with contrasting genome numbers of *F. glaucescens* in order to select overall the optimal *Lolium-Festuca* genome dosage required to ensure the most positive benefits. In addition to the assessment of variation in protein stability under simulated rumen conditions under various interspecific genome combinations, intraspecific variation both in *Lolium* and *Festuca* was also assessed including any intraspecific difference in *Lolium* that may have derived due to ploidy numbers (2x or 4x).

The introgression breeding approach enabled, for the first time, opportunities for the transfer of *F. glaucescens*-derived traits and genetic markers in *L. perenne* to be assessed. This employed a very similar introgression-mapping population to the one used by Shaw (2006). A nearly identical backcross breeding programme was used by Humphreys *et al.*, (2005), to transfer *F. glaucescens* genes for drought and heat tolerance into *L. multiflorum*. The final introgression line could withstand both drought and heat. All genotypes used in the introgression-mapping programme employed in the current work were used to determine whether the Backcross 2 (BC_2) introgression line had improved protein stability under rumen-simulated conditions compared with its parental genotypes.

Objectives for the research

(1) To determine optimal ryegrass and fescue genome complements required to maximise gene expression for protein stabilisation and to inform breeders of the strategies for their use in plant breeding.

(2) To test the hypothesis that natural adaptations in *F. glaucescens* to protect plant proteins can also protect protein in the rumen and so improve animal nutrition.

(3) To determine the efficacy for transfer of *F. glaucescens* genes into *L. perenne* through an introgression breeding approach.

3. Materials and methods

3.1. Plant materials and plant breeding

3.1.1 Stock plant material sources

Experimental plants were generally derived from established stock plants which were reproduced by cloning, except for those produced by a new backcross breeding program. Polycross progeny derived from F_1 *Lolium multiflorum* x *Festuca glaucescens* (LmFg 4x) and *Lolium perenne* x *Festuca glaucescens* (LpFg 4x) hybrid genotypes used in the current study for protein stability investigations were randomly selected from plants in the field grown as part of larger field trial not associated with this project at Gogerddan Campus, IBERS Aberystwyth University.

Plant materials used in this project

The plant materials used in this project are listed (Table 3.1) and show the totality genomes of the plants used for experiments described hereafter.

Table 3.1 Alternative genome dosage and composition used to assess protein stability under rumen-like conditions.

Forage grass species/species hybrid and gene dosage	Chromosome Number
F.arundinacea var glaucescens 4x (FgFgFgFg)	2n = 4x = 28
L.multiflorum 2x; 4x (LmLm);(LmLmLmLm)	2n = 2x =14; 2n = 4x = 28
L.perenne 2x; 4x (LpLp);(LpLpLpLp)	2n = 2x =14; 2n = 4x = 28
L.multiflorum x F.glaucencens F_1 4x (LmFgLmFg)	2n = 4x = 28
L.perenne x F.glaucescens F1 4x (LpFgLpFg)	2n = 4x = 28
Backcross 1; F_1 onto 2x *L.multiflorum* 3x (LmLmFg)	2n = 3x = 21
Backcross 1; F_1 onto 2x *L.perenne* 3x (LpLpFg)	2n = 3x = 21
Backcross 2; BC_1 onto 2x *L.multiflorum* 2x ($LmLm^{Fg}$) (Introgression lines)	2n = 2x = 14

A minimum of five distinct genotypes for each Fg, Lm, and Lp, parent group and for each Lm x Fg, or Lp x Fg hybrid group having contrasting genome dosage, were used to analyse the effect of genotype on protein stability. A complete list of plant project materials is shown (Table 3.2) including those used in the Lp x Fg backcross breeding program and for Lp x Fg molecular marker work.

Table 3.2 List of plant material including existing plants and those produced as part of the Lp x Fg breeding programme. Plants are given their cultivar name (cv) or accession number plus individual genotype reference number in parentheses.

Tetraploid parents (Whole genome) (2n = 4x = 28)			Diploid parents (Whole genome) (2n = 2x = 14)			
Festuca glaucescens	*Lolium multiflorum*	*Lolium perenne*	*L.multiflorum*	*L.perenne*	Mapping family diploids*	
Bn 354 (4)*	Bb 2264 (2) cv Roberta*	Ba 14075 (9)	Bb 2042 (12) cv AberComo*	Ba 14074 (33)	Ba 14087 (3)	Ba 14087 (25)
Bn 354 (8)	cv Gemini (2)	cv AberDell (5)	Bb 2205 (15) cv Atalja*	Ba 14087 (26)	Ba 14087 (4)	Ba 14074 (9)
Bn 354 (15)	cv Gemini (5)	cv AberDell (9)	Bb 2546 (2)	cv AberStar (1)*	Ba 14087 (5)	Ba 14074 (12)
Bn 354 (17)	cv Danergo (6)	cv Dunluce (5)	Bb 2546 (3)	cv AberStar (2)*	Ba 14087 (7)	cv AberFarrell (40)
Bn 354 (35)	cv Danergo (9)	cv Dunluce (6)	Bb 2546 (4)/ Bb 2546 (6)	cv AberStar (79)	Ba 14087 (18)	

L.multiflorum x F.glaucescens (Whole genome)		LmFg mapping family	*L.perenne x F.glaucescens* Mapping Family		
Tetraploid hybrids (2n = 4x = 28)	Triploid hybrids (2n = 3x = 21)	Parents, and hybrids (Whole genome)	Fg (4x) parents*	Lp4x parents*	LpFg F1 hybrids† (2n = 4x = 28)
P188/48 (1)†	P193/206 (3)	Fg 4x parent Bn 354 (4)*	Bn 354 (8)	cv AberDell (5)	P237/153 (10) cv AberDell (5) x Bn 354 (15)
P237/56 (8) cv Danergo (9) x Bn 354 (35)	P193/206 (4)^	Lm (4x) parent Bb 2264 (2) cv Roberta*	Bn 354 (15)	cv AberDell (5)	P237/154 (6) cv AberDell (5) x Bn 354 (8)
P237/119 (10) cv Gemini (2) x Bn 354 (8)	P193/206 (5)^	LmFg (4x) F1 hybrid P188/48 (1)†	Bn 354 (17)	cv Dunluce (5)	P237/164 (1) cv Dunluce (5) x Bn 354 (17)
P237/146 (4) cv Gemini (2) x Bn 354 (15)	P193/206 (10)	LmFg (3x) BC1 hybrids P193/206 (4)^ and 206 (5)^	Bn 354 (35)	cv Dunluce (6)	P237/164 (7) cv Dunluce (5) x Bn 354 (17)
P237/166 (8) cv Danergo (6) x Bn 354 (35)	P193/206 (16)	Introgression lines (BC2)‡ P194/208 (19) and P194/209 (28)			P237/180 (4) cv Dunluce (6) x Bn 354 (8)
P237/167 (10) cv Gemini (5) x Bn 354 (8)	P193/206 (20)				

LpFg (4x) F1 hybrids†	LpFg (4x) F1 polycross hybs†	LpFg BC1 hybs^ (2011) (2n = 3x = 21)		LpFg BC2 Introgression lines‡ (2012) Marker work only		
P237/153 (10)	95/3	P231/86 (1) 95/3 x cv AberStar(1)	P231/88 (1) 91/9 x cv AberStar (2)	P244 (1) (2) (3) (4) P231/87 (1) x Ba 14087 (7)	P244 (13) (14) P231/88 (5) x Ba 14087 (3)	P244 (21) (22) P231/88 (1) x Ba 14074 (12)
P237/154 (6)	91/10	P231/87 (1) 91/10 x cv AberStar (2)	P231/88 (5) 91/9 x cv AberStar (2)	P244 (5) (6) P231/97 (1) x cv AberFarrel (40)	P244 (15) (16) P231/97 (2) x Ba 14087 (25)	KEY: Mapping Family * Parent/s 2x/4x † F1 4x hybs ^BC1 3x hybs ‡Introgression line BC2
P237/164 (1)	91/9	P231/87 (2) 91/10 x cv AberStar (2)	P231/97 (1) 91/8 x cv AberStar (2)	P244 (7) (8) P231/88 (2) x Ba 14087 (18)	P244 (17) (18) P231/86 (1) x Ba 14087 (4)	
P237/180 (4)	91/8	P231/87 (3) 91/10 x cv AberStar (2)	P231/97 (2) 91/8 x cv AberStar(2)	P244 (9) (10) P231/87 (3) x cv AberFarrel (40)	P244 (18),(19) P231/88 (2) x Ba 14074 (12)	

The two *L.multiflorum* x *F.glaucescens* mapping families that produced the drought tolerant $LmLm^{Fg}$ (BC_2) introgression lines P194/208(19) and P194/209(28) were also used as part of the protein stability experiment. Both introgression lines have the same tetraploid (4x) and diploid (2x) parents until the BC_1 (3x) generation. For P194/208 (19) the LmFg BC_1 triploid (3x) parent was P193/206 (4) and the BC_2 LmLm (2x) diploid parent was Bb2042 (12) cvAbercomo. For P194/209(28) the LmFg BC_1 triploid parent was P193/206 (5) and the BC_2 LmLm (2x) diploid parent was Bb2070 (16) cv Trajan.

***Lolium perenne x Festuca glaucescens* backcross breeding programme**

Existing tetraploid F_1 Lp x Fg (2n=4x=28) hybrids (produced by Dr Mike Humphreys at Gogerddan campus, Aberystwyth University) were used as the initial stage in the backcross breeding programme and designed to transfer genetic material from Fg into *Lolium perenne*. The LpFg F_1 tetraploids (2n=4x=28) were backcrossed onto diploid Lp (2n=2x=14) to produce LpFg BC_1 triploids (2n=3x=21) which were in turn backcrossed onto diploid Lp (2n=2x=14) BC_2 introgression lines (2n=2x=14). Details of the genotypes used in the Lp x Fg marker-assisted introgression breeding programme were provided in Table 3.2. The initial LpFg (4x) F_1 hybrids (provided from the field trial by Dr Mike Humphreys) were backcrossed as male pollinators onto diploid Lp (2x) varieties to produce the triploid LpFg (3x) BC_1 hybrid plants and these were subsequently crossed again both as female and male parents onto diploid (2x) Lp varieties to produce the final BC_2 generation including possible introgression lines $LpLp^{Fg}$. The Lp x Fg backcross breeding programme (Table 3.3.) shows the crossing process of parents and hybrids involved in the production of the LpFg BC_2 introgression lines. Columns A + B contain the tetraploid Lp and Fg (4x) parents used to produce the LpFg tetraploid (4x) F_1 hybrids (Column C) which were then poly-crossed in a glasshouse to produce the LpFg (4x) seed used to produce plants employed in the field experiment (Column D). Four of the LpFg tetraploid (4x) polycross hybrids were selected at random from the field and backcrossed with LpLp diploid (2x) parents (Column E) to produce the LpLpFg triploid (3x) BC_1 hybrids (Column F). The LpFg triploid (3x) hybrids were then backcrossed onto LpLp diploid (2x) parents (Column G) to produce the putative $LpLp^{Fg}$ BC_2 (2x) introgression lines (Column H).

Table 3.3 Crossing programme to produce the $LpLp^{Fg}$ BC_2 generation including the tetraploid (4x), diploid (2x) and triploid (3x) parents.

A	B	C (A x B)	D (A x B)	E	F (D x E)	G	H (F x G)
L.perenne **(4x) parents**	***F.glaucescens*** **(4x) parents**	**LpFgLpFg (4x) hybrids**	**Polycross (4x) hybrids**	***L.perenne*** **(2x) parents**	**LpLpFg (3x) hybrids**	***L.perenne*** **(2x) parents**	**$LpLp^{(Fg)}$ (2x) intro. Lines**

Plants used in the Lp x Fg crossing programme had undergone preparatory vernalisation under prolonged exposures to low temperature and short days using natural conditions either in the field or in a non-heated glasshouse to ensure they flowered effectively. Plants were selected to ensure flowering phenology was synchronised between genotypes used together as intended male and female parental genotypes. Plants selected as females were maintained in 6" pots and 3 to 4 near-mature spikes of similar developmental stage were bagged together in a clear pollen-proof bag just prior to the start of anthesis. Any existing or subsequent heads undergoing anthesis were removed to encourage further vegetative growth, and to reduce opportunities for cross-pollination and entry of "foreign pollen" into the pollination bags. The selected spikes to be used as females were bound loosely together with green tie wire and also loosely to a supporting cane. A long circa 4" wide strip of cotton wool was then wrapped around the plant stems and tied again with green a green tie wire to prevent accidental entry of external pollen. A tall pollination bag was cut to size to cover the spikes from top to bottom of the cotton wool and tied onto the cane with more green wire. The "crossing unit" (Fig 3.3) was further attached to the cane with a paper clip and the corners of the bag folded and a label giving the plant number/names, bagging and crossing dates. Inflorescences of LpFg plants (4x) from the field near to anthesis were chosen as pollinators and removed ready for immediate use to produce the BC_1. Their precise identity was noted so that they could later be transplanted and maintained in the glasshouse. Four or five flowering heads were used as pollinators in each "crossing unit" all taken from the same genotype chosen as the male parent and were introduced simultaneously into a pollination bag at the stage when the oldest florets of the female inflorescences had begun to open and deemed receptive to pollen. The pollinators for each "crossing unit" were placed together into tubes filled with water attached to the cane which allowed them to persist and continue to pollinate over 2-3 days providing repeated opportunities for pollination to occur. The pollinators were removed from the "crossing units" once they had completed pollination to ensure that no seed in formation that they might accidentally carry could be deposited in the pollination bag.

Embryo rescue (Humphreys, 1989) was undertaken 16 days after pollination and prior to undertaking embryo rescue. Caryopses were surface sterilised in 7% sodium hypochlorite solution and washed 3x in sterile water to reduce incidents of fungal or bacterial contamination. Sterile conditions were employed throughout during embryo excision. Embryos were placed into sterilin tubes containing modified Gamborg and Miller B5 agar medium i.e. without 2, 4-D (growth hormone) or kinetin, and included 30% sucrose/L (Gamborg *et al.,* 1968) (Modified). Tubes with embryos were then placed in a dark incubator at 20°C until young plumules and radicles developed. They were then transferred to a lit growth room at 25°C, constant light for 2-3 weeks until young plantlets had developed and established successfully with developed young green leaves and root systems. Prior to transfer to soil, care was taken to remove agar from root surfaces to reduce opportunities for pathogen attack. The young plantlets were transferred to multi-trays filled with seedling compost in a glasshouse and

placed in a moist germination chamber until fully established. After a period of three months the young plantlets were transferred into 5" pots in standard potting compost and kept well watered and trimmed periodically to encourage tiller production and maintained to maturity in a frost-free glasshouse. Plants derived following embryo-rescue were tested for their ploidy (chromosome count) using flow cytometry. These numbered in total 15 plants produced from the following crosses:

P231/86 [LpFg 95/3 (4x) x Lp cv AberStar 1 (2x)] = 1 plant
P231/87 [LpFg 91/10 (4x) x Lp cv AberStar 2 (2x)] = 6 plants
P231/88 [LpFg 91/9 (4x) x Lp cv AberStar 2 (2x)] = 6 plants
P231/97 [LpFg 91/8 (4x) x Lp cv AberStar 2 (2x)] = 2 plants

All 15 LpFg BC_1 plants were compared with three relevant controls of known genome size and of different ploidy i.e. a known tetraploid, triploid and diploid genotype: *F. glaucescens* (2n=4x=28), *L. multiflorum x F. glaucescens* (2n=3x=21) and *Lolium perenne* (2n=2x=14). Genome ploidy levels were determined using a Partec PA CyFlow® Ploidy Analyser and the analysis was carried out in accordance with standard IBERS protocol. Two to three leaves were cut from each plant genotype into 1-2 cm lengths. Based on the flow cytometer readings for the known standards i.e. the 2x, 3x, and 4x genotype controls, chromosome number (± 1 chromosome) of the 15 BC_1 plants was determined using three leaf samples/genotype placed in a Petri dish. One millilitre of Buffer A1 (5 ml Tween 20, 10.5 g citric acid monohydrate/1L deionised water) was pipetted onto each sample in turn and the grass leaf blades finely chopped with a sharp razor blade which was wiped clean between samples. Another 1 ml of Buffer A was added to the Petri dishes followed by 0.5 ml Buffer B1 (50 g Na_2HPO_4 + 2 mg DAPI/L deionised water) and mixed thoroughly. The grass mixture was pipetted through a 30µm filter into a vial and left to stand for 1 minute after which time vials were inserted into the flow cytometer and the peak readings noted. LpFg BC_1 hybrids confirmed as triploid plants by flow cytometry were back-crossed onto diploid Lp (2x) plants to produce the $LpLp^{Fg}$ BC_2 generation. The backcross breeding programme for the BC_2 was carried out as described for the BC_1 (S2.3.2) but embryo rescue was not used to generate the BC_2 generation. The BC_1 LpFg plants were used in reciprocal as both male and female parents in crosses with Lp diploid (2x) cultivars. Lp x Fg BC_2 seed produced were incubated on a Copenhagen water incubator (or Jacobsen apparatus), (Kamra, 1972) at 25°C as in Morgan, (1975). Incubated seed that germinated successfully were removed from the incubation tank after 10 days and planted into 3 seedling trays filled with seedling compost. The seedlings were maintained at 15°C for 8 h in daylight and 16 h at 10°C at night and watered daily. After 8 weeks establishment the plants were transferred into 5" pots with standard potting compost, watered daily and trimmed as necessary to encourage tiller production.

3.2. Plant maintenance and phenotyping

3.2.1. Growth conditions

Clonal stock plants were maintained in pots of compost (Levingtons multipurpose) in a poly tunnel, under natural illumination and watered regularly. The plant materials used in this project were all maintained under optimal growth conditions in a frost-free glasshouse in potting compost in 6" pots at Gogerddan Campus, IBERS, Aberystwyth University in readiness for experimental use. They were cut, fertilised, and re-potted as required throughout the experiments to encourage maintenance of optimal vegetative growth and equivalent ontogeny. Any interactions that could arise such as with flowering physiology or differences in plant age were avoided or minimised. Prior to experimental use, plants were transferred to a controlled environment cabinet (CE) sourced from Gallenkamp PLC www.gallenkamp.co.uk and Skye Instruments Lighting www.skyeinstruments.com and maintained under identical growth conditions for a minimum of 6 weeks at all times in order to further assist achievement of a consistent growth habit and pre-treatment.

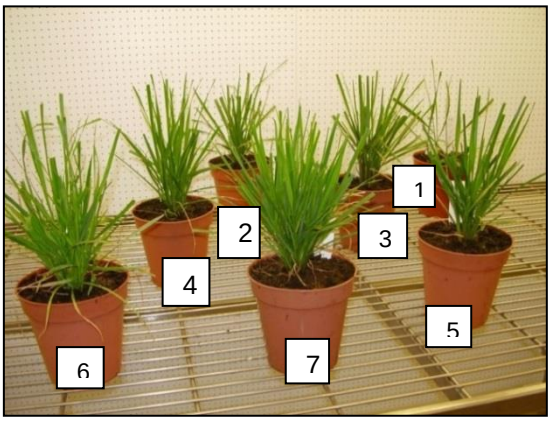
Plant maintenance included watering once daily in trays and feeding approximately once every 6 weeks with "Miracle-Gro" all-purpose soluble plant food (Scotts Miracle-gro UK) at the recommended concentration. The environmental parameters in the CE facility were equivalent to typical British summer conditions: Daylight (18 hours at 600 µmol.$m^{-2}s^{-1}$ illumination and at 22°C /72% humidity) and night time (6 hours darkness at 14°C/ 72% humidity).The plants were otherwise maintained as in the frost-free glasshouse. The plant material for use in phenotype and molecular characterisation was grown and maintained in a frost-free glass house as described for those used to assess protein stability, except that they were grown in 5" pots kept on three trestle tables and plants were rotated between trestles to ensure equal exposure to sunlight, ambient lighting, windows and temperature gradients. Matured plants were used after five months in the frost-free glasshouse for the *Festuca*-specific genetic marker study and phenotype characterisation after 6 months.

Fg 4x parent/ LmFg 4x hybrid/ Lm 4x parent

<u>Left:</u>
***Festuca glaucescens* tetraploid parent (2n=4x=28)**
<u>Centre:</u>
***L.multiflorum* x *F.glaucescens* tetraploid hybrid (2n=4x=28)**
<u>Right:</u>
***L.multiflorum* tetraploid parent (2n=4x=28)**

Fg 4x parent/ LpFg 4x hybrid/ Lp 4x parent

Left:
***Festuca glaucescens* tetraploid parent (2n=4x=28)**
Centre:
***L.perenne* x *F.glaucescens* tetraploid hybrid (2n=4x=28)**
Right:
***L.perenne* tetraploid parent (2n=4x=28)**



***L.multiflorum* x *F.glaucescens* mapping family**

1. Fg 4x parent (2n=4x=28)
2. Lm 4x parent (2n=4x=28)
3. LmFg 4x hybrid (2n=4x=28)
4. Lm 2x BC_1 parent (2n=2x=14)
5. LmFg 3x BC_1 hybrid (2n=3x=21)
6. Lm 2x BC_2 parent (2n=2x=14)
7. $LmLm^{Fg}$ Introgression line (2n=2x=14)

Figure 3.1 Tetraploid hybrids (top and middle) and Lm x Fg mapping family (bottom) used in protein stability experiment in CE facility at Aberystwyth University.

Phenotype characterisations

Physical trait measurements for phenotype characterisation of the original project plants used for the protein stability experiment and also the newly produced LpFg triploid (3x) BC_1 plants were carried out in the CE growth room where they were being maintained (S2.2.1). Measurements carried out were growth habit i.e. prostrate to erect, tiller count, leaf texture i.e. soft to firm and leaf width where the widths of five mature non-senescent leaves were measured (mm) (Table 2.4) and were ranked for principal component analysis (PCA) using GenStat version 14.2 (VSN International). The newly produced $LpLp^{Fg}$ BC_2 introgression lines which were maintained in the frost-free glasshouse were scored for physical traits as the rest of the plant material but also had their root volumes estimated and regrowth measured concurrently. Root volume was estimated by removing the plants from pots, shaking off the soil and visually estimating % roots occupying the pot from 2 cm below the leaf

stems. For regrowth measurements, leaves were cut back to 5 cm and after 3 weeks were cut back again to 5 cm and fresh weight measured (g).

Table 3.4 Ranked physical traits for project plant material

Habit = rank	Tiller Count = rank	Leaf Texture = rank	Leaf Width (mm) = rank	
Prostrate = 1	<10 =1	Soft = 1	2 to 3 = 1	5 to 6 = 7
Between = 2	11 to 20 =2	Soft – Med = 2	2 to 4 = 2	5 to 7 = 8
Erect = 3	21 to 30 =3	Med = 3	3 to 4 = 3	5 to 8 = 9
	31 to 50 =4	Med - Firm = 4	3 to 5 = 4	6 to 8 = 10
	>50 =5	Firm = 5	4 to 5 = 5	
		Very Firm = 6	5 = 6	

Rankings for the physical traits were established by observing for the natural range of the physical trait within the population and dividing that range into ranks.

3.3. Molecular marker selection

3.3.1 Screening for Festuca-specific alleles

Detailed and well-characterised *Lolium* and *Festuca* genetic linkage maps e.g. (Turner *et al*., 2008; King *et al.*, 2013) were used to select simple sequence repeat (SSR) markers that co-located with areas of high genome recombination and provide more genome dispersal. Choices of such SSR markers (from the *L.perenne* linkage group) based on their allelic polymorphism and amplification properties were made by Kirsten Skøt and operatives of IBERS ABI System. This culminated in four SSR markers being found suitable for use with Lp x Fg genotypes for Linkage Groups (LG) 1, 2, 4, and 5, and five SSR markers for use on LG3 and LG6. However LG7 was deficient in SSR markers with only one selected finally (Table 3.5).

Table 3.5 SSR markers from *L.perenne* linkage group selected for probing samples.

Chromosome	1	2	3	4	5	6	7	
L.perenne SSR markers	rv0913	M4-136	rv0863	rv0382	rv0250	rv1423	hd3agt	
	rv0244	rv1239	rv1332	rv0992	rv0757	rv1208		
	rv1391	rv0706	rv0154	rv0061	rv0260	rv1266		
	rv0327	rv0188	25ca1	rv1317	rv0340	rv0196		
			rv1046			rv0739		

In order to detect *Festuca* – specific SSR markers, initial screens were undertaken only on the Lp (4x), Lp (2x), and Fg (4x) parental genotypes used in generating the $LpLp^{Fg}$ introgression line mapping family. This ensured that SSR alleles selected were present only in the *F.glaucescens* tetraploid (4x) parents and were absent both in the *Lolium perenne* tetraploid (4x) and diploid (2x)

parents. Once *Festuca*-specific SSR markers were identified (Table 3.5.) they were then used to screen the LpFg F_1 tetraploid (4x) hybrids. The *Festuca*-specific SSR markers located in the F_1 were subsequently used to screen the LpFg BC_1 triploid (3x) hybrids. Similarly, those SSR markers still detected amongst the BC_1 were then used to screen the $LpLp^{Fg}$ BC_2 introgression lines in order to determine overall the Fg transmission across the generations.

The method used for marker screening was carried out as described in Turner *et al.,* (2008). The *L.perenne* tetraploid (4x) and diploid (2x) parents of the Lp x Fg mapping family (which produced the LpFg triploid (3x) hybrids and $LpLp^{Fg}$ introgression lines) were the first to be prepared for DNA extraction in preparation for *Festuca*-specific SSR marker screening. Approximately 50 mg of fresh mature leaves from each plant was cut into 1 cm lengths and placed into DNA collection tubes kept on ice and their position in the micro collection tube box recorded. The samples were then processed by the IBERS ABI service for DNA extraction and probed for the selected SSR markers. These DNA sequences were then genetically analysed using an Invitrogen ABI 3730 high through-put DNA analyser. This analysis produces DNA sequences labelled with four fluorescent dyes and an internal size standard dye and analysed using GeneMapper® V3.7 software (Fig 3.2).

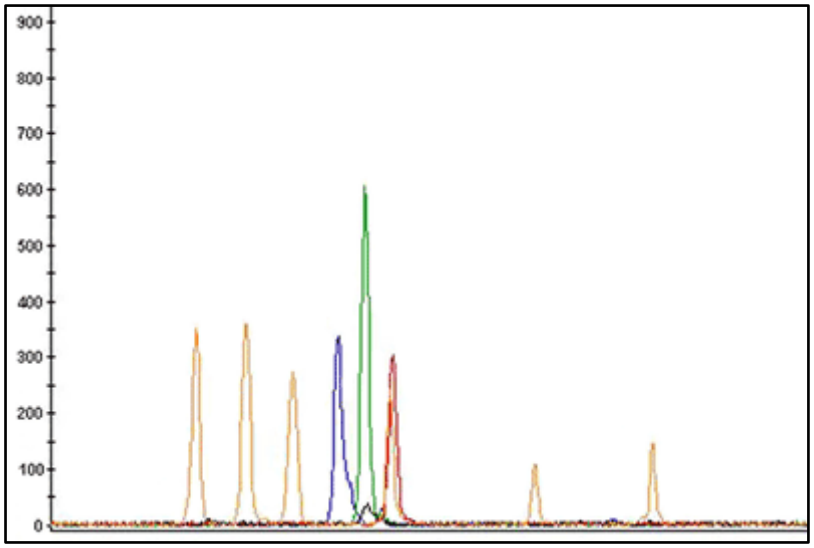


Figure 3.2 Image of colour coded DNA peaks using Gene Mapper software. The different colour dyes represent alternative genetic markers www.med.upenn.edu [Accessed on-line, 05 February 2014].

3.3.1. Biotechnologies for nutrient stability under rumen conditions

In-vitro protocols

In preliminary experiments to establish experimental protocols whole plants (maintained in a frost-free glasshouse were brought to the lab for sampling and experimental use for protein detection (Lowry *et al.,* 1951). For main experimentation plants were being maintained in the controlled

environment (CE) facility and protein determined by Bradford micro protein assay (Bradford 1976). On day of experimentation 0.5 g of fresh (non-senescent) leaf material from each plant was weighed and 0.1 g per time-point placed into individual Hungate tubes (www.sciquip.co.uk). Prior to incubation of the grass samples, warmed anaerobic medium (Van Soest, 1963) (Modified) was prepared and included**:** Micro-minerals: $CaCl_2H_2O$ (13.2g), $MnCl_24H_2O$ (10 g), $CoCl_2$ $6H_2$ (1 g) and $FeCl_2$ $6H_2O$ (8 g) made up to 100 ml and added at 0.1 ml/litre, Solution 'A' $NaHCO_3$ (39.253 g in 1L H_2O), Solution 'B' macrominerals (per litre H_2O): Na_2HPO_4 (9.45g), KH_2PO_4 (6.2g), $MgSO_4$ $7H_2O$ (0.6g) and Solution 'C' (reducing agent): DM water (95 ml), Cysteine HCl 0.625 and 1M NaOH (4 ml) and resazurin (2 ml). Working solution: DM water 1.1L, Solution 'A' 440 ml, Solution 'B' 440 ml, Solution 'C' 80 ml, resazurin 2ml and micro-minerals 0.2 ml. Method: Solutions 'A' and 'B' were brought to the boil and water, reducing agent (solution 'C') micro-minerals and resazurin were added to the gently boiling solution (buffer) and a stream of CO_2 piped into it. Once the buffer was reduced (turned from blue to pink) 5 ml of the buffer (kept warm) was pipetted into each of the Hungate tubes containing the freshly cut grass samples. They were then quickly sparged under a stream of CO_2 and capped , then placed into a warm water bath (39°C) with lid closed to block out the light (except for 0 h samples which had warmed Van Soest buffer added only and not sparged nor incubated). Incubated samples were then removed at the appropriate time-points and leaf residue was recovered following incubation, rinsed with Dm water in a Büchner apparatus and flash-frozen (in 2 ml sample tubes) and stored at -80º C in preparation for protein extraction.

3.3.2 Experimental design and sample processing for protein extraction

Due to the large number of samples being processed, incubations were carried out on subsequent days i.e. one rep of a group of samples was done per day and at four time-points: 0 h, 2 h, 6 h and 24 h. For the early experiments to establish protocols the Micro-Lowry protein assay method (Lowry *et al.*, 1951) was used. In preparation for milling, frozen incubated plant samples had Parafilm® placed over the microfuge tubes, pierced with a clean needle then placed in a desiccator over a week-end. There are two solutions used in the Micro-Lowry method for extraction of protein from the samples and they are known as the LE/LiDS. Solution1: LE is the Lithium Extraction buffer which extracts the protein from the intact plant cells. LE contains: orthophosphoric acid (1.7 ml), monoiodacetate (105 mg), glycerol (25g) 2-mercaptoethanol (5 ml) PMSF (85 mg) (dissolved in 5 ml methanol). Solution 1 was adjusted to pH 7.2 in a final volume of 500 ml. Solution 2: **LiDs** is Lithium dodecylsulphate and was added at 20g in 100 ml DM water is a detergent which breaks down the cell wall membranes to allow the release of proteins.

On the day of protein extraction, L.E. LiDS extraction buffer was made up and kept cold. The desiccated samples were removed from the desiccator and the Parafilm was removed from their tubes. Tungsten beads were added to the tubes containing the desiccated samples which were then snap-

frozen in liquid N and ground in a Retsch mill MM 300 at a frequency setting of 30s for 1 minute x 2 whilst being rotated on each arm of the mill.

Cold L.E buffer was added at 0.5 ml (Modified to include protease inhibitor cocktail PMSF). PMSF (phenylmethanesulfonylfluoride) (Sigma-Aldrich Co.LLC) was used to inhibit serine and cysteine proteases which may become active in the samples whilst they are being processed. The samples were ground again (as previously described in preparation for protein extraction) and were boiled for 5 minutes in a water bath and then centrifuged at 13,000g_{av} at room temperature for 10 minutes. The supernatant from the samples was retained and pellets discarded and tungsten beads collected. The supernatant from each sample (0.2 ml) was placed into a clean tube with 0.2 ml of 20% TCA, 0.4% PTA added to them and then left to precipitate at room temperature overnight. The samples were centrifuged again at 13,000g_{av} for 10 minutes at 20°C and supernatant discarded. The remaining pellets had 0.2 ml of 10% TCA and 0.2% PTA (1 x TCA/PTA) added to remove traces of mercaptoethanol and LiDS. Samples were centrifuged again at 13,000g_{av} at 20°C for 5 minutes and supernatant discarded. The pellets were then dried in a desiccator overnight for 24 hours and next day and were re-suspended in 0.1 ml of NaOH and after 1 hour were placed on a shaker. Sample pellets that were not fully re-suspended by shaking were then placed into a sonicator and remaining unsuspended samples following sonification were dissected using a sterile needle. Ultimately the sample supernatant was carefully pipetted out from the micro-centrifuge tube to avoid sucking up pellet fragments and was assayed for protein content according to the Micro-Lowry protein assay method.

For established protocols the Bradford micro protein assay method (Bradford 1976) was used. HEPES buffer (0.1 M) was calibrated to pH 7.5, along with 1 mM EDTA, Triton-X 100 made up to 100 ml in advance and kept in a fridge at 4°C. On day of protein extraction, protease inhibitors (PMFS, Sigma-Aldrich Co, Ltd, Dorset, England) (stored at - 20°C and thawed) and 2 mM DTT (0.1%) were added and mixed well with the HEPES buffer using a magnetic stirrer and kept on ice. The standard Bradford's micro-protein extraction method was modified because of some sample material being lost through milling. In the original method, 1.5 ml of HEPES extraction buffer was added to the complete dried and milled sample (from 0.1 g fresh weight). Partially milled samples were weighed individually in a tared plastic sample boat (after removal of tungsten beads) and then carefully scraped into a mortar cooled with liquid N. Cold HEPES extraction buffer was then added to the sample at a concentration of 40 µl per g dry weight and then hand ground to produce a homogenate in a cooled pestle and mortar. Following hand grinding a 20 µl aliquot (increased to 40 µl later on) from each sample homogenate was pipetted out for chlorophyll determination. The remaining homogenate was quickly and carefully scraped out from the mortar using the pestle into a labelled 2 ml micro-centrifuge tube and flash-frozen in liquid N and stored at - 80°C until determination of protein content.

3.4. **Biochemical analysis**

3.4.1 Micro-Lowry protein assays

Each incubated sample was placed into a well of a micro-titre plate and water added for a final volume of 150 µl. Lowry reagent C (Lowry *et al.*, 1951) was added at 50 µl and Folin-Coicalteu's reagent added at 50 µl. The samples (well mixed with the reagents) were incubated for 20 minutes before being assayed. The micro-titre plate containing the mixed samples was placed in the plate reader (BioTek model Elx808) and read at 750 nm absorbance and blanked against a 0.1 M NaOH control. The incubated samples were assayed against BSA protein standards at 1 mg/ml concentration. The protein assay was repeated at a range of sample concentrations (1 µl -20 µl) until they were within the range of the BSA standard curve (0 µg - 30 µg).

3.4.2 Bradford protein micro-assays

Each incubated sample was placed into a well of a micro-titre plate and water added to a final volume of 200 µl. A BSA standard curve using Bradford's concentrated dye reagent from a Bio-Rad Protein Assay kit (Bio-Rad UK Ltd., Hemel Hempstead UK) was produced before measuring the protein content of the samples. The initial BSA standard assay was carried out using a 1 mg/ ml concentration of BSA (working range 0 µg – 20 µg) but was diluted down to 0.1 mg/ml concentration to achieve the working range for the samples (0 µg - 5 µg). DM water was added to the BSA solution make up 160 µl and then 40 µl of Bradford's undiluted dye concentrate was added to make a total volume of 200 µl and well mixed before being assayed and any bubbles caused by the mixing dispersed using a clean hypodermic needle. The standards were then read at absorbance 595 nm (A595) and repeated at alternative concentrations until a standard curve was achieved. Protein concentration in samples was calculated by being assayed (in aliquots 1 µl – 20 µl) against the BSA standard. Sampling for SDS-Page was carried out at this point.

Chlorophyll determination

Samples for chlorophyll determination were extracted from the sample homogenates produced by hand grinding for protein determination (as described in S2.4.4) and using a cut pipette tip, 20 µl of homogenate sample was pipetted into a labelled 1.5 ml micro-centrifuge tube and 980 µl of 80% (v/v) acetone (and later a 40 µl homogenate/960 µl of 80% (v/v) acetone ratio) added to make up a total volume of 1 ml. The tube was inverted once to mix the sample plus acetone solution, placed on ice in the dark or stored at -20 °C. For chlorophyll determination, samples were removed from the minus 20°C freezer and thawed on ice and then gently agitated using a vortex mixer before being centrifuged at 13,000g_{av} for 10 minutes. Samples were then read at 663 nm and 645 nm (Pharmacia Ultrospec

4000: Amersham plc. Little Chalfont, UK). To determine the chlorophyll content the following calculation was performed: (Arnon, 1949).

20.2 x (A645) + 8.02 x (A663) = mg/l chlorophyll

Corrections for dilutions: mg/l x 1/1000 x 1000/100 = mg chlorophyll/ml

3.5. **Protein electrophoresis**

3.5.1 Separation of proteins by SDS-PAGE

Polypeptide composition of protein extracts was analysed by SDS-PAGE. Equal volumes of extract from each of three biological replicates were combined and an equal volume of x2 Laemmli buffer (Laemmli 1970) added. Samples were boiled for 2 minutes and stored at - 20°C if not used immediately. Samples were run on 4 – 20 % mini-PROTEAN® TGX™ pre-cast gels (BioRad UK Ltd., Hemel Hempstead UK) (Fig. 2.8) with either 10 or 15 wells alongside a molecular weight ladder (Pre-stained broad range markers Precision Plus ™ Dual color standards; BioRad UK Ltd, Hemel Hempstead UK.) added at 5 µl to allow for molecular weight comparison of the separated polypeptides.

For high through-put analysis a maximum of four gel cassettes with the pre-cast gels were placed into the Bio-Rad mini-PROTEAN® Tetra cell (tank) and samples loaded into the gel wells. Pre-made electrode buffer (10x made up to 1l) working solution 1 x 25 mM Tris,192 mM glycine 0.1% (w/v) pH 8.3 SDS buffer (BioRad UK Ltd, Hemel Hempstead UK) was added to the Tetra cell and filled according to the amount of gels being run. The Gels were run at 4°C for 45 - 50 minutes at 200V constant voltage according to the manufacturer's instructions (BioRad UK Ltd, Hemel Hempstead UK).

Gels were stained with Coomassie Blue stain (1 g Coomassie Brilliant Blue G-250, 90 ml methanol, 90 ml deionised water, 20 ml glacial acetic acid) for 1 hour on a shaker. Gels were then de-stained using de-staining solution (500 ml methanol, 140 ml glacial acetic acid 1360 ml deionised water) to show blue-stained polypeptide bands. The stained gels were scanned (GS 800 calibrated imaging densitometer (BioRad UK Ltd, Hemel Hempstead UK) and scanned images saved for peptide band analysis. Where indicated, peptide bands of interest were excised from stained gels with a new razor blade previously cleaned in 70% ethanol and placed in a 1.5 ml tube containing 1% acetic acid. Samples were sequenced by Tandem Mass Spectrophotometry (Professor Ian Brewis, Cardiff University).

3.5.2 Electrophoretic transfer of proteins and detection of HSP polypeptides

Specific immunological detection of proteins was performed by western blotting. Polypeptides were separated by SDS-PAGE gels as previously described but not stained with Coomassie blue stain.

Following electrophoresis the unstained gels were removed from their cassettes as per manufacturer's instructions (BioRad UK Ltd, Hemel Hempstead UK), and protein bands were transferred onto pre-cut nitrocellulose transfer blotting packs and placed into the TransBlot® Turbo™ transfer system for 7 minutes (BioRad UK Ltd, Hemel Hempstead UK). The blots were removed from the TransBlot® Turbo™ transfer system and placed into clean clear plastic square dishes where Ponceau S red Bio-Reagent ready-to-use stain (Sigma-Aldrich Co Ltd, Dorset England) was poured onto the blotted filters to confirm protein transfer.

Alternatively where higher sensitivity was required filters were rinsed with deionised water and Amido black stain (0.1 % Amido black, 10 % acetic acid, 25 % Isopropanol) was poured onto the filters until protein bands appeared. Filters were then de-stained (20 % methanol, 7.5 % acetic acid) three times in 15 minutes. Blots were rinsed in TBST (Tris-Buffered Saline and Tween 20) x 1 rinse buffer and scanned (GS 800 calibrated imaging densitometer: BioRad UK Ltd, Hemel Hempstead UK). Following scanning, the filters were then blocked overnight in TBST-B blocking buffer (1.5 mg BSA in 50 ml TBS-T x 1). Next day the filters were transferred into fresh transparent square dishes with 20 ml HSP–70 antiserum solution (Rabbit anti-HSP-70 IgG; Calbiochem Ltd, California, USA) at a 1:25,000 dilution in TBST-B for 4 h at room temperature on an orbital shaker. After 4 hours the HSP antiserum solution was removed and filters rinsed in TBST x 1 three times for 15 minutes each before being placed into the second antiserum solution (Swine anti-rabbit IgG horseradish peroxidase conjugate at 1:2,000 dilution; Dako, Glostrop, Denmark) for 1 h and then washed again in TBST x 1 three times for 15 minutes each. Bands of cross-reacting polypeptides were identified by chemiluminescence (ECL: Amersham Biosciences, Buckinghamshire, UK). ECL reagents were mixed in equal parts and 5 ml of mixture applied to each 10 x 10 cm filter for 1 minute. The filters were then lightly blotted with filter paper and wrapped in cling film without creasing or air bubbles and placed in a dark cassette. Under Safelite (in a dark room) light-sensitive film (Hyperfilm™ ECL, Amersham Biosciences, Buckinghamshire UK) was placed on top of the cling filmed blot filters with the top right hand corner bent. Individual films were developed for 30s and at intervals of 1, 2, 5, 10 and 15 minutes. The developed films were scanned as grey film on the GS 800 calibrated imaging densitometer (BioRad UK Ltd, Hemel Hempstead UK) and stored for further analysis.

3.6. **Statistical methods**

Apart from the protein degradation analyses (see below) all data was analysed according to standard procedures with the menu-driven options within Genstat 14.2 for Windows (VNS International Ltd. (2011) and also Microsoft Excel where appropriate.

Measures of protein degradation

Half-life curves for protein stability. The protein degradation time courses were fitted with exponential decay curves of the form:

$$(1) \qquad c = br^t$$

Where: c is protein content mg g^{-1} dry weight

t is time from start of incubation, hours

b is a fitted parameter describing protein content at time zero

r is a fitted parameter describing the rapidity of the decay of protein

This equation was re-arranged to allow calculation of the time taken for protein to decay to half its value at time 0. ($t^{1/2}$)

$$(2) \qquad t^{1/2} = \frac{log_e 0.5}{log_e r}$$

Non-linear curve fitting was performed using a Maximum Likelihood Program (Ross, 1987) and used parallel curve analysis (Ross, 1990) to determine significant differences between fitted curves, and to estimate standard errors of fitted parameters and $t^{1/2}$.

4.0 Results

4.1 Transfer and expression of *Festuca arundinacea var glaucescens* traits to hybrids with *Lolium*

4.1.1Production of Backcross 1 (BC_1) from *Lolium perenne* x *Festuca glaucescens* F_1 hybrids

F_1 LpFg (4x) hybrids as male parents were backcrossed onto diploid Lp cv AberStar to produce 15 Backcross 1 (BC_1) progeny (Table 4.1). Embryo rescue was required as a precursor to their establishment as described in Chapter 3. The 15 new BC_1 (3x) plantlets produced by the LpFg (4x) x Lp (2x) crosses were analysed for fecundity and were found to have an average seed set of 11.25% (Table 4.1.).

Table 4.1 Percentage seed set and genotypes recovered by embryo-rescue in a BC_1 plant population produced from *L. perenne* (Lp) (2x) (male) x Lp x Fg F_1 (2n=4x=28) (Female).

Female parent *L. perenne* (2x)	Male parent (F_1) Lp x Fg (4x)	BC_1 Lp x Fg (3x) Crossing Code No.	Seed set (% of florets pollinated) prior to embryo rescue	No. of BC_1 plants established
AberStar (1)	LpFg 95/3	P231/86	10	1
AberStar (2)	LpFg 91/10	P231/87	10	6
AberStar (2)	LpFg 91/9	P231/88	15	6
AberStar (2)	LpFg 91/8	P231/97	10	2
			Total BC_1 plants	15

The 15 LpFg BC_1 putative triploids were screened for their ploidy (chromosome count) to confirm that they were all triploids (3x) and not diploids (2x) or tetraploids (4x) when compared against known genetically equivalent diploid, triploid and tetraploid grass genotypes screened as controls. Of the 15 LpFg BC_1 genotypes that were tested for ploidy number using a flow cytometer, nine (60%) were shown to have triploid 2n=3x=21 (±1) genome constitutions. One of the plants was a diploid and presumably a result of

Lp self-pollination whist the remainder were aneuploids, likely to be either deficient in Fg chromosomes (i.e < 1 Fg genome chromosome complement if <3x) or containing duplicate Fg chromosomes (i.e. >1 Fg genome chromosome complement with additional Fg chromosomes if >3x).

Table 4.2 Flow cytometry DNA readings of *L. perenne x F. glaucescens* BC_1 triploids (3x) compared against, *L. perenne* diploid (2x), known Lm x Fg triploid (3x) and *F .glaucescens* tetraploid (4x) controls.

Species Controls	Peak	Approx. Ploidy (Chr/no.)
LmLmFg BC1 - P193/206 (16)	83	3x
F.glaucescens - Acc. Bn354(35)	103	4x
L..perenne - cv Liprior	55	2x
LpLpFg BC1	**Peak**	**Approx. Ploidy (Chr/no.)**
P231/86 (1)	86	3x
P231/87 (1)	82	3x
P231/87 (2)	87	3x
P231/87 (3)	84	3x
P231/87 (4)	88	>3x
P231/87 (5)	76	<3x
P231/87 (6)	50	2x
P231/88 (1)	85	3x
P231/88 (2)	87	3x
P231/88 (3)	49	2x
P231/88 (4)	120	>4x
P231/88 (5)	87	3x
P231/88 (6)	75	<3x
P231/97 (1)	78	3x
P231/97 (2)	82	3x

4.1.2 Production of diploid Backcross 2 (BC_2) introgression lines $LpLp^{Fg}$ (2n=2x=14) from *L. perenne* (2x) (Lp) x LpFg (BC_1).

The Backcross 2 (BC_2) derived from a further backcross stage that involved backcrossing LpFg BC_1 triploid (2n=3x=21) hybrids onto Lp (2x) plants and produced a total of 276 BC_2 genotypes that established successfully without recourse to embryo rescue. The average seed to head ratio was 3.22 overall. Seed set was significantly greater when the BC_1 hybrid was used as the male parent ($p<0.0001$).

4.1.3 Physical characterisations and *Festuca*-specific markers

4.1.3.1 Comparisons of physical traits measured in project plants

The physical traits selected for screening amongst the Lp, Lm, and Fg parents and hybrid progeny are described in Chapter 3 (Materials and Methods). A Principal Component Analysis (PCA) component biplot (Fig.4.1) illustrates results of the physical traits measured on all the tetraploid (4x) Lp, Lm, and Fg parents and the tetraploid (4x) and the BC_1 triploid (3x) hybrid progeny. PCA was used as a multivariate method for exploring the data as it is useful when more than two or more observations have been made on the same samples (Dytham, 2003) and in this case there were four observations; a) Habit, b) Tiller Count, c) Texture and d) Leaf Width. PCA, although a non-statistical method, can synthesise and process the interrelationships between sample observations the results of which can be interpreted in a statistical way. PCA changes the variants (which may or may not be correlated) and converts them into uncorrelated components. Two sets of results are produced; eigenvalues which are the variances for the components and eigenvectors which give "weighting" to the original variants on each of the new components and are properties of matrices. A total of 79% of the variation was accounted by the current PCA (Fig. 4.1).

The *F. glaucescens* (4x) parents (Fg) (black spots) are clustered to the left of the PCA plot (Fig. 4.1.) indicating that they generally have firmer texture, wider leaves and more erect habit than Lm and Lp with Bn 354(8) as an outlier showing some variation was present within the five Fg genotypes. The *L. multiflorum* (4x) parents (Lm) (red spots) are positioned more centrally in the plot indicating that they are less erect and have narrower, softer leaves than the Fg genotypes. However there was more variation within these genotypes than within the Fg genotypes. The *L. perenne* (4x) parents (Lp) (blue spots) are clustered to the right of the plot indicating that they are generally more prostrate with more tillers, narrower leaves and have softer texture. The LmFg (4x) hybrids (red diamonds) mostly cluster to the centre and right of the plot with one notable exception (P188/48(1)) which has traits more consistent with the Fg parent. Overall, whilst the majority of the LmFg (4x) hybrids are more Lm than Fg-like, they are in the main distinct from either parent group. The LmFg BC_1 (3x) hybrids (red triangles) are loosely clustered around the centre of the plot and were more Lm than Fg-like and could be distinguished from their LmFg (4x) parent hybrids. The Lp Fg F_1 tetraploid (4x) hybrids (blue

diamonds) are clustered to the upper right section of the PCA plot indicating that they are more Lp-than Fg-like with more tillers, narrower/softer leaves and were more prostrate in habit. The Lp Fg BC_1 (3x) hybrids (blue triangles) are clustered in the lower right section of the PCA plot also indicating that they are more *Lolium*-like, except that they on average have lower numbers of tillers.

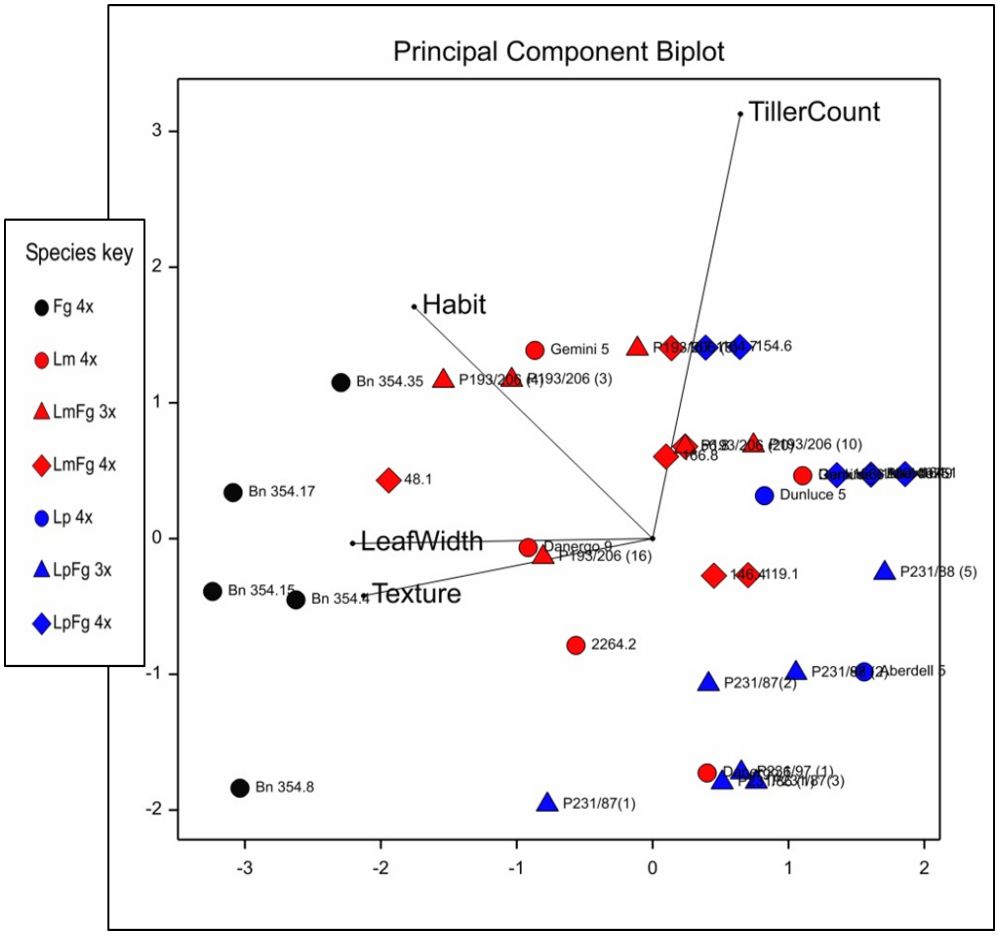


Figure 4.1 Principal component analysis (PCA) plot showing physical characterisation comparisons of habit, tiller count, leaf texture and leaf width (ranked) within and between tetraploid parent groups (*F.glaucescens, L.multiflorum, L.perenne*) tetraploid F_1 hybrid groups (LmFg and LpFg) and triploid (3x) hybrid groups (LmFg and LpFg). Variation accounted for 79% derived from first two principal component roots: (52% + 27%).

Scores for foliar traits for *L. perenne* (4x) (Lp), *L. multiflorum* (4x) (Lm), and *F. glaucescens* (4x) (Fg) and their expression in F1 hybrids: *L. multiflorum x F. glaucescens* (4x) (LmFg), *L. perenne x F. glaucescens* (4x) (LpFg) are provided in Table 4.3and significance of variation for physical trait scores given in Table 4.4.

Table 4.3. Scores for foliar traits for *L. perenne* (4x) (Lp), *L. multiflorum* (4x) (Lm), and *F. glaucescens* (4x) (Fg) and their expression in F1 hybrids: *L. multiflorum x F. glaucescens* (4x) (LmFg), *L. perenne x F. glaucescens* (4x) (LpFg).

Foliar trait scores for *F.glaucescens* (4x), *L.multiflorum* (4x) and *L.perenne* (4x) parents and their *Lolium* sp. *x F.glaucescens* hybrids

	F. glaucescens (4x) parent					*Lolium sp.* (4x) parent					*Lolium* sp. x *F.glaucescens* (4x) hybrid			
Fg (4x)	Habit	Tiller Count	Leaf Texture	Leaf Width	Lp (4x)	Habit	Tiller Count	Leaf Texture	Leaf Width	LpFg (4x)	Habit	Tiller Count	Leaf Texture	Leaf Width
Bn 354 (15)	3	3	5	10	Aberdell (5)	1	3	1	1	P237/153 (10)	1	5	1	3
Bn 354 (8)	3	1	5	8	Aberdell (5)	1	3	1	1	P237/154 (6)	3	5	1	2
Bn 354 (17)	3	4	5	10	Dunluce (5)	1	5	3	2	P237/164 (1)	1	5	1	1
Bn 354 (17)	3	4	5	10	Dunluce (5)	1	5	3	2	P237/164 (7)	3	5	1	3
Bn 354 (8)	3	1	5	8	Dunluce (6)	1	5	1	4	P237/180 (4)	1	5	1	2
					Lm (4x)					LmFg (4x)				
Bn 354 (35)	3	5	4	9	Danergo (9)	3	3	1	7	P237/56 (8)	3	4	1	3
Bn 354 (8)	3	1	5	8	Gemini (2)	3	5	1	8	P237/119 (10)	1	4	1	5
Bn 354 (15)	3	3	5	10	Gemini (2)	3	5	1	8	P237/146 (4)	1	4	1	6
Bn 354 (35)	3	5	4	9	Danergo (6)	1	2	1	5	P237/166 (8)	3	4	2	2
Bn 354 (8)	3	1	5	8	Gemini (5)	1	5	1	4	P237/167 (10)	3	5	1	4
Bn 354 (4)*	3	3	6	6	Bb 2264 (2)* cvRoberta	3	2	1	5	48 (1)*	3	4	4	7

L. multiflorum x F.glaucescens mapping family*

Table 4.4 Physical trait scores (mean and S.E.) for each plant group: Fg, Lm and Lp (4x) parents, LmFg and LpFg (4x) F_1 hybrids, LmFg and LpFg (3x) BC_1 hybrids and LpFg BC_2 introgression lines.

Plant species		Habit		P	Tiller		P	Leaf Texture		P	Leaf Width		P
Groups	(n)	Mean	S.E.	value	Mean	S.E.	value	Mean	S.E.	value	Mean	S.E.	value
F.glaucescens 4x	5	3.0	0	ns	3.25	0.66	ns	4.75	0.32	ns	9.25	0.75	ns
L.multiflorum 4x	5	2.2	0.49	ns	3.40	0.68	ns	1.00	0.00	ns	5.80	0.73	ns
L.perenne 4x	5	1.0	0.42	ns	4.50	0.07	ns	1.50	0.49	ns	2.25	0.76	ns
LmFg 4x F_1	6	2.3	0.42	ns	4.17	0.07	ns	1.67	0.49	ns	4.50	0.76	ns
LpFg 4x F_1	5	1.8	0.49	ns	5.00	0	ns	1.00	0	ns	2.20	0.37	ns
LmFg 3x BC_1	6	3.0	0	ns	4.33	0.33	ns	2.17	0.60	ns	4.00	0.73	ns
LpFg 3x BC_1	9	1.0	0	ns	2.71	0.24	ns	1.71	0.35	ns	3.10	0.43	ns
All LpFg BC_2	257	1.7	0.04	***	3.17	0.05	**	2.48	0.07	***	3.62	0.13	***

4.1.4 Recovery of *Festuca*-specific morphological traits in LpFg BC_2 plants

The newly generated LpFg BC_2 plants were screened for fescue specific morphological traits identified in (S4.2.1) but in addition were screened for their foliar regrowth (fresh weight) and root volume (%), as described in Chapter 3. The majority of the LpFg BC_2 plants ranged between 2 – 10g leaf fresh weight and between 20 - 60% root volume (Figure 4.2). There are small numbers of

genotypes that weighed less than 2 g (Leaf Fwt) and above 50% root volume. Comparison of physical characteristics between the tetraploid *F. glaucescens* (n=4) and *L. perenne* parent genotypes (n=4) and their Lp x Fg BC_2 population (2n=2x=14), (n=200), and includes the aneuploids (2n = >14), (n=44), which were more *Festuca*-like due to having a complete *Festuca* chromosome as well as two Lp chromosomes (Fig.4.3).

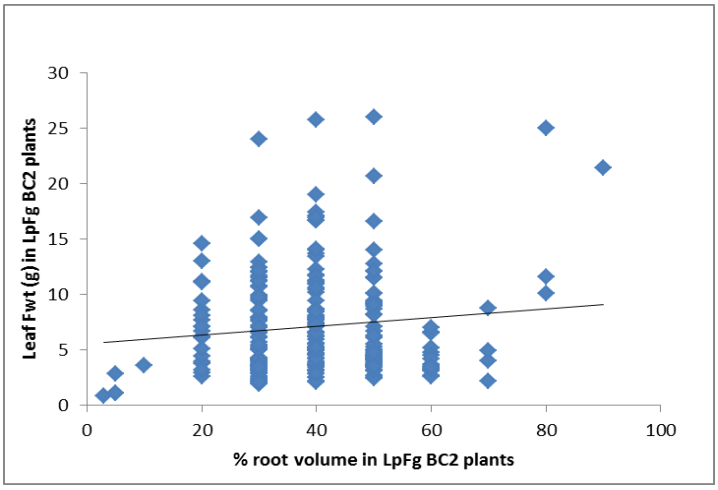


Figure 4.2 Correlation analysis between root volume (%) and fresh leaf weight (g) in the LpFg BC_2 population (n=256) showing a positive correlation overall ($y = 0.0393x + 5.5527$ $r^2 = 0.0124$) between root

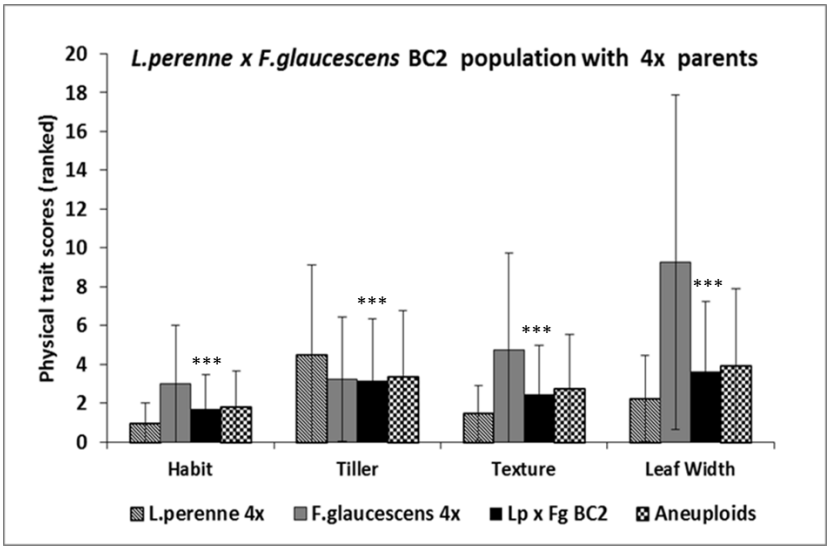


Figure 4.3 Comparison of physical characteristics between the *F. glaucescens* (n=4) and *L. perenne* (4x) parent genotypes (n=4) and their Lp x Fg BC_2 population derivatives comprising diploids (2n=2x=14); n=200) and aneuploids (2n = >14; n=44).

The score ranges for each of the measured traits; tiller count, habit, leaf texture and leaf width in the Lp x Fg BC_2 lines are shown in Table 4.5 and compared with their parental Lp and Fg (4x) scores in

order to assess the overall transmission of the relevant Fg traits into Lp-like BC_2 (2x) genotypes. The overall tiller number of the LpFg BC_2 diploid and putative introgression line plants were within the Lp (4x) parental range. In other cases, the LpFg BC_2 lines measurements fell outside or did not overlap with either parental ranges. For the habit trait, the parents had no score rating of [2] and the BC_2 were scored as intermediate in habit between the Lp and Fg parent groups. For leaf width, the parents had no score rating [5] making the BC_2 again intermediate between Lp and Fg. For leaf texture, the BC_2 had a score rating of 0 indicating there was no intermediate phenotype compared against the parent species (i.e no evidence for a Lp Fg species-interaction).

Table 4.5 Physical trait scores (ranked) for the Lp x Fg BC_2 introgression lines. Score ranges expected in *F. glaucescens* parents (Fg) phenotype, in *L. perenne* (4x) parents (Lp) and those intermediate or independent scores found exclusively in the LpFg BC_2 introgression lines (Lp x Fg) and absent in either *Lolium* or *Festuca* parent (i.e. *Festulolium*-like).

	Trait Score Range [Ranks]			
Plant species groups	Habit	Tiller Count	Leaf Texture	Leaf Width
Fg (4x) parents	[3]	[1-5]	[4-6]	[6-10]
Lp (4x) parents	[1]	[3-4]	[1]	[1-4]
LpFg BC_2 Introg. Lines	[2]	[1-5]	[1-5]	[1-8]
Festulolium traits	[2]	[0]	[2-3]	[5]

Figure 4.4 shows for each trait category the BC_2 introgression lines that have either no Fg-like trait expression (i.e. are Lp-like), those that are Fg-like (i.e. distinct from Lp) and also the BC_2 population and putative introgression lines that show a phenotype within the range of both parent groups. The Tillers and Habit trait measurements showed a high percentage (76.1% and 50.2% respectively) of the LpFg BC_2 plants having scored in the overlapping ranges with their tetraploid (4x) parents. The lowest score for tiller count in the LpFg BC_2 plants (7.1%) was that found in the Lp parental range, whilst 17.3% of the BC_2 displayed the Fg- phenotype, the remainder being overlapped with both parents. For growth habit, a greater number of the BC_2 plants were Lp-like (38%) with only 12% Fg - like. For the Leaf Width and Leaf Texture trait measurements all BC_2 were either Lp-like or Fg-like with 14% and 22% having Fg parent-like phenotype, respectively.

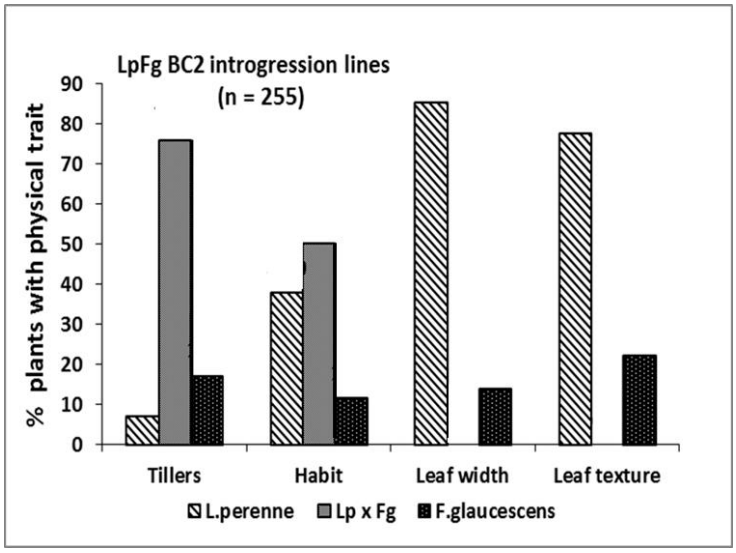


Figure 4.4 Percentage transmission of foliar traits in the Lp x Fg BC_2 lines. *Lolium*-specific traits (Diagonal lines), *Festuca*-specific traits (Black). Lp x Fg BC_2 lines either overlapped their parents (Tiller count/grey) or were *Festulolium*-specific (Habit/grey). In leaf width leaf texture traits LgFg BC_2 lines were either *Lolium*-specific or *Festuca*-specific and did not overlap with their tetraploid parents (No grey).

Analysis of variance (ANOVA) of physical trait comparisons (ranked scores) between the Lp Fg BC_2 lines and their tetraploid (4x) parents (Table 4.6) shows that there is significant variation (F probability highlighted in grey) within the LpFg BC_2 population in all four parameters measured, particularly for tiller count. Spearman's rank analysis shows that there was a strong negative correlation (0.003) between habit and tiller count indicating that the hybrids which are more prostrate (less erect) are likely to have more tillers (*L. perenne* trait) or *vice versa* (*F. glaucescens* trait). There is a positive correlation (0.591) between habit and leaf texture indicating that firmer-leaved hybrids are more inclined to be more erect (*F. glaucescens* trait) and *vice versa* (*L. perenne* trait). The correlation between tiller number and texture is negative (0.06) indicating that hybrids with less tillers are more likely to have firmer leaves. Conversely, a positive correlation (0.79) between habit and leaf width indicates that more erect hybrids are much more likely to have wider leaves (*F. glaucescens* trait) and *vice versa* that prostrate hybrids will have narrower leaves (*L. perenne* trait).

The association between tiller count and leaf width has a very strong negative correlation (<0.001) indicating that the Fp x Fg BC_2 population with higher tiller count are much more likely to have narrower leaves (*L. perenne* trait) and *vice versa* that hybrids with lower tiller count will have wider leaves (*F. glaucescens* trait). The leaf width versus leaf texture correlation is also negative (0.01) indicating that BC_2 with firm leaves are more likely to have narrower leaves. In this case the Fg-like trait for firm leaves is present together with the Lp-like trait for narrower leaves

Table 4.6 Analysis of variance and Spearman's Rank correlation coefficients of ranked and scored physical traits of Lp x Fg BC_2 lines compared with their *L. perenne* and *F. glaucescens* (4x) parents.

GenStat ANOVA table for physical trait comparisons													
Trait	Species group	n (obs)	Mean	Med'n	Min.	Max.	S.e.d	S.O.V	d.f.	S.S.	m.s.	v.r.	F pr.
Habit	Lp x Fg BC2 intro lines	256	1.72	2	1	3	0.64	Species	2	8.597	4.298	10.7	<0.01
	Fg 4x parent	4	3.00	3	3	3	0.00	Residual	261	105.3	0.404		
	Lp 4x parent	4	1.00	1	1	1	0.00	Total	263	113.9			
Tiller Count	Lp x Fg BC2 intro lines	256	3.17	3	1	5	0.81	Species	2	6.96	3.48	5.04	0.007
	Fg 4x parent	4	3.25	3.5	1	5	1.71	Residual	261	180.2	0.69		
	Lp 4x parent	4	4.50	5	3	5	1.00	Total	263	187.1			
Leaf Texture	Lp x Fg BC2 intro lines	256	2.48	2	1	5	1.05	Species	2	24.39	12.19	11.1	<0.01
	Fg 4x parent	4	4.75	5	4	5	0.50	Residual	261	285.6	1.094		
	Lp 4x parent	4	1.50	1	1	3	1.00	Total	263	310			
Leaf Width	Lp x Fg BC2 intro lines	256	3.62	4	1	8	1.99	Species	2	133.2	66.64	17.1	<0.01
	Fg 4x parent	4	9.25	9.5	8	10	0.96	Residual	261	1018	3.9		
	Lp 4x parent	4	2.25	2	1	4	1.26	Total	263	1151			
Spearman's rank correlation coefficient	Habit = 1	*											
	Tiller count = 2	0.003	*										
	Leaf texture = 3	0.591	0.055	*									
	Leaf width = 4	0.79	0.000	0.014	*								
	(n = 264 d.f. 262)	1	2	3	4								

4.1.5 Inheritance of *Festuca*-specific markers in the LpFg backcross breeding programme

Details of the presence or absence of the 7 selected *Festuca*-specific markers (one for each chromosome initially) employed in the marker screening of the Lp x Fg mapping family are contained in Tables 4.7 (a) – 4.7 (c) inclusive. Tables 4.7 (a) and 4.7 (b) (relating to Chromosomes 1–3 and 4–7 respectively) contain the details of the marker frequencies in the Fg tetraploid (4x) parents (alleles), Lp x Fg (4x) F_1 hybrids (presence/absence of markers) and Lp x Fg BC_1 (3x) hybrids (presence/absence of markers). Table 4.7 (c) contains the frequency of the three *Festuca*-specific markers that could be monitored through into the Lp x Fg BC_2 generation as their alleles were never observed within any Lp parent genotype. The Fg allelic variants were included for comparison. It should be noted that the overall transmission scores for the Fg alleles into the BC_2 generation are almost certainly lower than the actual number present as in many cases alleles were found that were common to both Lp and Fg parental genotypes. Progeny were never scored if there was any possibility that an allelic variant for a marker originated from an Lp source and not from an Fg parent

Table 4.7(a) Transmission of *Festuca*-specific markers in the LpFg backcross breeding programme within chromosomes 1-3 (C1-C3). *Festuca*-specific markers and alleles present in *F. glaucescens* (4x) (Bn354) parents but not present in *L. perenne* (4x) or (2x) parents are shown in phase 1 marker screen. Presence (✓) and absence (x) of *Festuca*-specific markers in the F_1 LpFg (4x) hybrids and BC LpFg (3x) hybrids are shown as phases 2 and 3 marker screens respectively.

Inheritance of *Festuca*-specific markers in Backcross 2 LpFg introgression lines and intermediates (C1 -C3)													
Marker + Chromosome (x) / Marker alleles	rv0244 (C1)	rv0327 (C1)	rv0913 (C1)	rv1391 (C1)	M4-136 (C2)	rv0188 (C2)	rv0706 C2)	rv1239 (C2)	25ca1 (C3)	rv0154 (C3)	rv0863 (C3)	rv1046 (C3)	rv1332 (C3)
a	235	131	80	156	194	185	200	120	280	98	250	155	298
b	-	141	180	160	-	-	-	137	-	141	-	-	-
c	-	-	-	-	-	-	-	-	-	86	-	-	-
d	-	-	-	-	-	-	-	-	-	-	-	-	-
Phase 1 Screen : *Festuca*-specific markers absent in *Lolium* sp. tetraploid (4x) parents but present tetraploid (4x) Fg parents													
Bn354 (8)	x	x	x	x	a	x	x	x	x	b	x	x	x
Bn354 (15)	x	x	x	a	x	x	x	x	x	a	a	x	x
Bn354 (17)	a	a	x	x	x	a	x	x	x	c	x	x	a
Bn354 (35)	x	a b	x	b	x	a	x	b	x	x	x	x	x
Phase 2 Screen : *Festuca*-specific markers (presence/absence) in F1 (polycross) generation (tetraploid (4x) LpFg hybrids)													
95 (3) [Family 1]	✓	✓	x	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
91 (10) [Family 2]	✓	✓	x	x	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
91 (9) [Family 3]	✓	✓	x	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
91 (8) [Family 4]	✓	✓	x	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
Phase 3 Screen : *Festuca*-specific markers (presence/absence) in BC1 generation (triploid (3x) LpFg hybrids)													
P231/86 (1) [Family 1]	✓	✓	n/a	x	✓	✓	n/a	✓	n/a	✓	x	n/a	x
P231/87 (1) [Family 2]	✓	✓	n/a	x	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
P231/87 (2)	✓	x	n/a	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
P231/87 (3)	✓	✓	n/a	x	x	✓	n/a	✓	n/a	✓	x	n/a	✓
P231/88 (1) [Family 3]	✓	✓	n/a	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	x
P231/88 (2)	✓	✓	n/a	x	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
P231/88 (5)	✓	✓	n/a	x	✓	✓	n/a	✓	n/a	✓	x	n/a	✓
P231/97 (1) [Family 4]	x	✓	n/a	✓	✓	x	n/a	✓	n/a	✓	x	n/a	✓
P231/97 (2)	✓	✓	n/a	✓	✓	✓	n/a	✓	n/a	✓	x	n/a	x

Table 4.7(b) Transmission of *Festuca*-specific markers in the LpFg backcross breeding programme within chromosomes 4-7 (C4-C7). *Festuca*-specific markers and alleles present in *F.glaucescens* (4x) parents but not present in *L perenne* (4x) or (2x) parents are shown in phase 1 marker screen. Presence and absence of *Festuca*-specific markers in the F_1 LpFg (4x) hybrids and BC_1 LpFg t (3x) hybrids are shown as phases 2 and 3 marker screens respectively.

Inheritance of *Festuca*-specific markers in Backcross 2 LpFg introgression lines and intermediates (C4 -C7)														
Marker + Chromosome (x) /Marker alleles	rv0061 (C4)	rv0382 (C4)	rv0992 (C4)	rv1317 (C4)	rv0250 (C5)	rv0260 (C5)	rv0340 (C5)	rv0757 (C5)	rv0196 (C6)	rv0739 (C6)	rv1208 (C6)	rv1266 (C6)	rv1423 (6)	hd3agt (C7)
a	199	129	167	263	261	138	100	208	131	200	85	178	111	68
b	-	135	177	-	271	-	104	214	146	-	140	-	120	90
c	-	179	186	-	275	-	118	-	156	-	-	-	124	156
d	-	185	193	-	-	-	-	-	-	-	-	-	127	-
Phase 1 Screen : *Festuca*-specific markers (alleles) absent in *Lolium* sp. tetraploid (4x) parents but present tetraploid (4x) Fg parents														
Bn354 (8)	x	b	a b c d	a	a b	a	a b c	a b	x	x	x	a	a b	b
Bn354 (15)	a	c d	b	x	a b c	x	a b c	a	a	x	x	a	a b c	a b
Bn354 (17)	x	x	x	x	x	x	x	b	b	x	x	x	x	x
Bn354 (35)	x	a	d	x	x	x	x	x	b c	x	x	x	x	c
Phase 2 Screen : *Festuca*-specific markers (presence/absence) in F1 (polycross) generation (tetraploid (4x) LpFg hybrids)														
95 (3) [Family 1]	✓	x	✓	✓	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
91 (10) [Family 2]	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
91 (9) [Family 3]	x	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	x	✓	✓
91 (8) [Family 4]	x	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
Phase 3 Screen : *Festuca*-specific markers (presence/absence) in BC1 generation (triploid (3x) LpFg hybrids)														
P231/86 (1) [Family 1]	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/87 (1) [Family 2]	✓	x	✓	✓	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/87 (2)	x	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/87 (3)	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	x	✓	✓
P231/88 (1) [Family 3]	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/88 (2)	✓	x	✓	✓	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/88 (5)	✓	x	✓	x	x	✓	✓	✓	✓	n/a	n/a	x	✓	✓
P231/97 (1) [Family 4]	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓
P231/97 (2)	✓	x	✓	x	✓	✓	✓	✓	✓	n/a	n/a	✓	✓	✓

Table 4.7(c) Transmission of *Festuca*-specific markers in the LpFg backcross breeding programme: *Festuca*-specific marker alleles present in the LpFg BC_2 lines following screening out common alleles found later in parental *L. perenne* 4x genotype AberDell (5) shown as phase 4 marker screen. Presence and absence of fescue specific marker alleles never recovered in Lp genotypes are shown specific to each *F. glaucescens* parent. The total number of the *Festuca*-specific marker alleles is indicated with respect to their family groups. Only in three instances (for hd3agt) is there a genotype where more than a single fescue allelic variant was observed (within a family) for the same marker type.

Phase 4 screen : *Festuca* -specific markers (alleles) remaining in BC2 LpFg introgression lines. Markers found later in Lp 4x parent AberDell (5) were not included : rv0327; M4-136; rv0757; rv0196													
Marker	rv0154 (C3)				rv0061 (C4)				hd3agt (C7)				
Allele (Fg 4x parents)	a	b	c	a+c	a	b	c	a+c	a	b	c	a+c	
Bn354 (8)	x	✓	x	x	x	x	x	x	x	✓	x	x	
Bn354 (15)	✓	x	x	x	✓	x	x	x	✓	✓	x	x	
Bn354 (17)	x	x	✓	x	x	x	x	x	x	x	x	x	
Bn354 (35)	x	x	x	x	x	x	x	x	x	x	✓	x	
Family 1 (95/3) n = 7	2	x	x	x	6	x	x	x	1	x	5	x	
Family 2 (91/10) n = 44	28	x	x	x	7†	x	x	x	4	x	4	x	
Family 3 (91/9) n = 93	27	x	x	x	67	x	x	x	28	x	21	2 (n = 4)	
Family 4 (91/8) n = 112	59	x	x	x	44	x	x	x	6	x	10	1 (n = 2)	
Total n = 256	116	0	0	0	117	0	0	0	39	0	40	6	
% transmission per allele	45.31	0	0	0	48.44	0	0	0	15.23	0	15.63	3.85	
% transmission per marker	45.31				48.44				34.71				

†Family 2 (91/10) transmission of rv0061 was from a population of 251 as it was not present in BC1 P237/87 (2)

A Pearson's Chi square test for independence and goodness-of–fit (Pearson, 1900) was carried out to establish effectiveness of transmission of the *Festuca* – specific markers rv0154, rv0061 and hd3agt from the *F. glaucescens* parents to the Lp Fg BC_2 population (Table 4.8).

Table 4.8 Chi-square cross tabulation grid showing the frequency of *Festuca* – specific markers in the LpFg BC_2 population. (Expected recovery of Fg markers if transmitted from the BC_1 into the BC_2 at equal frequency to those of Lp)

	Markers Observed (Expected)			
	rv0154	rv0061	hd3agt	Total
Present	116 (109)	124 (107)	85 (109)	325
Absent	140 (147)	127 (144)	171(147)	438
Total	256	251	256	763

Results show that transmission of all three markers in the Lp Fg BC_2 population was significant ($p<0.001$). Transmission of rv0154 (Chromosome 3) and rv0061 (Chromosome 4) was higher than

that of hd3agt (Chromosome 7) and both had a higher frequency than expected whereas the transmission of hd3agt was lower than expected. The rv0061 marker came from a smaller population than rv0154 and hd3agt because it was absent in the triploid Lp Fg (3x) BC_1 parent P237 /87 (2) (Table 4.7b).

Associations between foliar traits and *Festuca*-specific SSRs in the LpxFg BC2 introgression lines are shown in Table 4.9.

Table 4.9 Association between foliar traits and *Festuca*-specific SSR markers in the Lp x Fg BC_2 introgression lines using REML.

Physical trait analysis forLp x Fg BC2 introgression lines using REML variances component analysis (n=256)

Variate =TRAIT	Factors=SSR Markers	Estimate σ^2	Std.err.	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr	Predicted mean for constant	Std.err. constant	Mean: marker absent	Mean: marker present	Std.err. diffs
Habit	rv0154	0.408	0.0362	3.95	1	3.95	254	0.048*	1.715	0.0401	1.794	1.635	0.0803
(Ranked score)	rv0061	0.405	0.036	5.93	1	5.93	254	0.016*	1.726	0.0398	1.629	1.823	0.0796
	hd3agt	0.402	0.0356	8.15	1	8.15	254	0.005**	1.767	0.0426	1.646	1.889	0.0852
Tiller	rv0154	0.66	0.0586	1.09	1	1.09	254	0.296ns	3.166	0.0511	3.22	3.113	0.1021
(Ranked score)	rv0061	0.66	0.0586	1.06	1	1.06	254	0.304ns	3.174	0.0508	3.121	3.226	0.1016
	hd3agt	0.663	0.0588	0.02	1	0.02	254	0.879ns	3.169	0.0547	3.177	3.16	0.1094
Leaf texture	rv0154	1.07	0.095	9.39	1	9.36	254	0.002**	2.497	0.065	2.298	2.696	0.13
(Ranked score)	rv0061	1.074	0.095	8.45	1	8.45	254	0.004**	2.471	0.0648	2.659	2.282	0.1296
	hd3agt	1.098	0.097	2.61	1	2.61	254	0.107ns	2.435	0.0704	2.549	2.321	0.1408
Leaf width(mm)	rv0154	3.905	0.347	2.31	1	2.31	254	0.129ns	3.629	0.1242	3.44	3.817	0.2483
(Ranked score)	rv0061	3.939	0.35	0.12	1	0.12	254	0.732ns	3.611	0.1241	3.568	3.653	0.2482
	hd3agt	3.937	0.349	0.25	1	0.25	254	0.619ns	3.585	0.1333	3.651	3.519	0.2666
	rv0154	1.698	15.1	1.11	1	1.11	254	0.293ns	39.73	0.819	38.87	40.59	1.637
% root volume	rv0061	167.5	14.9	4.54	1	4.54	254	0.034*	39.59	0.809	41.31	37.86	1.619
	hd3agt	167.6	14.9	4.35	1	4.35	254	0.038*	38.97	0.87	40.79	37.16	1.74
	rv0154	0.0701	0.00622	1.69	1	1.69	254	0.194ns	0.7696	0.0166	0.7912	0.7479	0.0333
Log_Leaf FWT (g)	rv0061	0.0656	0.00582	19.1	1	19.1	254	<0.001***	0.7739	0.016	0.7039	0.844	0.032
	hd3agt	0.0682	0.00605	8.84	1	8.84	254	0.003**	0.7909	0.0176	0.7387	0.8431	0.0351
	rv0154	0.081	0.00719	2.54	1	2.54	254	0.112ns	-0.7998	0.0179	-0.7713	-0.8283	0.0358
LogLR	rv0061	0.074	0.0066	27.03	1	27.03	254	<0.001***	-0.7941	0.017	-0.8826	-0.7057	0.034
	hd3agt	0.0773	0.0069	14.79	1	14.79	254	<0.001***	-0.7705	0.0187	-0.8424	-0.6987	0.0374
	Habit	0.397	0.0357	17.22	7	2.46	248	0.019**	1.744	0.0605			
	Tiller	0.648	0.0582	11.91	7	1.7	248	0.109ns	3.281	0.0772			
SSR Markers	Leaf texture	1.072	0.096	14.83	7	2.12	248	0.042*	2.432	0.0994			
(combined)	Leaf width(mm)	3.903	0.351	8.44	7	1.21	248	0.300ns	3.582	0.1896			
	% root volume	167.6	15	10.49	7	1.5	248	0.168ns	39.01	1.242			
	Log_Leaf FWT	0.0642	0.00576	31.23	7	4.46	248	<0.001***	0.8026	0.0243			
	LogLR	0.072	0.0065	40.76	7	5.82	248	<0.001***	-0.7616	0.0257			

	Predicted means and comparisons between means (bonferroni test) for combined markers O=Absent 1=Present								Std.err.diffs		
Traits\marker	000	001	010	011	100	101	110	111	Avge	Max/Min	Avge var.
Habit	1.62a	2.29a	1.75a	1.91a	1.59a	1.25a	1.72a	1.82a	0.227	0.395/0.113	0.0585
Tiller	3.29a	3.14a	3.39a	3.06a	2.99a	3.75a	3.28a	3.35a	0.291	0.51/0.14	0.095
Leaf texture	2.49abc	2.29abc	2.19a	2.21ab	2.80ac	2.50abc	2.33abc	2.65abc	0.374	0.65/0.185	0.158
Leaf width(mm)	3.53a	3.57a	3.31a	3.43a	3.65a	2.50a	4.67a	4.00a	0.713	1.24/0.353	0.354
% root volume	42a	31.43a	39.58a	36.7a	41.62a	45a	36.67a	39.12a	4.671	8.11/2.32	24.68
Log_Leaf FWT	0.753ab	0.757ab	0.766ab	0.845b	0.665a	0.793ab	0.958b	0.884b	0.0914	0.159/0.045	0.00945
LogLR	-0.855	-0.731	-0.8	-0.694	-0.92	-0.847	-0.589	-0.666	0.0968	0.168/0.05	0.0106

4.2 **Optimal genome composition in hybrids between *Festuca* and *Lolium* for expression of protein stability under rumen conditions**

4.2.1 **Protein stability between and within the tetraploid parent groups**

The tetraploid *Lolium* and *Festuca* spp. parents (Fig.4.5) have four genomes per genotype and five genotypes per group and they are; *L.multiflorum* (Lm 4x), *L.perenne* (Lp 4x) and *F.glaucescens* (Fg 4x). There was considerable intraspecific variation found for all three tetraploid species (Fig.4.5, Table 4.10). Overall it was the Fg (4x) parents (all genotypes) that had the longest mean half-life at 8.8 h (±1.48). The Fg were significantly different from one-another at 0 h ($p<0.001$) but they were not significantly different after 24 hours incubation. The Lp (4x) parents, had a shorter half-life on average (than the Fg (4x) parents) at 4.5 h (± 0.86). The Lp (4x) parents were significantly different at 0 h ($p<0.001$) and also at 24 h ($p<0.001$). The Lm (4x) parents with an average half-life of 4.1 h (±0.91) were also significantly different at 0 h ($p<0.001$) and at 24 h ($p<0.001$). The Lm (4x) parent cv Gemini (5) had the longest half-life (on average) and also had the most variability of all the tetraploid parents groups with a half-life of 18.55 h (±18.93). The genotypes with the shortest half-lives were both *Lolium* but genotypes from different species: Lm (4x) cv Danergo (9) at 1.53 h (±0.40) and Lp (4x) cv Dunluce (6) at 1.96 (±0.41). The shortest half-life in the Fg (4x) parent group was genotype Bn 354 (17) with 3.71 h (±1.02).

When the Lm (4x) parents were compared with the Fg (4x) parents as groups (genotypes used in the formation of the LmFg (4x) hybrids only) they differed significantly at 0 h ($p<0.001$) but not in terms of residual protein (Lm = 10.35 mg/g DW; Fg = 11.62 mg/g DW; Table 4.10). However, when the Lp (4x) parents were compared with the Fg (4x) parents as groups (i.e. only the genotypes used in the formation of LpFg (4x) hybrids) they differed significantly at both in terms of protein content at 0 h ($p<0.05$) and after 24 h (residual protein, $p<0.05$).

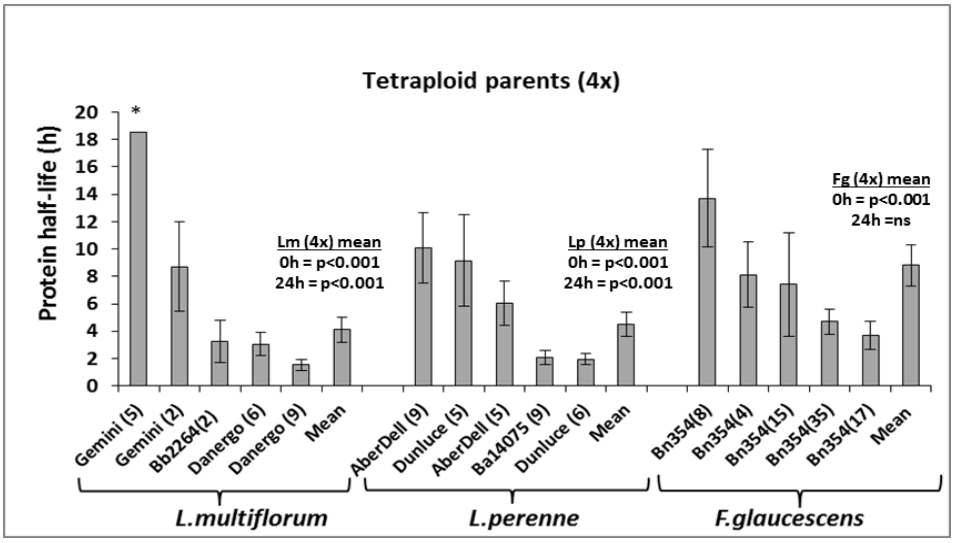


Figure 4.5 Comparison of protein stability (half-life) within and between the three tetraploid (2n=4x=28) parental groups *L.multiflorum* (Lm 4x), *L.perenne* (Lp 4x) and *F.glaucescens* (Fg 4x) (* Large s.e. 18.93h; bars). Intraspecific mean protein half-life variation at 0 h and 24 h also shown for groups.

Protein stability in the diploid parental *Lolium* species and in triploid LmLmFg and LpLpFg hybrids was calculated (data not shown).

4.2.2 Protein stability between and within the tetraploid (F_1) hybrid groups

Protein stability was also investigated in two tetraploid hybrid groups; LmFg (4x), (6 genotypes) and LpFg (4x), (5 genotypes) each with a balanced complement of two genomes of *Lolium* and two genomes of *F. glaucescens.* These were the F_1 generation derived from the hybridisation of autotetraploid *Lolium* (genomes from a common ancestor) and allotetraploid *Festuca* parents (genomes from more than one ancestor). The tetraploid LmFg and LpFg (4x) hybrids (Figs 4.6a and 4.6b) and their *Lolium* and *F. glaucescens* (4x) parents were compared in terms of protein half-lives. Interspecific comparisons were made between the two LmFg and LpFg F_1 (4x) hybrid groups and the variation sought within each group. Comparisons were also made between the F_1 (4x) hybrids and their respective Lm, Lp and Fg tetraploid (4x) parents to determine if the protein stability trait observed in the parental genotypes was maintained in their hybrid progeny.

On average, the LmFg (4x) hybrid group (Fig. 4.6a) had the longer half-life (Table 4.10) and had the greatest within group variability at 21.21 h (±12.89). The shortest half-life was 9.2 h (±2.15) (P188/48.1), the F_1 used in the LmFg introgression line family (Humphreys, *et al.*, 2005) and the longest half-life was 61.65 h (±173.60) (P237/56.8). There was considerable variation between replicate samples taken from certain genotypes from within the LmFg (4x) group, at 0 h ($p<0.001$) and at 24 h ($p<0.001$). Also the predicted half-life values extended beyond the 0-24 h sampling range and was likely to have led to the large observed Standard Errors. The LpFg (4x) hybrids as a group had comparatively shorter half-lives on average at 17.85 h (± 3.31) with the shortest half-life at 9.91 h (± 2.46) (P237/180.4) and the longest half-life recorded as 26.63 h (±12.19) (P237/164 (1)). The mean protein half-life variability for the LpFg (4x) hybrids was less significant at 0 h ($p<0.05$) and at 24 h ($p<0.05$) when compared to the LmFg (4x) hybrids. The LmFg hybrids (4x) individually all had longer half-lives (mean of 3 replicates) than their Lm (4x) and Fg (4x) parents whereas one LpFg (4x) hybrid (P237/180.4) had a shorter half-life at 9.91 h (±2.46) than its Fg (4x) parent Bn 354 (8) (half-life 13.70 h ± 3.57). However all the other LpFg (4x) hybrids had longer half-lives than their respective Lp and Fg (4x) parents.

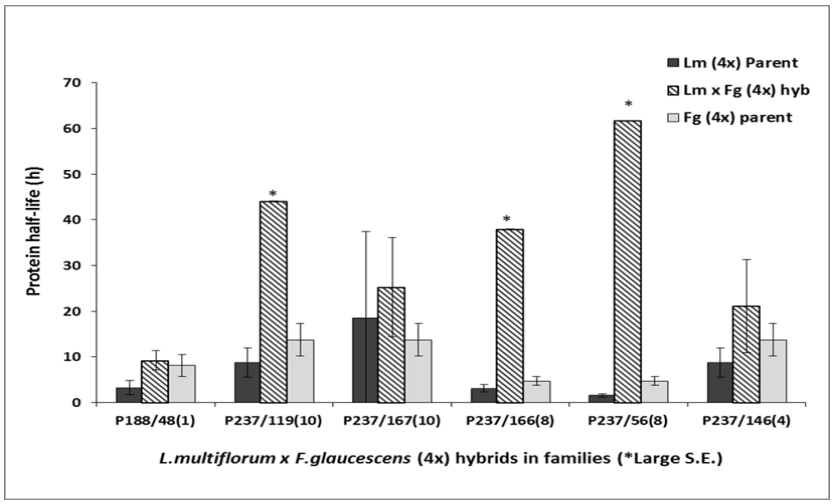


Figure 4.6(a). Comparison of protein stability (half-life) within and between tetraploid (2n=4x=28) *L.multiflorum x F.glaucescens* hybrids and their tetraploid (2n=4x=28) Lm and Fg parents. * SE not shown as very high:- P237/119(10) [±11681 hrs]; P237/166(8) [± 90 hrs]; P237/56(8) [±61 hrs].

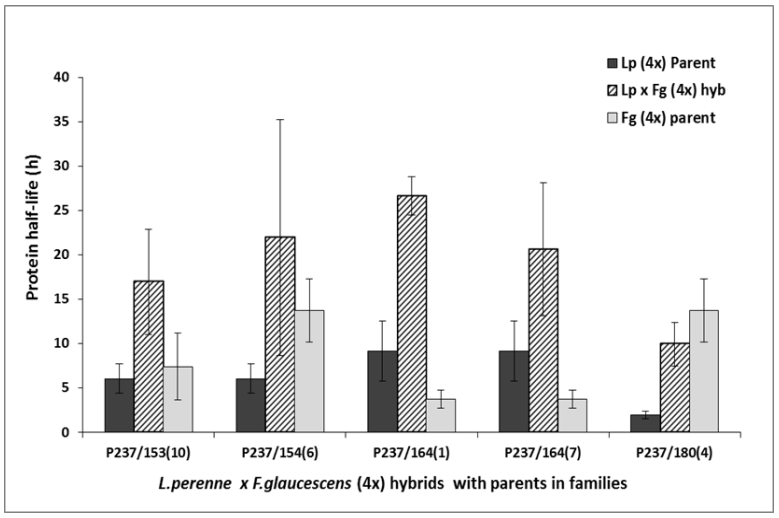


Figure 4.6(b). Comparison of protein stability (half-life) within and between tetraploid (2n=4x=28) *L.perenne* x *F.glaucescens* hybrids and their tetraploid (2n=4x=28) Lp and Fg parents. Breeding line codes for F_1 hybrids shown on x axis.

Group comparisons were also made between the tetraploid parents and their relevant tetraploid hybrids for protein half-life: Lm (4x) parents as a group when compared with their LmFg (4x) F_1 hybrid progeny as a group were highly significantly different from each other both at 0 h ($p<0.001$) and in terms of residual protein at 24 h ($p<0.001$; Table 4.10; Table 4.11) and this was the same with or without inclusion of the mapping family F_1 hybrid P188/48 (1). Comparisons of the Lp (4x) parents as a group with their LpFg F_1 (4x) hybrids as a group also showed significant differences from each other at 0 h ($p<0.001$) but less so after 24 h ($p<0.01$) (Table 4.10; Table 4.11). The Fg (4x) parents as a group were also significantly different from their LmFg (4x) F_1 hybrid group (mapping family F_1 P188/48 (1) not included) at 0 h ($p<0.001$) and 24 h ($p<0.05$). The same comparison (mapping family

F_1 P188/48 (1) included) whilst significantly different at 0 h ($p<0.001$) was not significantly different in terms of residual protein (Table 4.10). The protein half-life comparisons between Fg (4x) parents as a group against their LpFg (4x) F_1 hybrid group were significantly different at 0 h ($p<0.01$) and were also significantly different in terms of residual protein ($p<0.05$) (Table 4.10; Table 4.11).

Table 4.10 Residual protein at 24h (mean of species and hybrid groups, mg/g/DW)

Plant group	Residual protein at 24 h mean of groups (mg/g DW)
Fg (4x) parents	11.62
Lm (4x) parents	10.35
Lp (4x) parents	12.44
LmFg (4x) hybrids	15.48
LpFg4x hybrids	16.45
LmLm (2x) parents	14.88
LpLp (2x) parents	15.84
LmFg (3x) hybrids	13
LpFg (3x) hybrids	9.44

Table 4.11 Comparison of protein half-life within and between groups at 0 hours and residual protein at 24 hours

Group Number	Group 1 (n)	Half-life/h (S.E)	Group 2 (n)		Comparison of protein at 0h within/between groups	Comparison of residual protein at 24 h within/between groups
	Protein stability within groups comparisons					
1a	Lm 2x parents (4)	6.3 (1.07)	n/a		ns	ns
1b	Lm 2x parents (6)*	8.2 (1.22)	n/a		ns	ns
1c	Lp 2x parents (5)	4.8 (0.66)	n/a		<0.01	<0.05
1d	LmFg 3x hybrids (6)	4.4 (0.64)	n/a		<0.01	<0.001
1e	LpFg 3x hybrids (9)	5.4 (0.93)	n/a		<0.01	ns
1f	Lm 4x parents (5)	4.1 (0.91)	n/a		<0.001	<0.001
1g	Lp 4x parents (5)	4.5 (0.86)	n/a		<0.001	<0.001
1h	Fg 4x parents (5)	8.8 (1.48)	n/a		<0.001	ns
1i	LpFg 4x hybrids (5)	17.9 (3.31)	n/a		<0.05	<0.05
1j	LmFg 4x hybs (6)*	21.2 (12.9)	n/a		<0.001	<0.001
	Protein stability between groups comparisons			Half-life/h (S.E)		
2a	Lm 2x parents (4)	6.3 (1.07)	Lm 4x parents (5)	4.1 (0.91)	<0.001	<0.05
2b	Lp 2x parents (5)	4.8 (0.66)	Lp 4x parents (5)	4.5 (0.86)	<0.01	ns
2c	Lm 4x parents (5)	4.07 (0.91)	LmFg 4x hybrids (5)	32.2 (27.2)	<0.001	<0.001
2d	Lm 4x parents (5)	4.07 (0.91)	LmFg 4x hybrids (6)*	21.2 (12.9)	<0.001	<0.001
2e	Lm 4x parents (5)	4.07 (0.91)	Fg 4x parents (3)†	9.0 (3.80)	<0.001	ns
2f	Lp 4x parents (5)	4.5 (0.86)	LpFg 4x hybrids (5)	17.9 (3.31)	<0.001	<0.01
2g	Lp 4x parents (5)	4.5 (0.86)	Fg 4x parents (3)†	10.3 (3.92)	<0.05	<0.05
2h	Fg 4x parents (5)	8.8 (1.48)	LmFg 4x hybrids (5)	32.2 (27.2)	<0.001	<0.05
2i	Fg 4x parents (5)	8.8 (1.48)	LmFg 4x hybrids (6)*	21.2 (12.9)	<0.001	ns
2k	Fg 4x parents (3)†	10.3 (3.92)	LpFg 4x hybrids (5)	17.9 (3.31)	<0.01	<0.05
	Protein stability of LmLm (Fg) introgression line P194/208 (19) compared with each mapping family parent					
3a	P194/208 (19)	6.9 (2.68)	Fg 4x parent: Bn354 (4)	8.1 (2.41)	<0.001	ns
3b	P194/208 (19)	6.9 (2.68)	Lm 4x parent: 2264(2)	3.2 (1.53)	<0.05	ns
3c	P194/208 (19)	6.9 (2.68)	LmFg 4x F1 hybrid:P188/48 (1)	9.2 (2.15)	ns	ns
3d	P194/208 (19)	6.9 (2.68)	Lm 2x parent Bb 2205 (15)	7.2 (1.98)	ns	ns
3e	P194/208 (19)	6.9 (2.68)	LmFg 3x BC1 hybrid 3x P193/206 (4)	2.4 (0.34)	<0.001	<0.01
3f	P194/208 (19)	6.9 (2.68)	Lm 2x parent Bb 2042 (12)	3.7 (0.95)	<0.05	ns

* Includes mapping family parents. † Fg parents of LmFg/LpFg hybrids

4.2.3 Relationship between initial protein and protein half-life

Protein content (mg/g DW) in each sample group was related to protein half-life (Fig. 4.7). There was a significant negative correlation (P<0.0001) between initial protein content and protein half-life i.e. the sample groups with higher initial protein content at 0 h were significantly more likely to have shorter protein half-lives on average. Most of the sample groups including tetraploid parents, diploid parents and triploid BC_1 hybrids clustered to the top left of the graph, indicating that they generally had higher initial protein and shorter half-lives. In contrast the tetraploid hybrid groups (LmFg 4x illustrated × and LpFg 4x illustrated ✳) clustered lower down and more centrally (LpFg 4x) or to the bottom right (LmFg 4x) of the graph indicating that they had generally less initial protein content and longer protein half-lives. This was particularly noticeable for LmFg (4x) hybrids in comparison with any other group assessed.

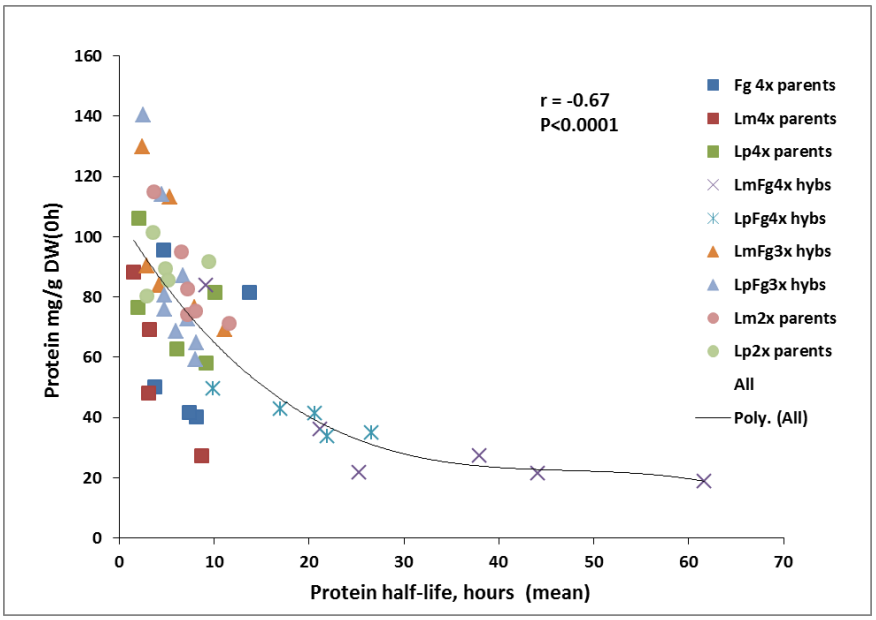


Figure 4.7 Correlation ($y=-0.0008x^3+0.1068x^2-5.1439x+106.49$) between protein content at 0 h and protein half-life in hours (mean of genotypes in each group) showing a significantly negative relationship (P<0.0001).

Although in all cases recovered protein content decreased with incubation time (by 24 h) as a group, the LmFg (4x) hybrids were more stable in their protein content (Fig.4.9). By 2 h they had 102.8% ± 2.6% protein remaining, 6 h (108.6% ± 26.5%), and at 24 h (57% ± 13.6%). The LpFg (4x) hybrid group also maintained protein stability, but overall protein retention was lower than for LmFg (4x): By 2 h (103.2% ± 21.9%) remained, 6 h (81.4%), 24 h (41.5% ± 15.2%) (Fig 4.8).

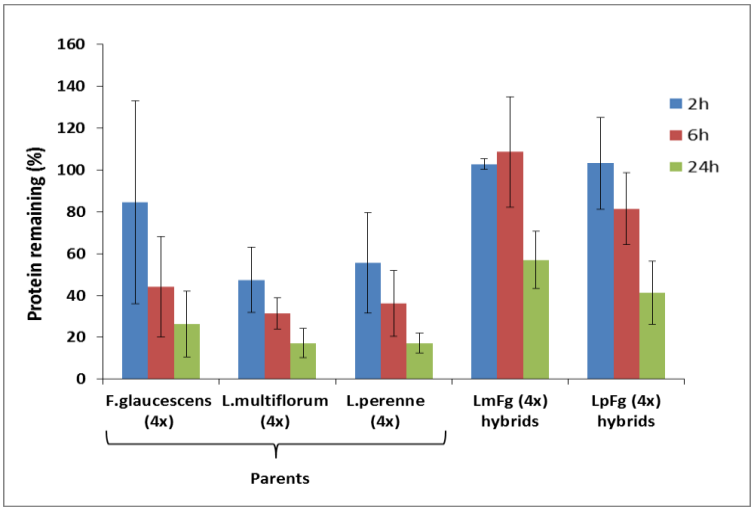


Figure 4.8 Protein degradation (% remaining) in tetraploid (4x) parents and *Lolium spp.* x *Festuca* hybrids with time of incubation in rumen like conditions

A comparison of the protein degradation curves of all contrasting genome groups arranged according to their respective Lm x Fg and Lp x Fg species is shown in Figure 4.9 (a) and (b) including significance of initial and residual (0 h/24 h) protein half-lives. Within the Lm x Fg genome groups (Fig. 4.9a) the Fg (4x) parents ($p<0.001$/ns), the Lm (2x) parents (ns/ns) and the LmFg (3x) hybrids ($p<0.01$/$p<0.001$) all had similar protein half-life curves i.e. they had high initial protein content and a similar shaped protein degradation curve (more gradual protein loss) and similar amounts of residual protein. The Lm tetraploid (4x) parents ($p<0.001$/$p<0.001$), showed lower initial protein content but also a steeper curve (indicating more rapid protein loss) with lower residual content than the other groups. The LmFg (4x) hybrids ($p<0.001$/$p<0.001$) had comparatively low initial protein content, and had a distinctly flattened curve indicating little change in protein content over 24 h. These hybrids also had the highest residual protein. Comparisons within the Lp x Fg groups (Fig. 4.9b) shows that their protein half-life curves are highly similar. The Lp (2x) parent group ($p<0.01$/$p<0.05$) has the steepest curve starting with the highest overall protein content and ending with the lowest residual protein indicating rapid protein loss over 24 h. The Lp (4x) parent group ($p<0.001$/$p<0.001$) also has a similar steep curve to the Lp (2x) parents but had less initial protein content. The LpFg (3x) hybrid group ($p<0.01$/ns) also had relatively high initial protein but a more gradually declining curve indicating a slower loss of protein content over 24 h. The Fg (4x) parents ($p<0.01$/$p<0.05$) have a flatter but still declining curve and lower initial protein content indicating slower protein loss. The tetraploid LpFg hybrids ($p<0.05$/$p<0.05$) have the flattest curve with the lowest initial protein content and highest residual protein indicating they had the slowest rate of protein loss in this group. Comparisons overall between the two groups (Lm x Fg and Lp x Fg) generally show that Lm x Fg group had lower initial protein content and that the LmFg (4x) tetraploid hybrids had the slowest rate of protein degradation. Fitted plots (Fig. 4.10) of the group that had the fastest rate of protein decay

(Lm 4x parents) were compared with the group that had the slowest rate of protein decay (LmFg 4x hybrids). This showed that there was a much wider range of foliar protein content amongst the Lm tetraploid (4x) parents than amongst the LmFg tetraploid (4x) hybrids at 0 h.

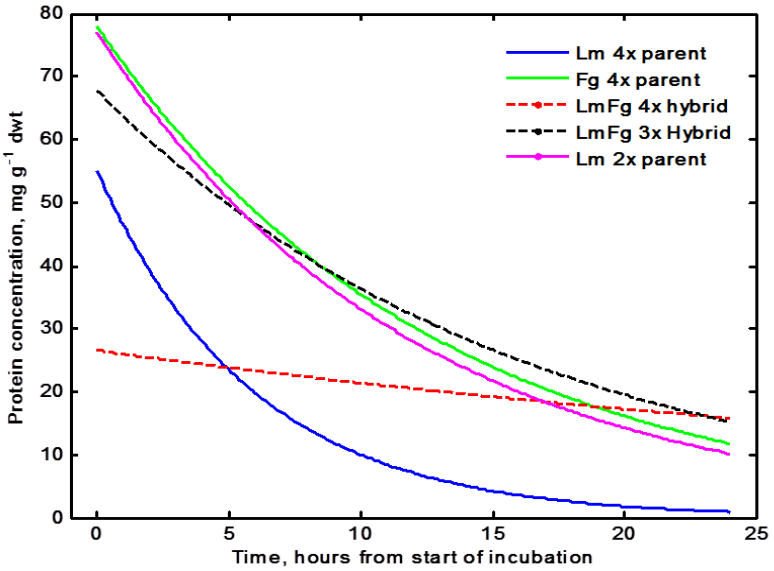


Figure 4.9a Comparison of fitted protein degradation curves (equation) arising from incubation of Lm x Fg groups in rumen like conditions from 0 – 24 h. Curve fitted by Matlab, n = 60 independent replicates from three replicated experiments (Introgression lines not shown)

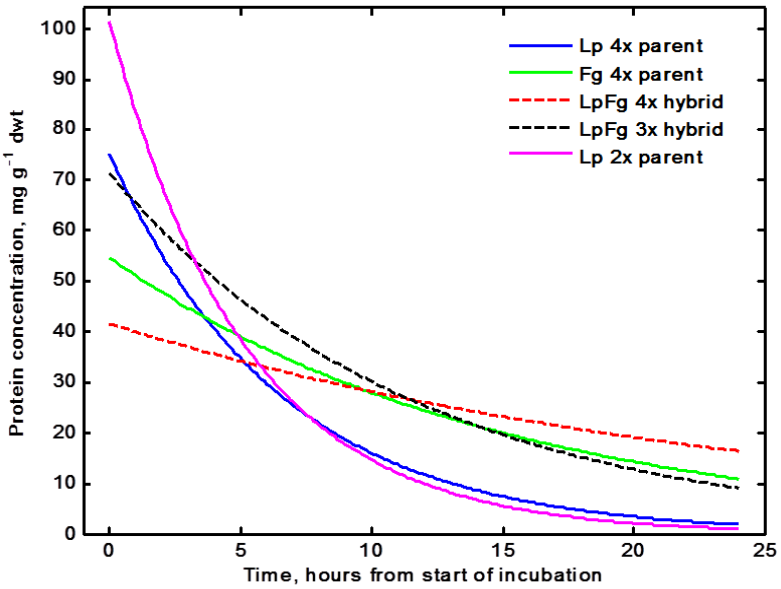


Figure 4.9b Comparison of fitted protein degradation curves (equation) arising from incubation of Lp x Fg groups in rumen like conditions from 0 – 24 h. Curve fitted by Matlab, n = 60 independent replicates from three replicated experiments.

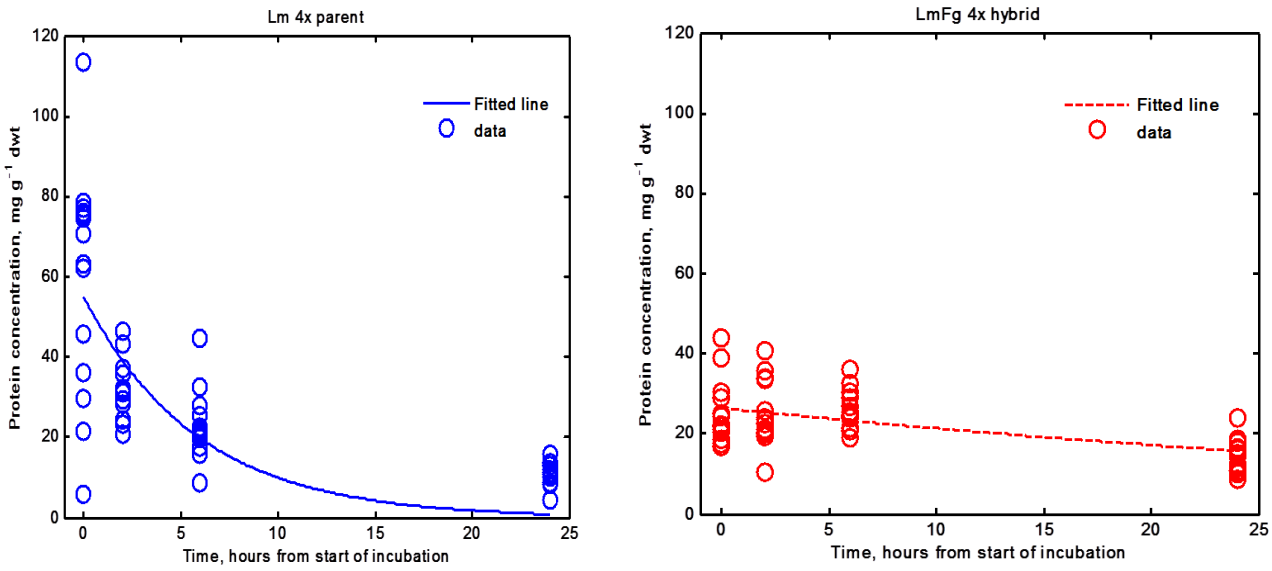


Figure 4.10 Fitted plots comparing the groups with the quickest rate of protein decay: Lm (4x) parents (blue circles, left) with the group with the slowest rate of protein decay LmFg (4x) hybrids (red circles, right). The tetraploid Lm (4x) parents have a much wider spread of sample results at 0 h than the tetraploid LmFg (4x) hybrids.

4.3 Potential alternative indicators of protein stability in *Lolium* and *Festuca*

4.3.1. The effect of *in vitro* incubation on the polypeptide profiles of *Lolium* spp, *Festuca glaucescens* and their hybrids

The aim was to determine if there were differences in the polypeptide profiles associated with differential protein stability in the leaves from *F. glaucescens* and *Lolium* spp. plants. Qualitative protein analysis was performed by polyacrylamide gel electrophoresis (SDS-PAGE). Sampling of selected incubated *F. glaucescens* and *Lolium spp.* plants was carried out for protein analysis using SDS-PAGE initially to visualise proteins stained with Coomassie blue dye followed by western blotting (Fig. 4.11; 4.12) of selected plant samples probed for heat shock proteins (Hsp 70 and Hsp 101) as described in Chapter 3 (Materials and Methods).

Protein composition assessed by SDS-PAGE reflected the protein abundances shown previously (Chapter 4) where triploid hybrids contained three times (LmFg) and four times (LpFg) the foliar protein of tetraploid hybrids. This was most noticeable where hybrids comprised *L. perenne* as compared to those with *L. multiflorum.* In group one the LmFg (4x) tetraploid hybrids (Fig. 4.11A.)

had very few, faint polypeptide bands (arrows) visible at 50 kDa at 0 and 2 h, while a 25 kDa protein band was visible up to 6 h.

By contrast, in the LmFg (3x) triploid hybrids (Fig. 4.11B) bands were clearly visible at 50 kDa, 25 kDa and 15 kDa at 0 h and 2 h with no bands visible at 6 h or 24 h. The LpFg (4x) hybrids (Fig.4.11 C) also had very faint bands at 50 kDa, 25 kDa and 15 kDa which were strongest at 2 h. By contrast, in the LpFg (3x) hybrids (Fig. 4.11D), polypeptide bands were visible at approximately 145 kDa at 0 h and 2 h. Polypeptide bands at 50 kDa could be seen at 0 h, 2 h and 6 h and bands of ~40 kDa at 0 h. Bands were also visible at 25 kDa and 15 kDa at 0 h, 2 h and 6 h with no bands visible at 24 h. Overall the LpFg (3x) hybrids combined contained more abundant protein bands than the LpFg (4x) hybrids with more visible bands at the 6 h time point. No polypeptide bands were visible at 24 h in either group.

Protein samples from the tetraploid parents (combined genotypes) of *L. multiflorum* (4x), *L. perenne* (4x) and *F. glaucescens* (4x) and also their tetraploid hybrids (combined genotypes) (Lm x Fg and Lp x Fg, 4x) which were incubated in simulated rumen conditions over a period of 24 hours, were run on SDS-PAGE gels and probed for expression of heat shock protein 70 (Hsp70) by western blotting (as described in Materials and Methods). An exposure time of 5 minutes was found to reveal the cross-reacting bands most clearly with minimum background (Figure 4.12). The detection of cross-reactive peptides at around 70 kDa was not obvious for most samples although bands at other molecular weights were present representing non-specific or spurious cross-reaction. A weak band at around 70 kDa could be seen in the *F. glaucescens* parents (4.12. E) at 0 h.

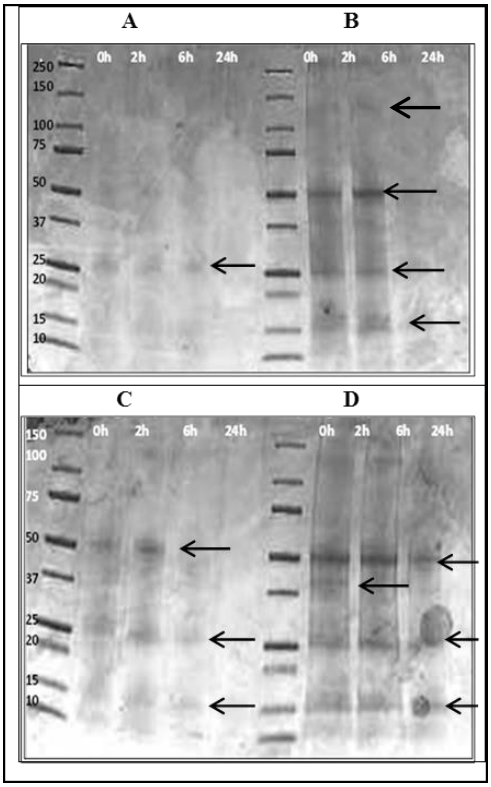


Figure 4.11 *Lolium spp*. x *F.glaucescens* hybrids (Combined genotypes) incubated in simulated rumen conditions *in vitro* and run on SDS-PAGE. Gels were stained with Coomassie blue dye for visualisation of polypeptide bands.-Plant groups: *L.multiflorum* x *F.glaucescens* (4x) hybrids (A), *L.multiflorum x F.glaucescens* (3x) hybrids (B), *L.perenne x F.glaucescens* (4x) hybrids (C) and *L.perenne x F.glaucescens* (3x) hybrids (D). Polypeptide bands of interest (arrows): 50 kDa, 37 kDa, 25 kDa and 15 kDa.

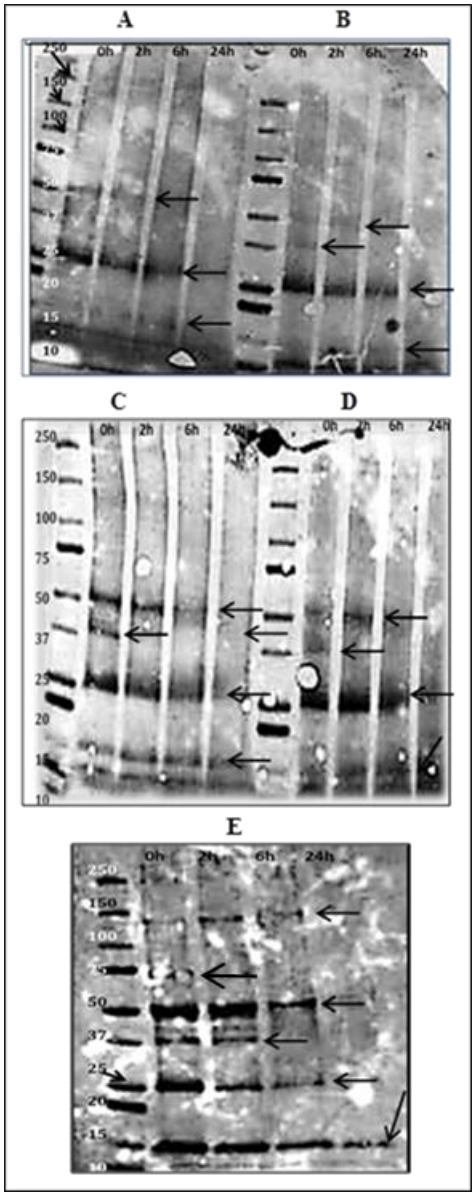


Figure 4.12 Tetraploid *Lolium* spp. and Fg (4x) parents and *Lolium spp.* x Fg (4x) hybrids probed for detection of Hsp 70. Combined samples (100 µl per group) were loaded in 20µl aliquots/lane plus molecular weight standard (M). Plant groups: Lm (4x) parents (A), LmFg (4x) hybrids (B), Lp (4x) parents (C), LpFg (4x) hybrids (D) and Fg (4x) parents (E). Polypeptide bands of interest (arrows): 150 kDa, 75 kDa, 50 kDa, ~45 kDa, 37 kDa, 25 kDa and 15 kDa.

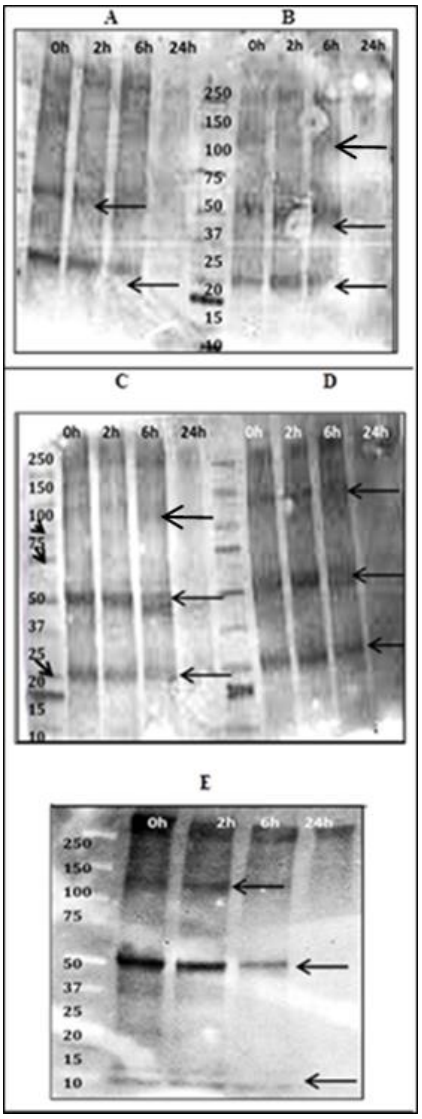


Figure 4.13 Tetraploid *Lolium* spp. and *F.glaucescens* parents (4x) and *Lolium spp.* x *F .glaucecens* hybrids (4x) probed for detection of heat shock protein Hsp 101 using western blotting. Plant groups: Lm (4x) parents (A) LmFg (4x) hybrids (B), Lp (4x) parents (C), LpFg (4x) hybrids (D) and Fg (4x) parents (E). Polypeptide bands of interest 100-150 kDa, 50 kDa, 25 kDa and 15 kDa.

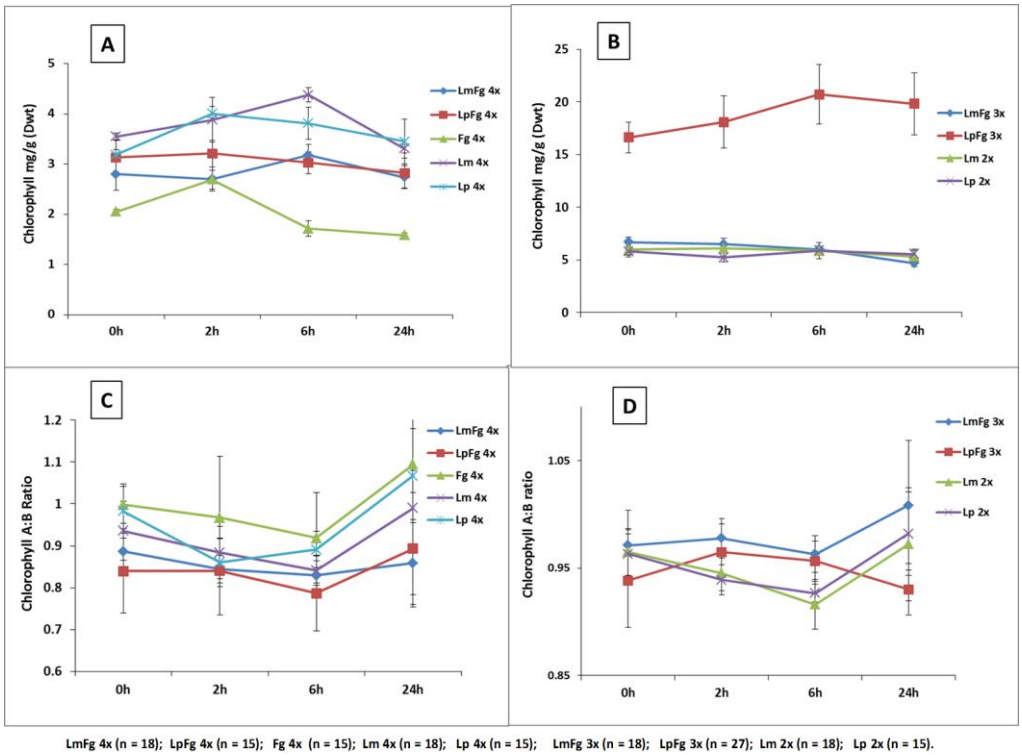
The tetraploid parents (combined genotypes) of Lm (4x), Lp (4x) and Fg (4x) and the tetraploid hybrids (combined genotypes) LmFg and LpFg (4x) were also probed for detection of heat shock protein 101 (Hsp101). Here a 2 minute exposure time was optimal (Figure 4.13, above). As with Hsp 70, the detection of cross-reactive peptides at around 100 kDa was not very obvious but some weak bands are visible around 110 kDa from 0 h - 6 h (Fig.4.13 A and B). However, bands of between 100 -

150 kDa molecular weight could be seen in the LpFg (4x) hybrids at 0 – 6 h (Fig. 4.13 D) and in the *F.glaucescens* (4x) parents at 0 – 2 h (4.13 E).

4.3.2 Investigations of chlorophyll degradation in incubated plants *in vitro*

Aliquots of the extracts prepared for protein analysis (as described in Materials and Methods) were also analysed for chlorophyll content. The change of protein content (mg/g dwt), chlorophyll content (mg/g/dwt) and chlorophyll A:B ratios in the plant material over the 24 h incubation period were determined for each sample group. Comparisons of chlorophyll content between the tetraploid (4x) parent groups (Fig. 4.14) showed that it was highest overall in the two *Lolium* (4x) parent groups (Fig. 4.14A). The lowest chlorophyll content on average was found in the Fg (4x) parent group. The tetraploid hybrid groups had a similar pattern to their respective *Lolium* (4x) parents i.e. the Lp (4x) parent and the LpFg (4x) hybrid groups both appeared to peak at 2 h (ns) whilst the Lm (4x) parents and the LmFg (4x) hybrids appeared to peak at 6 h (ns). The Fg (4x) parents behaved differently as while they appeared to peak at 2 h (ns) they dropped in chlorophyll content relatively sharply at 6 h and to 24 h unlike the LpFg 4x and Lp 4x which dropped more gradually to the 24 h time point. Comparisons of chlorophyll content (mg/g DW) in the diploid parents and triploid hybrid groups (Fig. 4.14B) show that the LpFg (3x) hybrid group had markedly higher chlorophyll content (16.6 -19.8 mg/g DW) on average than any of the other groups which are all within a similar range.

The chlorophyll A:B ratios in the tetraploid parent and (4x) hybrid groups (Fig. 4.14.C) show that the Fg (4x) parents had the highest chlorophyll A:B ratio overall (0.91-1.09) but were significantly lower at 6 h ($p<0.05$). The Lm (4x) parents showed a similar overall pattern to the Fg (4x) parents but had a lower range of chlorophyll A:B ratio values (0.84-0.99) and were also significantly lower at 6 h ($p<0.01$). The Lp (4x) parents had the second highest overall chlorophyll A:B ratios (0.86-1.06) but were significantly lower at 2 h ($p<0.001$) and at 6 h ($p<0.01$). The tetraploid hybrid groups (LmFg and LpFg) both had lower overall chlorophyll A:B ratios (0.83-0.89/0.79-0.89) than any of the tetraploid parent groups and LmFg and LpFg behaved differently to each other and to the tetraploid parent groups. The LpFg (4x) hybrid group had the lowest chlorophyll A:B ratio on average but was only significantly different at 6 h ($p<0.05$). The diploid (2x) parents were similar to each other (0.92-0.97/0.93-0.98) and the LmFg (3x) hybrids (0.97-1.01) and LpFg (3x) hybrids (0.93-0.97) in their chlorophyll A:B ratios. However, there was not much change overall in the chlorophyll A:B ratios over 24 hours .



LmFg 4x (n = 18); LpFg 4x (n = 15); Fg 4x (n = 15); Lm 4x (n = 18); Lp 4x (n = 15); LmFg 3x (n = 18); LpFg 3x (n = 27); Lm 2x (n = 18); Lp 2x (n = 15).

Figure 4.14 Chlorophyll content (mg/g DW) (A and B) and chlorophyll A:B ratios (C and D) in *Lolium* spp. (2x and 4x), *F.glaucescens* parents (4x) and *Lolium* spp. x Fg hybrids (3x and 4x) incubated in simulated rumen conditions over 24 hours (p=0.05).

An analysis of variance (ANOVA) was carried out to assess any differences in the chlorophyll content between the parental and hybrid groups as depicted in Fig. 4.14 Results for the tetraploid parental groups showed that there was a significant difference in change of chlorophyll content between the three groups ($p<0.001$). There was a just significant difference over 24 h ($p<0.05$) when looking at time alone in the three groups together but there was no significant difference within groups over 24 h. The diploid parent groups and tetraploid hybrid groups had no significant differences in the change of chlorophyll content over time. The triploid hybrids did differ significantly in chlorophyll between the two groups ($p<0.001$) and there was a marginal significant difference over 24 h overall and within groups ($p<0.05$).

4.3.3 The relationship between chlorophyll and protein degradation in *in vitro* incubations

On a dry weight basis chlorophyll content was remarkably stable throughout and any apparent peaks are due to within-species variation at given time points as there were not any real differences. Changes in protein content (Fig. 4.15A and B) were more significant in contrast to changes in chlorophyll content following 24 hours incubation Fig. 4.15C and D).

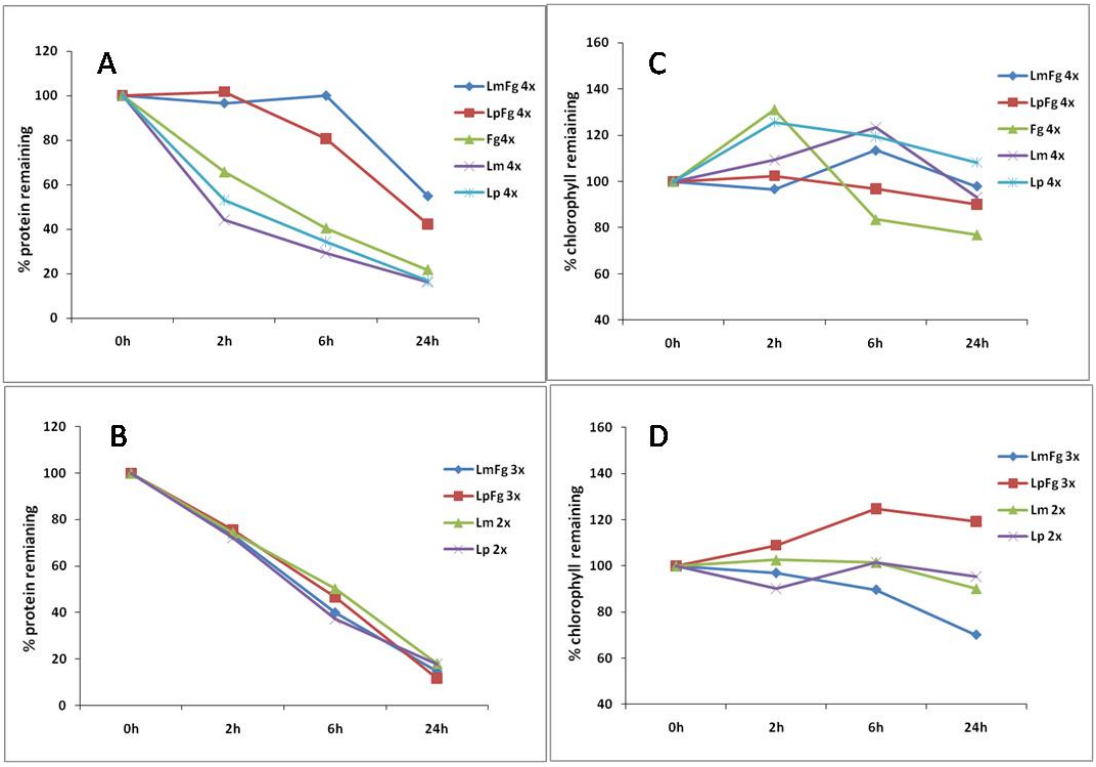


Figure 4.15 Comparisons between protein degradation and chlorophyll degradation in incubated plant material *in vitro* over 24 hours. Protein content (% loss) in the tetraploid (4x) parent groups combined (A) and triploid hybrid (3x) and diploid (2x) parent groups combined (B). Chlorophyll content (% loss) in the same incubated plants over 24 hours (C and D).

A correlation analysis was carried out between % protein degradation and % chlorophyll degradation of plant samples incubated for 24 hours with sampling time-points at 0 h, 2 h, 6 h and 24 h. Fig. 4.16 shows that there was no correlation between protein and chlorophyll degradation when leaves of these plants were incubated under rumen like conditions. The (weakly) positive linear relationship $r^2 = 0.154$ was non-significant ($p = 0.310$), ($y= 0.1996x +73.45$). It can be seen that most of the samples saw little or no real change in chlorophyll content from 0 h- 24 h relative to protein loss. The tetraploid hybrids were noticeably lower down on the x axis (protein % loss) which shows their noticeably lower protein loss relative to their chlorophyll loss. The LpFg (3x) hybrids (blue triangles) are clustered high up on the x axis indicative of their higher protein loss relative to chlorophyll loss. The LmFg (3x) hybrids on the other hand are clustered towards the centre and top right of the graph indicating they have greater protein chlorophyll loss relative to protein loss on average. One Fg (4x) parent (blue square) is placed among the LmFg (4x) hybrids (purple crosses) indicating lower protein loss and no loss of chlorophyll but two of the remaining Fg (4x) parents and Lm (4x) parents are in the centre/top of the graph indicating that they have lost more protein and chlorophyll combined.

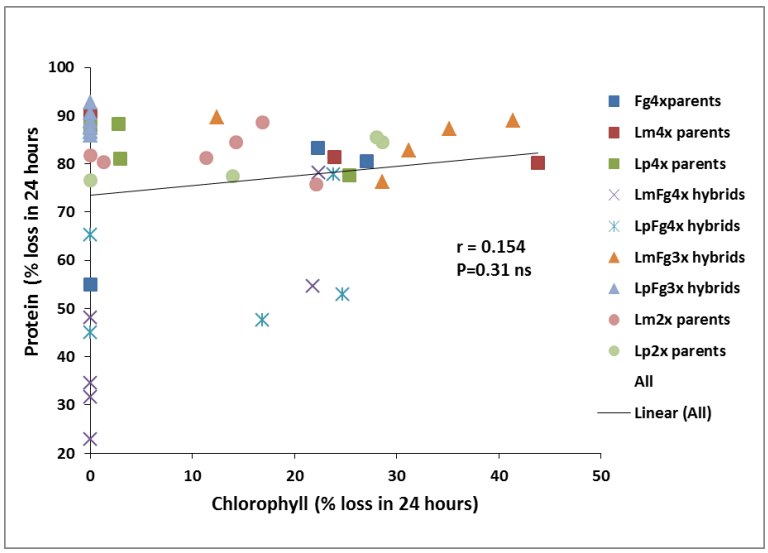


Figure 4.16 Correlation analysis between % loss of protein (mg/g DW) and % loss of chlorophyll (mg/g DW) in groups with individual genotypes. The weakly positive linear relationship $r^2 = 0.154$ is not significant (ns, p = 0.310), (y= 0.1996x +73.45).

5. Discussion

5.1 Effects of plant genome dosage on rumen initiated plant-mediated proteolysis

Over a 24h period the LmFg and LpFg F_1 hybrids were significantly more stable than their tetraploid (4x) Lm ($p<0.001$), Lp ($p<0.01$), and Fg ($p<0.05$) parental genotypes and there was evidence of highly significant *Lolium-Festuca* genome interaction and heterosis. Indeed, it would be impossible to predict the protein stability of the F_1 based solely on the protein half-lives of their respective *Lolium* or *Festuca* parents. Protein half-lives in the majority of the LmFg F_1 hybrids studied frequently far exceeded 24h, whereas the protein stability in both their Lm (4x) and Fg parents were consistently lower. When the same genotypes used by Shaw (2006), were incorporated in the current study, the protein half-lives were remarkably consistent. The LmFg F_1 hybrid used by Shaw (2006) and here, P188/48(1) had a protein half-life of 9.2 h and was the most unstable of the LmFg F_1 hybrids assessed. Despite a circa 10 year time difference, the consistency in result for protein stability with those obtained previously by Shaw (2006), using the same plant genotypes but different protocols, provided excellent support to the rigour of procedures used both herein and previously. Kingston-Smith *et al*., (2006), showed in white clover when given similar rumen-simulated conditions that 50% of protein had degraded under 6 h and 80 % after 24 h.

In future, more accurate measures for protein stability for LmFg F_1 would be achieved through the use of a greater numbers of sample time points and these could be extended beyond the current 24 hours. However, as ingested forage is not retained within the rumen for more than 24h, testing for protein retention >24h may not have relevance to achieving improved efficiency in ruminant nutrition.

An alternative method to measure protein stability in all the plant material used in this study was to calculate percentage loss of protein content from 0 h to each subsequent time point (2 h, 6 h, and 24 h). This was a useful measure of protein stability when making comparisons between the plant groups particularly at 2 h and up to the 6 h time point as most of the protein degradation activities by the ingested forage and the rumen microbial communities has taken place (Kingston-Smith *et al.*, 2003; Huws *et al*., 2013). The calculation of the protein half-lives of the ingested plant material is a more accurate measure for protein stability over 24 hours (and beyond) and may give an estimate of the availability of protein that may be indigestible to the rumen microbes but be used later on in the digestion system in the Abomasum (true stomach) which benefits the host animal (Kingston-Smith *et al.*, 2003).

Microbial community activities in the rumen ecosystem are a major factor to consider when addressing the challenge of reducing the environmental footprint of ruminant livestock agriculture (Edwards *et al*., 2008). Forage quality in terms of structure (soluble/insoluble fibre) and nutrients (carbohydrates, proteins and lipids) to provide fermentable energy to breakdown proteins and produce more microbial protein can affect the balance of the microbial communities and have contrasting benefits or unwanted effects with regard to environmental protection. Studies are being carried out to observe the transformations and successional processes of these microbial communities over time and identify the species involved in response to changes in the ruminants feed (Edwards *et al*., 2008). The structural component of the *Lolium spp.* x *F.glaucescens* hybrids used and produced in this study may be an important aspect (in future investigations) when considering the possible effects on the rumen microbial populations due to the inheritance of more firmer leaf texture from the Fg parents in some of the *Lolium* spp. x Fg hybrids. Another aspect to consider are the increases in numbers of certain ruminal bacteria species such as the hyper-ammonia-producing species *Peptostreptococcus anaerobius* and *Clostridium sticklandii* which can increase the amount of ammonia lost to the environment when fermentable energy is limiting in the ingested forage which slows down microbial activities (Russell *et al.*, 1992; Russell & Rychlik, 2000; Edwards *et al.*, 2008). The *Lolium* spp. x Fg hybrids which are the most protein-stable in simulated rumen conditions i.e. the LmFg/LpFg tetraploid hybrids, could be investigated to see if they would reduce the populations of hyper-ammonia-producing bacteria in the rumen due to increased time available to them for assimilation of available fermentable energy.

The asynchrony between protein and carbohydrate availability is only in the first few hours of post ingestion and Huws *et al.*, (2013), demonstrated a successional change in microbial diversity associated with newly ingested forage and a recent Next Generation Sequence approach at IBERS has shown this to be associated with a change in microbial function between 2 h and 4 h (unpublished). Little is still known about the nature of rumen microbial biofilm communities that attach to freshly

ingested forage and how the type of forage will affect their behaviour. Future research will aimed at understanding more about these processes (Huws *et al.*, 2013).

There was considerable variation in protein stability within the Lm (4x) and within the Fg (4x) ($p<0.001$) parent genotype groups but when compared collectively as groups there was no significant difference between Lm and Fg. Indeed individual Lm (4x) were identified with greater stability than certain Fg genotypes. As mentioned previously, the protein stability of the parental Lm (or Lp) and Fg genotypes was generally a poor indicator of the extent of protein stability available within their F_1 hybrid progeny. These results show an opportunity to achieve a real benefit in use of *Festulolium* hybrids in crop improvement programmes where interactions between *Lolium* and *Festuca* genomes can provide benefits unavailable to either parental species on their own.

For the LpFg F_1, protein half-lives were more consistent and in some cases much lower than those calculated for the LmFg F_1. As with LmFg F_1, the LpFg F_1 were significantly more stable than their Lp ($p<0.01$) and their Fg ($p<0.05$) parent genotypes. Therefore in both F_1 hybrid groups comprising balanced *Lolium* and *Festuca* genomes, heterosis was evident. However, unlike the Lm and Fg genotypes investigated, there was a significant difference found in protein stability between the Lp and Fg parents ($p<0.05$). However, plant numbers sampled are probably too small and intraspecific variation for protein stability too great to infer much as to whether there is a real difference between Lm and Lm in protein protection and retention when subjected to rumen-like stress conditions.

Of potential interest in terms of attempting to explain the greater stability found in the LmFg and LpFg F_1 hybrids compared to their respective parents was their consistently lower protein content at 0 h prior to exposure to rumen conditions. Whilst the initial protein content of the F_1 hybrids was lower than their respective parents, following 24 h exposure to rumen-simulated conditions, the protein content of both parent and F_1 hybrid genotypes was very similar. Therefore, the difference between parents and hybrids was due to the greater rate of protein decline by the parent genotypes as compared to the F_1 hybrids. Indeed when percentage protein loss was measured across the 24 h time-points very little if any decline was observed amongst the LmFg and LpFg F_1 hybrid genotypes. Protein degradation in the early time period following ingestion of forage is considered to be important but despite the availability of peptide and amino acid substrates for microbial growth at this stage, the availability of energy will be determined (and possibly limited) by the extent of microbial colonisation and cell wall degradation (Johnson, 1976; Edwards *et al.*, 2008). Hence, the use of *Festulolium* hybrids that can decrease the rate of plant mediated proteolysis (perhaps through delayed cell wall degradation by the biofilms) has the potential to improve delivery of protein and energy and therefore benefit the host animal with increased nutrition and reduce nitrogenous waste to the environment.

The backcross 1 (BC_1) LpFg and LmFg hybrids derive from hybridisation between *Lolium* spp. (2x) and the amphiploid F_1 (4x) hybrids. Consequently they are triploids with unbalanced genomes comprising 2 genomes of *Lolium* and 1 genome of Fg. In terms of their protein stability when under

rumen-simulated conditions, all observable benefits evident in the F_1 were lost. Overall the LpFg (3x) hybrids had protein half-lives of only 5.4 h whilst the LmFg (3x) hybrids' protein half-life was 4.5 h. The LmFg (3x) were siblings (described in Humphreys *et al.*, 2005) but nevertheless were significantly different in their protein retention ($p<0.001$), an observation found previously by Shaw (2006). Despite their greater genetic diversity having derived from alternative parental genotypes, the protein stability of the LpFg (3x) genotypes was not significantly different.

Two genomes of *F. glaucescens* would appear to be required in combination with *Lolium* in order to optimise expression of the trait for protein stability under rumen conditions, or at least a balanced *Lolium-Festuca* genome complement. Whilst this would support an amphiploid breeding approach to ensure the presence of a full Fg genome complement, it would still not necessarily preclude the alternative introgression-breeding approach where heterozygous diploid introgression lines were produced that were balanced for relevant *Lolium* and Fg heteroalleles. An introgression breeding approach had been used previously for transfers of complex traits from *Festuca* into *Lolium* e.g. (Humphreys & Pašakinskienė, 1996) where a heterozygous combination of *Lolium* and *Festuca* alleles in an otherwise undisturbed diploid *Lolium* genotype provided for improved drought tolerance compared to its *Lolium* parent.

In order to assess whether increasing the genome complement of Lm or Lp from diploid to an autotetraploid complement would make a difference to protein stability, genotypes of different ploidy levels were compared. In fact, for Lm the opposite was the case with overall Lm (2x) having a protein half-life of 8.2 h compared with the Lm (4x) which was 4.5 h and significantly different ($p<0.05$). Of course genotype numbers examined were small and further examination and greater plant numbers will be required before any conclusions as to whether differences in protein stability exist between Lm (2x) and Lm (4x) genotypes. No significant difference was evident in protein stability between the Lp (2x) and Lp (4x) which might imply if more genotypes were investigated that a similar conclusion would be found for Lm. At this stage of our understanding the converse should also be considered that genome dosage within *Lolium* may be relevant to protein stability, and that Lp (2x and 4x) may, like Lm (2x and 4x) differ in protein stability. Further research is necessary to confirm the impact of genome number on the variability in protein stability between Lp and Lm (2x and 4x) with increased sample sizes required to elucidate whether they truly differ (or not).

5.2 Factors affecting plant-mediated proteolysis under rumen-simulated conditions

From the evidence obtained from the current investigation, the F_1 LmFg and LpFg hybrids should be the best material for increasing our understanding of the mechanisms involved in reducing plant mediated proteolysis in ingested plant cells under rumen conditions. Proxies as alternative measures for protein retention in the rumen would be very useful both from the aspect of their incorporation in high throughput phenotype screens and also to obtain deeper understanding of the mechanisms involved in mitigating impacts of plant mediated proteolysis. The identification of any proxy for

protein retention suitable for any practical use in procedures for plant selection would of necessity have to be apparent within the living crop itself and prior to its use as feed to livestock. The research on protein degradation described herein found that balanced genomes of Fg and either Lm and Lp would interact together to both achieve benefits to protein retention as compared to alternatives where either their *Lolium* or *Festuca* parent genomes were alone exposed to the same rumen-simulated stresses. A consistent and quite possibly important observation was the lower initial protein content of the LmFg and LpFg F_1 hybrids compared to their parental genotypes, and further investigations will be required to establish the importance and relevance of this observation in terms of its possible effect on protein retention. A similar finding was reported in white clover by Kingston-Smith *et al*., (2006) who stated that the extent of protein degradation in rumen conditions was dependent on the initial protein content.

Shaw (2006), recognising that Fg would more likely be adapted to heat stress than either Lm or Lp due to its Mediterranean origin, considered that some co-adaptation might exist within the fescue species to equivalent high temperatures found within the rumen. Several attempts using gel-based electrophoresis were undertaken in the current work aimed at differentiating protein composition of the F_1 hybrids and their *Lolium* and *Festuca* parents when exposed to rumen stresses. Indeed distinct protein bands were observed in the F_1 hybrids and/ or Fg that were not expressed in Lp or Lm over the four time points of exposure to rumen conditions. However, it is acknowledged that only very tentative conclusions could be drawn at this stage from this preliminary investigation. It is also acknowledged that any new protein bands that were evident could be the outcome of protein breakdown and not themselves necessarily indicative of the presence of an active protein-protective mechanism induced by tissues exposed to rumen-simulated conditions. However, their presence in Fg and/or the F_1 hybrids which have known greater protein stability, might suggest otherwise and would indicate a possible role in protein protection. In order to attempt to identify a specific protein protection mechanism for the F_1 hybrids, specific heat shock protein (HSP) expression was also investigated using SDS-PAGE and two antibody probes specific for Hsp 70 and Hsp 101. These antisera were raised and had been tested for specificity in *Arabidopsis*. However, it was clear from the work reported here that there was significant cross-reaction with non-target protein when grass proteins were under investigation. To continue this line of enquiry it is therefore suggested that new, antisera are raised to specifically target grass HSP proteins. Nevertheless certain polypeptide bands were apparent consistently in Fg, and particularly in the LmFg and LpFg F_1 hybrids that were not detectable in *Lolium*. In particular, a consistent increased expression of a protein band of approximately 100 kDa was evident over the 2 h time point in LpFg which has already stimulated further investigations with the polypeptide band extracted and now undergoing comparisons with known protein databases. At this stage, it can be concluded that the preliminary protein analyses have revealed definite differences in the protein composition of *Festulolium* F_1 hybrids and *Lolium* thereby adding support to their distinction and contrasting protein stability. Future research could involve

looking at the genes expressed as a result of the plants being exposed to the abiotic stresses they encountered when incubated in simulated rumen conditions which may also reveal which HSP's were expressed.

In the rumen, ingested plant tissue comprises a bolus of living cells reported to enter into a process of cell death which is distinct from that observed during the course of normal plant senescence (Beha *et al.,* 2002). In the normal course of events and plant development in the field, chloroplasts remain intact until late senescence (3 to 6 weeks) when there is loss of greenness. This is due to extensive degradation of chlorophyll when it is converted to tetrapyrolles, called non-fluorescent chlorophyll catabolites (NCC's), which are the end product of chlorophyll breakdown (Hörtensteiner, 2006). Whilst the loss of chlorophyll occurs naturally in plants undergoing senescence (Lim *et al*., 2007), it is also known to occur sometimes prematurely when plants are exposed to heat stress (Reynolds *et al.,* 1994; Fokar *et al.,* 1998; Al-Khatib & Paulsen, 1984). This led to Ristic *et al.,* (2007), to suggest the measure of chlorophyll content at the beginning of heat stress to be used as a proxy in order to determine the likely rate of chlorophyll loss and to assess overall plant heat tolerance. The research described in the current work showed that the more heat-tolerant Fg parents had significantly lower chlorophyll content than either the Lm (4x) or the Lp (4x) parent genotypes but not one of the three species showed evidence of any chlorophyll loss during their 24 h exposure to simulated rumen conditions, including heat stress at a rumen temperature of 39°C. Also, despite their different protein stability, no significant difference was found in chlorophyll content between the LmFg and LpFg F_1 hybrids and their respective *Lolium* parent genotypes. Also like their parental genotypes, there was no evidence of any chlorophyll degradation in the LpFg and LmFg F_1 over the 24 h rumen-simulation treatment. A correlation was undertaken between % protein and % chlorophyll loss over 24h exposures to rumen-like conditions using all the Lm (4x and 2x), Lp (4x and 2x), Fg, LmFg F_1, LpFg F_1, LmFg BC_1 and LpFg BC_1 genotypes studied. Collectively, despite their genome diversity, and contrasting protein retention, no correlation could be detected. It can be concluded that initial plant chlorophyll content measures were ineffective as proxies of protein stability in *Lolium*, *Festuca,* and *Festulolium* hybrids. Chlorophyll degradation over the 24 h time points was negligible (with the possible exception of LmFg BC_1) in any of the plant groups tested. The chloroplasts as reported previously in Beha *et al.,* (2002) when incubated under simulated rumen conditions may have remained intact without the loss of chlorophyll whilst elsewhere extensive protein breakdown had occurred.

Whilst plant chlorophyll content provided no obvious indicator of its protein stability, the chlorophyll a:b ratio present in Photosystem II (PSII) may provide us with greater scope. The chlorophyll a/b ratio in the LmFg and LpFg F_1 hybrids commenced lower and remained lower than in any other plant group assessed throughout the 24 h exposure to rumen conditions. According to the theory of optimal N partitioning within a leaf, the chlorophyll a/b ratio is expected to increase when leaf N content

decreases (Katajima & Hogan, 2003). The lower chlorophyll a/b ratio found in the LmFg and LpFg F_1 may be indicative of greater protein retention than that observed in their parent species and in the BC_1.

5.3 Assessing the potential value of gene introgression from *F. arundinacea var glaucescens* into *L. perenne*

The requirements for future grass variety development place ever greater emphasis on their efficiency in terms of their crop production capabilities, their nutrient and water requirements, their persistency in the face of increasing climatic stresses, and the impact their use may have over the environment as a whole, and in particular their impact on livestock nutrition. In this context *Festulolium* varieties are considered to contribute an important future role as they provide resilience to extreme weather events and efficiency in crop production based on their water and nutrient requirements and uptake capabilities borne in part from their characteristically deep root systems (Durand *et al*., 2007). The deep rooting trait was originally selected as an aid to an improved drought resistance (Durand *et al*., 2007; Alm *et al*., 2011), but also provides other ecosystem services, such as improving soil water retention and hence reduced runoff providing a new option for flood mitigation (Gregory *et al*., 2010; Macleod *et al.*, 2013). The cultivation of *Festulolium* with large and deep-rooting systems as an aid to increase the input deep into soils of atmospheric CO_2 in order to reduce the "carbon footprint" of grassland agriculture is being assessed currently (Humphreys *et al.,* 2013). The *Festulolium* hybrids described in the current work are based on both *Lolium multiflorum* and *Lolium perenne* combinations with the fescue species *Festuca arundinacea var glaucescens,* a species derived from Mediterranean regions with adaptations to drought and high temperatures (Humphreys *et al.,* 1997). The potential for agricultural use of the *Festulolium* combination of *L. multiflorum* and the fescue species had been considered previously and had led to its commercial development and use in France (Ghesquière *et al.,* 2010). However, the *L. perenne* x *Festuca arundinacea var glaucescens* amphiploid hybrid combination outside of IBERS as far as known (Humphreys, pers. comm.) does not exist. Similarly, the backcross breeding programme described in the current study that involved introgression of *Festuca arundinacea var glaucescens* genes into *L. perenne* has, it was thought (Humphreys, pers. comm.), never previously been attempted neither at IBERS nor elsewhere. The agronomic performance of the allotetraploid *L. multiflorum* x *Festuca arundinacea var glaucescens* and the *L. perenne* x *Festuca arundinacea var glaucescens* hybrid combination and also equivalent allotetraploid combinations that involved another fescue species derived from North Africa, *F. mairei* was assessed in a field trial at IBERS in 2013 and is described in Humphreys *et al.,* (2014). From a forage production and a forage quality perspective all *Festulolium* combinations compared very favourably with highly regarded current commercially available control *Lolium* varieties. Interestingly from the perspective of the work described in the current thesis based on three harvest cuts was that the plots of amphiploid *Lolium* with *Festuca arundinacea var glaucescens* genome combinations had low %total

protein and high %water soluble carbohydrate (WSC), considered an ideal combination to enhance ruminant nutrition and livestock gain and to reduce emissions of greenhouse gases by livestock.

In the current plant breeding work, fescue-specific phenotypic traits were identified that could relatively easily discriminate between *Lolium* and *Festuca* genotypes and their transfer and expression in *Festulolium* hybrids was monitored. Wide leaves, glaucous leaf colour, coarse leaf texture, and erect growth habit particularly evident in Fg made them distinguishable from Lm and especially Lp tetraploid genotypes whose high tiller number, more prostrate growth habit, narrow leaves, and soft leaf texture were quite distinct from Fg. These distinctions helped to make the Fg morphological traits readily identifiable in LpFg F_1 hybrids and in their BC_1 and BC_2 derivatives, following their backcross onto Lp (2x), demonstrating their heritability.

Although not scored amongst the parent genotypes, root growth as a known Fg trait was recorded amongst the BC_2 and was compared with their foliar growth at the same developmental stage. The majority of the BC_2 were Lp-like in terms of their high foliar growth and their root development but around 3% of the BC_2 had both high foliar growth and very large Fg-like root systems. Three of the BC_2 were rhizomatous, a trait not observed in Lp but identified previously by Morgan *et al.*, (2001) in Lm following a backcross breeding programme involving Fg genome transfers.

Simple sequence repeat (SSR) markers were selected initially that gave broad genome coverage with 2 SSR markers chosen to represent a chromosome arm of their each of the 7 linkage groups (i.e. 28 SSRs). The SSRs chosen were located at the distal regions of each linkage group, genome areas considered to be recombination "hot-spots" (King *et al.*, 2013) and as such, regions most likely to include Fg chromosome introgressions. Clearly, the numbers of markers selected initially was small and any conclusions subsequently drawn could only be indicative of the total frequencies of Fg introgressions that were actually present within the $LpLp^{Fg}$ BC_2 populations. Nevertheless the fescue-derived SSR markers taken together with the morphological screen for Fg traits provided convincing evidence that transfers of Fg genes and traits into Lp was highly amenable to a backcross breeding approach such as that described here. The SSRs selected were in the main polymorphic and suitable for discriminating between Lp and Fg genome sequences. Only three fescue-specific SSR markers were used in the final analysis: rv0154, rv0061, and hd3agt. These represented locations on three individual linkage groups, i.e. chromosomes 3, 4, and 7. As with all marker-assisted breeding it speeds up the plant breeding process because it removes the need to phenotype in the field and allows selection from within genetically segregating populations to ensure continued presence of the early heading trait (Humphreys *et al.*, 2005).

5.4 Conclusion

The Lp-like BC_2 population provides convincing evidence for the successful transmission of a wide range of Fg genes and traits and should provide a valuable resource for further genetic studies and germplasm of value for future use in plant breeding. It remains to be seen whether an Fg introgression

breeding approach will provide in a diploid Lp genotype an equivalent protein stability to that achieved by the F_1 LpFg hybrids under rumen conditions described in the current work. The introgression-mapping approach described in Shaw, (2006) failed to recover genotypes amongst the BC_2 that expressed the slow plant-mediated proteolysis trait. Identical parental genotypes were screened for protein half-lives under simulated rumen conditions in the current study and the results reported previously by Shaw (2006) largely verified. It was clear from observations throughout the current investigation, that the protein stability found in parental genotypes provided little or no indication for maintained expression in subsequent generations. For the fescue-derived trait for slow plant-mediated proteolysis an introgression-breeding approach, based solely on phenotypic selection will probably be unfeasible. If such an approach is ever to succeed, it will be necessary to find specific markers for marker-assisted-selection (ideally SNPs) from within the major gene sequences involved in relevant protein protection mechanisms. However, it was also evident that optimal expression for the protein stability trait may depend on complex interactions involving both *Lolium* and *Festuca* genomes. A genome-wide association study (GWAS) may prove more appropriate than a targeted introgression-mapping approach to achieve the desired and predicted expression for the slow plant-mediated proteolysis trait over generations. For many other desirable Fg traits, such as deep rooting, an introgression breeding approach would seem suitable.

The main premise of the current study was to establish the optimal procedures necessary to incorporate the Fg trait into *Lolium* forage grass species for improved protein retention by ruminants with the current knowledge and technologies available. The outcome from the work has shown that the amphiploid approach in both Lm x Fg and Lp x Fg F_1 hybrid combinations would be the most appropriate compared with the introgression approach. The agronomic performance of both hybrid combinations described in Humphreys *et al.,* (2014), provides further support for future commercial exploitation. The main requirements for future commercial varieties are distinctiveness uniformity and stability and whilst there is no question over their distinctiveness and potential for livestock farming, only time will tell whether the F_1 LmFg and LpFg hybrids have the required stability and uniformity necessary to permit their future cultivar development. The F_1 LmFg and LpFg had characteristically lower protein content than their parent genotypes and combined with their high sugar content (shown in Humphreys *et al.,* 2014) as a high energy resource for rumen micro-organisms provides a good agronomic trait combination for efficient protein stability. Evidence of novel proteins were evident in the F_1 hybrids when exposed to simulated rumen conditions and these may be indicate protein-protective mechanisms such as HSPs. A low initial and maintained chlorophyll a/b ratio in the F_1 hybrids may provide a "proxy" for their initial selection and for protein stable genotypes. The research presented provides a good foundation for further successful exploration of the potential of *Festulolium* for improved ruminant nutrition**.**

6. References

References:

Al-Khatib, K., & Paulsen, G.M. (1984). Mode of high temperature injury to wheat during grain development. *Physiologia Plantarum* 61: 363-368.

Alm, V., Busso, C., Ergon, Å., Rudi, H., Larsen, A., Humphreys, M. & Rognli, O. (2010). A QTL analysis and comparative genetic mapping of frost tolerance, winter survival and drought tolerance in meadow fescue (*Festuca pratensis*). *Theoretical and Applied Genetics*, 123: 369-382.

Armstead, I.P., Skot, L., Turner, L.B., Skot, K, Donnison, I.S., Humphreys M.O., & King I.P. (2005). Identification of perennial ryegrass (*Lolium perenne* L.) and meadow fescue (*Festuca pratensis* Huds) candidate orthologous sequences to the rice *Hd1(Se1)* and barley *HvCo1 CONSTANS* like genes through comparative mapping and microsynteny. *New Phytologist*, 167:239-247.

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts, polyphenol oxidase in *Beta vulgaris. Plant physiology* 24:1-15

Badreldin, N. & Goossens, R. (2013). A satellite-based disturbance index algorythm for monitoring mitigation strategies effects on desertification change in an arid environment. *Mitigation and adaptation strategies for global change* [1381-2386] Springer Science +Business media Dordrecht 2013.

Beauchemin, K.A., Kreuzer, M., O'Mara, F.P. & McAllister, T.A. (2008). Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture.* 48: 21-27.

Beha, E., Theodorou, M., Thomas, B. & Kingston Smith, A. (2002). Grass cells ingested by ruminants undergo autolysis which differs from senescence: implications for grass breeding targets and livestock production. *Plant, Cell & Environment,* 25: 1299-1312.

Bellarby J., Tirado R., Leip A., Weiss F., Lesschen J.P. &Smith P. (2013). Livestock greenhouse gas emissions and mitigation potential in Europe**.** *Global Change Biology* 19: 3-18, doi 10.1111/j.1365-2486.2012.02786.x

Bera, P., Francisco, J. & Lee, T. (2009). Identifying the molecular origin of global warming. *Journal of Physical Chemistry A,* 113: 12694-12699.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254

Brewer, M. & Prestat, C (2002). "Consumer attitudes toward food safety issues." Journal of Food Safety 22: 67-83.

Breese, E., Lewis, E. & Evans, G. (1981). Interspecies hybrids and polyploidy. *Philosophical Transactions of the Royal Society London. B, Biological Science*, 292: 487.

Buchanan-Wollaston, V (1997). The molecular biology of leaf senescence. *Journal of Experimental Biology*, 48: 181-199.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page,T and Pink, D (2003). The molecular analysis of leaf senescence: A genetic approach. *Plant Biotechnology Journal*, 1: 3-22.

Buckner, R.C., Boling, J.A., Burrus, II, P.B., Bush, L.P., & Hemken, R.A. (1983). Registration of "Johnstone" tall fescue. *Crop Science* 23: 399-400.

Buckner, R.C., Burrus, II, P.B., & Bush, L.P. 1977. Registration of "Kenhy" tall fescue. *Crop Science* 17: 672-673.

Canter, P., Pašakinskiene, I., Jones, R. & Humphreys, M. (1999). Chromosome substitutions and recombination in the amphiploid *Lolium perenne*× *Festuca pratensis* cv Prior (2n= 4x= 28). *Theoretical and Applied Genetics*, 98: 809-814.

Cao, M., & Sleper, D.A. (2001). Use of genome-specific repetitive DNA sequences to monitor chromatin introgression from *Festuca mairei* into *Lolium perenne*. *Theoretical and Applied Genetics* 103: 248-253.

Casler, M.D., Pitts, P.G., Rose-Fricker, C., Bilkey, P.C., & Wipff, J.K. (2001). Registration of "Spring Green" *Festulolium*. *Crop Science* 41: 1365-1366.

Casler, M.D., Van Santen, E., Humphreys, M.W., Yamada, T., Tamura, K., Ellison, N.W., Jackson, R.D., Undersander, D.J., Gildersleeve, R., Stendal, C., Reiter, M., & Opitz, C. (2009). Remnant oak savanna acts as refugium for meadow fescue introduced during nineteenth century human migration

in the USA, *In* T. Yamada, Spangenberg, G., ed. *5th International Molecular Breeding of Forage and Turf,* 2007, Sapporo, Japan. Springer Science + Business Media, LLC.p. 91-101.

Causton, D.R., (1987). *A Biologist's Advanced Mathematics.* Allen and Unwin.

Charmet, G., Ravel, C., & Balfourier, F. (1997). Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA. *Theoretical and Applied Genetics* 94: 1038-1046.

Cheong, H.Y., Chang, H-S., Gupta, R., Wang, X., Zhu, T., and Sheng, L (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress and hormonal responses in Arabdopsis. *Plant Physiology* 29: 661-677.

Cherdthong, A. & Wanapat, M. (2010). Development of urea products as rumen slow-release feed for ruminant production: a review. *Australian. Journal of Basic and Applied Science* 4: 2232-2241.

Church, D. (1988). *The Ruminant Animal: Digestive Physiology and Nutrition.* Prentice Hall Englewood Cliffs, NJ.

Dalgaard, R., Schmidt, J., Halberg, N., Christensen, P., Thrane, M. & Pengue, W. (2008). LCA of soybean meal. *International Journal of Life Cycle Assessment,* 13: 240-254.

Davies, W. (1960). The grass crop, its development, use and maintenance. London, UK: E & FN Spon Ltd.

De Batista, J.P., Bouton, J.H., Bacon, C.W. & Siegel, M.R. (1990): Rhizome and herbage production of endophyte-removed tall fescue clones and populations. *Agronomy Journal* 82: 651-654.

Debbarama. C., Khanna, V.R., Tyagi W., Rai M., & Meetei N.T. (2013). Wide hybridisation and embryo rescue for crop improvement in *Capsicum*. *Agrotechnology* S11:003. doi:10.4172/2168-9881.S11-003.

Dewhurst, R.J., Mitton, A.M., Offer, N.W., Thomas, C. (1996). Effects of the composition of grass silages on milk production and nitrogen utilisation by dairy cows. *Animal Science* 62: 25-34.

Draper, J., Mur, L. A. J., Jenkins, G., Ghosh-Biswas, G. C., Bablak, P., Hasterok, R. & Routledge, A. P. M. (2001). Brachypodium distachyon. A New Model System for Functional Genomics in Grasses. *Plant Physiol.* 127: 1539-1555.

Drew, M.C. (1997). Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annual Review of Plant Physiology and Plant Molecular Biology*.48: 3-250.

Durand, J.L., Bariac, T., Ghesquière, M., Biron, P., Richard, P., Humphreys, M., & Zwierzykowski, Z. (2007). Ranking of the depth water extraction by individual grass plants using natural ^{18}O isotope abundance. *Environmental and Experimental Botany* 60: 137-144.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A.G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., Younes, M. (2002). Hazard characterisation of chemicals in food and diet: dose response, mechanisms and extrapolation issues. *Food and Chemical Toxicology* 40, 237–282.

Dytham, C. (2003). *Choosing and Using Statistics: A Biologist's Guide* (2nd ed.) Blackwell Publishing Ltd.

Edwards, J.E., Huws, S.A., Kim, E.J., Lee, M.R.F., Kingston-Smith, A.H. and Scollan, N.D. (2008). Advances in microbial ecosystems and their consequences for ruminant agriculture. *Animal* 2:5 653-660.

Essad S. (1964). Etude comparative des hybrides reciproques *Lolium perenne* L. x *Festuca pratesis* Huds. et de leur descendre. *Annals Amelioration des Plantes* 14: 295-308.

Evans G.M., & Aung T. (1986). The influence of the genotype of *Lolium perenne* on homoeologous chromosome association in hexaploid *Festuca arundinacea*. *Heredity* 56: 97-103.

Fang, Z., BouwKamp, J.C. & Solomos, T. (1998). Chlorophyllase acivities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild-type of *Phaseolus vulgare* L. *Journal of Experimental Botany*. 49: 503-510.

Firkins, J L. (1996). Maximizing microbial protein synthesis in the rumen. *Journal of Nutrition* 126: 1347- 1354

Fokar, M., Blum, A., & Nguyen, H.T. (1998). Heat tolerance in spring wheat. II. Grain filling. *Euphytica*, 104: 9–15.

Gamborg O.L., Miller R.A. & Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50: 151-158.

Ghesquière, M., Humphreys, M. & Zwierzykowski, Z. (2010). *Festulolium* Hybrids: Results, Limits and Prospects. *Sustainable Use of Genetic Diversity in Forage and Turf Breeding*: 495-507.

Gordon, G.L. & Phillips, M.W.(1998). The role of anaerobic gut fungi in ruminants. *Nutrition Research Reviews*, 11:133-168.

Gregory, A.S., Webster, C.W., Watts C.W., Whalley, W.R., Macleod, C.J.A.., Joynes, A., Papadopoulos, A., Haygarth, P.M., Binley, A., Humphreys, M.W., Turner, L.B., Skot, L., & Matthews, G.P. (2010). Soil management and grass species effects on the hydraulic properties of shrinking soils. *Soil Science Society of America Journal*, 74: 753-761

Grigg,D.(1995). The pattern of world protein. *Geoforum*. 26: 1-17.

Haeckel, E. (1882) *Gramineae*. W.A. Engler & K. Prantl., Die natürlichen Pflanzenfamilien, 2:1-97. W. Engelmann, Leipzig.

Harper, J., Thomas, I., Lovatt, J. & Thomas, H. (2004). Physical mapping of rDNA sites in possible diploid progenitors of polyploid *Festuca* species. *Plant Systematics and Evolution.* 245: 163-168.

Hayward, M.D., Forster, J.W., Jones, J.G., Dolstra, O., Evans, C., Mcadam, N.J., Hossain, K.G., Stammers, M., Will, J., Humphreys, M.O., & Evans, G.M. (1998) Genetic analysis of *Lolium*. I. Identification of linkage groups and the establishment of a genetic map *Plant Breeding* 117: 451- 455

Hegarty, M., Yadav, R., Lee, M., Armstead, I., Scollan, N., Powell, W.A. & Skøt, L. (2013). Genotyping by sequencing enables mapping of fatty acid composition traits in *Lolium perenne* (L.). *Plant Biotechnology Journal*, 11:572-581.

Hoekstra, N., Schulte, R., Struik, P. & Lantinga, E. (2007). Pathways to improving the N efficiency of grazing bovines. *European Journal of Agronomy*, 26: 363-374.

Hörtensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual Review of Plant Biology*, 57: 55-77.

Howarth, C. & Ougham, H. (1993). Gene expression under temperature stress. *New Phytologist*, 125: 1-26.

Hume D.E. & Brock J.L. (1997). Morphology of tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) plants in pastures under sheep and cattle grazing. *The Journal of Agricultural Science* 129: 19-31.

Humphreys, J., Harper, J. A., Armstead, I. P. & Humphreys, M. W. (2005). Introgression-mapping of genes for drought resistance transferred from *Festuca arundinacea* var. *glaucescens* into *Lolium multiflorum Theoretical and Applied Genetics,* 110:, 579-587

Humphreys, M.O. (1989). Water-soluble carbohydrates in perennial ryegrass breeding. *Grass and Forage Science*: 44: 237-244.

Humphreys, M.O. & Thomas, H. (1993). Improved drought resistance in introgression lines derived from *Lolium multiflorum*× *Festuca arundinacea* hybrids. *Plant Breeding*: 111: 155-161

Humphreys, M.O. (2005). Genetic improvement of forage crops–past, present and future. *Journal of Agricultural Science,* 143: 441-448.

Humphreys, M.W. (1989). The controlled introgression of *Festuca arundinacea* genes into *Lolium multiflorum*. *Euphytica* 42: 105-116.

Humphreys, M. & Thomas, H. (1993). Improved drought resistance in introgression lines derived from *Lolium multiflorum*× *Festuca arundinacea* hybrids. *Plant Breeding* 111: 155-161.

Humphreys, M.W., & Ghesquière, M. (1994). Assessing success in gene transfer between *Lolium multiflorum* and *Festuca arundinacea*. *Euphytica* 77: 283-289.

Humphreys, M.W. & Thorogood, D. (1993). Disturbed Mendelian segregations at isozyme marker loci in early backcrosses of *Lolium multiflorum x Festuca pratensis* hybrids to *L.multiflorum*. *Euphytica,* 66: 11-18.

Humphreys, M.W., Thomas, H.M., Morgan, W.G., Meredith, M.R., Harper, J.A., Thomas, H., Zwierzykowski, Z. & Ghesquiere, M. (1995). Discriminating the ancestral progenitors of hexaploid *Festuca arundinacea* using genomic *in situ* hybridization. *Heredity,* 75: 171-174.

Humphreys, M.W., Pašakinskienė, I. (1996). Chromosome painting to locate genes for drought resistance transferred from *Festuca arundinacea* into *Lolium multiflorum*. *Heredity,* 77: 530-534.

Humphreys, M.W., Thomas, H., Harper, J., Morgan, G., James, A., Ghamari-Zare, A. & Thomas, H. (1997). Dissecting drought-and cold-tolerance traits in the *Lolium-Festuca* complex by introgression mapping. *New Phytologist*, 137: 55-60.

Humphreys, M.W., Canter P.J., & Thomas H.M. (2003) Advances in introgression technologies for precision breeding within the *Lolium-Festuca* complex. *Annals of Applied Biology*, 143:1-10.

Humphreys, M.W. Yadav, R., Cairns, A., Turner, L., Humphreys, J. & Skøt, L. (2006). A changing climate for grassland research. *New Phytologist*, 169: 9-26.

Humphreys, M.W., Gasior, D., Lesniewska Bocianowska, A., Zwierzykowski, Z. & Rapacz, M. (2007). Androgenesis as a means of dissecting complex genetic and physiological controls: selecting useful gene combinations for breeding freezing tolerant grasses. *Euphytica* 158: 337.

Humphreys, M.W. & Harper, J. (2008).*Festulolium loliaceum*, an understudied natural UK grass hybrid species that may provide benefits to UK grasslands withstanding the onsets of climate change. In *European Crop Wild Relatives* Jan 6., pp 7-9.

Humphreys MW, MacLeod CJA, Whalley WR, Turner LB, Farrell, MS, Ghesquiere M, Haygarth PM (2011). Designing grass cultivars for droughts and floods. In: *"Breeding strategies for sustainable forage and turf grass improvement"* Proceedings 29th Eucarpia Fodder Crops and Amenity Section Meetin 4th – 8th Sept 2011, Dublin Ireland.

Humphreys, M.W., Marshall, A., Collins, R. & Abberton M.A. (2012). Exploiting genetic and phenotypic plant diversity in grasslands. (Chapter 16) *Grassland Productivity and Ecosystem Services.* Lemaire G., Hodgson J., Chabbi A. (Eds). CAB International ISBN-13:978 1 84593 809 3 pp 148-157

Humphreys, M.W., Farrell, M.S., Detheridge, A., Scullion, J., Kingston-Smith, A. & O'Donovan S.A. (2013). Resilient and multifunctional grasslands for agriculture and environmental service during a time of climate change. In: *The Role of Grasslands in a Green Future. Threats and Perspectives in Less Favoured Areas*. *Grass. Sci. Eur.* Vol. 18, Eds. Helgadottir, A., Hopkins, A. ISBN 978-9979-881-20-9 pp 335-337.

Humphreys, M.W., O'Donovan, S.A., Farrell, M. S., Gay, A.P., & Kingston-Smith, A.H. (2014). Novel *Festulolium* (2n=4x=28) hybrids and their potential as productive, nutrient-use-efficient fodder for ruminants. *Journal of Food and Energy Security* (In press).

Huws, S. A**.,** Mayorga, O.L., Theodorou, M.K., Kim, E. J., Newbold C.J., & Kingston-Smith, A.H. (2013) Successional colonisation of perennial ryegrass by rumen bacteria. *Letters in Applied Microbiology*, 56: 186-196

Jauhar, P.P. (1975). Chromosome relationships between *Lolium* and *Festuca* (Gramineae). *Chromosoma* (Berl.) 52: 103-121.

Jauhar, P. (1993). Cytogenetics of the *Festuca-Lolium* complex: relevance to breeding. Springer-Verlag Berlin.

Jenkin, T.J. (1933). Interspecific and intergeneric hybrids in herbage grasses. Initial crosses. *Journal of Genetics* 28: 205-264.

Johnson R.R. (1976). Influence of carbohydrate solubility on non-protein nitrogen utilization in the ruminant. *Journal of Animal Science* 43, 184–191.

Jones, E., Mahoney, N., Hayward, M., Armstead, I., Jones, J., Humphreys, M., King, I., Kishida, T., Yamada, T. & Balfourier, F. (2002). An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. *Genome* 45: 282-295.

Jones, N., Ougham, H. and Thomas, H. (1997) Markers and mapping: we are all geneticists now. *New Phytologist* 137: 165-179.

Kamra, S. K. (1972) Comparative studies on germinability of *Pinus silvestris* and *Picea abies* seed by the indigo carmine and x-ray contrast methods. No. 99:1-21.

Katajima, K, & Hogan, K.P. (2003). Increases in chlorophyll a/b ratios during acclimation of tropical woody seedlings in nitrogen limitation and high light. *Plant Cell and Environment*, 6:857-865.

Kebreab, E., France, J., Beever, D. & Castillo, A. (2001). Nitrogen pollution by dairy cows and its mitigation by dietary manipulation. *Nutrient Cycling in Agroecosystems*, 60: 275-285.

Kell D. B. (2011) Breeding crop plants with deep roots: their role in sustainable carbon, nutrient and water sequestration. *Annals of Botany*, 108: 407–418.

King, J., Roberts, L., Kearsey, M., Thomas, H., Jones, R., Huang, L., Armstead, I., Morgan, W. & King, I. (2002). A demonstration of a 1: 1 correspondence between chiasma frequency and recombination using a *Lolium perenne/Festuca pratensis* substitution. *Genetics,* 161: 307

King, J., Armstead, I., Harper, J., Ramsey, L., Snape, J., Waugh, R., James, C., Thomas, A., Gasior, D., Kelly, R., Roberts, L., Gustafson, P. & King, I. (2013a). Exploitation of interspecific diversity for monocot crop improvement. *Heredity* 110: 475-483.

King, J., Armstead, I., Harper, J. & King, I. (2013b).Transmission Frequencies of Introgressed *Festuca pratensis* Chromosome Segments in *Lolium perenne*. *Crop Science* 53:1968-1373

Kingston-Smith, A. & Theodorou, M. (2000). Post-ingestion metabolism of fresh forage. *New Phytologist,* 148: 37-55.

Kingston-Smith, A.H., Bollard, A.L., Humphreys, M.O., & Theodorou, M.K. (2002). An assessment of the ability of the stay-green phenotype in Lolium species to provide an improved protein sypply for ruminants. *Annals of Botany*, 89, 731-740.

Kingston-Smith, A.H. & Thomas, H.M. (2003). Strategies of plant breeding for improved rumen function. *Annals of Applied Biology*. 142:13-24

Kingston-Smith, A., Merry, R., Leemans, D., Thomas, H. & Theodorou, M. (2005). Evidence in support of a role for plant-mediated proteolysis in the rumens of grazing animals. *British Journal of Nutrition,* 93: 73-79.

Kingston-Smith, A.H., Bollard, A.L., & Minchin, F.R. (2005). Stress-induced changes in protease composition are determined by nitrogen supply in non-nodulating white clover. *Journal of Experimental Botany*, 56, 745-753.

Kingston-Smith, A.H., Bollard, A.L., & Minchin, F.R. (2006). The effect of nitrogen status on the regulation of plant-mediated proteolysis in ingested forage; an assessment using non-nodulating white clover. *Annals of Applied Biology*, 149:35-42.

Kingston-Smith, A., Davies, T., Edwards, J. & Theodorou, M. (2008). From plants to animals; the role of plant cell death in ruminant herbivores. *Journal of Experimental Botany,* 59: 521.

Kingston-Smith, A.H., Edwards, J.E., Huws, S.A., Kim, E.J. & Abberton, M. (2010). Plant-based strategies towards minimising 'livestocks' long shadow'. *Proceedings of the Nutrition Society*, 2000 : 613-620.

Kingston-Smith, A.H., Marshall, A.H. & Moorby, J.M. (2013). Breeding for genetic improvement of forage plants in relation to increasing animal production with reduced environmental footprint. *Animal*, 7:79-88.

Kopecký, D., Loureiro, J., Zwierzykowski, Z., Ghesquière, M., & Doležel, J. (2006). Genome constitution and evolution in *Lolium* × *Festuca* hybrid cultivars (*Festulolium*). *Theoretical and Applied Genetics* 113: 731-742.

Kopecký D, Havránková M, Loureiro J, Castro S, Lukaszewski AJ, Bartoš J, Kopecká J, Doležel J (2010) Physical distribution of homoeologous recombination in individual chromosomes of *Festuca pratensis* in *Lolium multiflorum*. *Cytogenetic Genomemic Research*, 129: 162–172

Kotak, S., Vierling,E., Bäumlein, H., & von Koskull-Döring, P.(2007). A novel transcriptomic cascade regulating expression of heat stress proteins during seed development of *Arabidopsis*. *Plant Cell*. 19:182-195.

Krishna, P. (2003). Plant responses to heat stress. *Topics in Current Genetics*.4:73-101.
Lack, G. (2002). Clinical risk assessment of GM foods. *Toxicology Letters*, 127: 337-340.

Laemmli, U.K. (1970).Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lee, M.R., Jones, E.L., Moorby, J.M., Humphreys, M.O., Theodorou, M.K., MacRae, J.C. & Scollan, N.D. (2001). Production responses from lambs grazed on *Lolium perenne* selected for an elevated water-soluble carbohydrate concentration. *Animal Research* 50: 441-450.

Lee, M.R., Tweed, J.K., Cookson, A. & Sullivan, M.L. (2010). Immunogold labelling to localize polyphenol oxidase (PPO) during wilting of red clover leaf tissue and the effect of removing cellular matrices on PPO protection of glycerol-based lipid in the rumen. *Journal of Science, Food and Agriculture*, 90:503-510.

Lewis, E., Tyler, B. & Chorlton, K. (1973). Development of *Lolium-Festuca* hybrids. In *Annual Report of the Welsh Plant Breeding Station*, 1972, pp. 34-37.

Lewis, E., Humphreys, M. & Caton, M. (1980). Disomic inheritance in *Festuca arundinacea* Schreb. *Zeitschrift fur Pflanzenzüchtung*. 84: 335-341.

Levitt, J. (1972). *Responses of Plants to Environmental Stresses.*Physiological Ecology, Academic Press, New York, NY, USA.

Licker, R., Johnston, M., Foley, A.J., Barford, C., Kucharik, C.J., Monfreda, C., & Ramankutty, N (2010). Mind the Gap: How do climate and agricultural management explain the 'yield gap' croplands around the world? *Global Ecology and Biogeography.* (pp1-14) DOI: 10.1111/j.1466-8238.2010.00563.x

Lim, O.P., Kim, H.J. and Nam, G.H. (2007). Leaf Senescence. *Annual review of Plant Biology.* 58: 115-136.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry*, 193: 265-275.

MacLeod, C.J.A., Humphreys, M.W., Whalley, R., Turner, L., Binley, A., Watts, C.W., Skot, L., Joynes, A., Hawkins, S., King, I.P., O'Donovan, S. & Haygarth, P.M. (2013). A novel grass hybrid to reduce flood generation in temperate regions. *Nat. Sci. Rep.* DOI: 10.1038/srep01683

MacRae, J.C., & Ulyatt, M.J. (1974). Quantitative digestion of fresh herbage by sheep. 2. Sites of digestion of some nitrogenous constituents. *Journal of Agricultural Science*, 82: 309-319.

Malik CP, Thomas PT (1966): Karyotypic studies in some *Lolium* and *Festuca* species. *Caryologia* 19:167-196.

Matile P. (1982). *Protein degradation.* In: *Encyclopedia of Plant Physiology 14A:Nucleic Acids and Proteins in Plants* (eds D. Boulter & B. Parthier),pp.169-188. Springer Verlag, Berlin, Germany.

Matile, P., Hörtensteiner, S. &Thomas, H (1999). *Chlorophyll Degradation. Annual Review of Plant Physiology and Plant Molecular Biology.* 50:67-95.

McDonald, P., Greenhalgh, J. & Morgan, C. (2002). *Animal Nutrition.* Addison-Wesley Longman Ltd.

Merry, R., Lee, M., Davies, D., Dewhurst, R., Moorby, J., Scollan, N. & Theodorou, M. (2006). Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. *In vitro* and *in vivo* studies of nitrogen utilization. *Journal of Animal Science,* 84: 3049.

Miles, S., Brennan, M., Kuznesof, S., Ness, M., Ritson, C. & Frewer, L. (2004). Public worry about specific food safety issues. *British Food Journal,* 106: 9-22.

Miller, B.L. & Huffaker, R.C. (1985). Differential Induction of Endoproteinases during Senescence od Attached and Detached Barley Leaves. *Plant Physiology* 78*:* 442-446.

Miller, L., Moorby, J., Davies, D.R., Humphreys, M.O., Scollan, N.D., Macrae, J. C. & Theodorou, M.K. (2001). Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.): milk production from late-lactation dairy cows. *Grass and Forage Science*, 56(4): 383-394.

Mittler, R., Finka, A & Goloubinoff, P (2012). How do plants feel the heat? *Trends in Biochemical Sciences* 3: 118-125.

Moorby, J.M., Kingston-Smith, A.H., Abberton, M.T., Humphreys, M.O. & Theodorou, M.K. (2008).Improvement of forages to increase the efficiency of nitrogen and energy use by ruminants. In *42nd University of Nottingham Feed Conference.* University of Nottingham.

Moore, B.J., Donnison I.S., Harper, J.A., Armstead, I.P., King, J., Thomas, H., Jones. R.N., Jones, T.H., Thomas, H.M., Morgan, W.G., Thomas, A., Ougham, H.J., Huang, L., Fentem, T., Roberts, L.A., & King, I.P. (2005). Molecular tagging of a senescence gene by introgression mapping of a stay-green mutation from *Festuca pratensis*. *New Phytologist* 165: 801-806.

Morgan, W.G., Thomas, H., & Lewis, E.J. (1988). Cytogenetic studies of hybrids between *Festuca gigantea* Vill. and *Lolium multiflorum* Lam. *Plant Breeding* 101: 335-343.

Morgan, W.G., King, I.P., Koch, S., Harper, J.A., & Thomas, H.M. (2001). Introgression of chromosomes of F*estuca arundinacea var. glaucescens* into *Lolium multiflorum* revealed by genomic *in situ* hybridisation (GISH). *Theoretical and Applied Genetics,* 103:, 696-701

Morris, K., Thomas, H &Rogers, L.J. (1996). Endopeptidases during the development and senescence of *Lolium temulentum* leaves. *Phytochemistry.* 41:377-384.

Moss, A.R. Jouany, J-P. & Newbold J. (2000). Methane production by ruminants: its contribution to global warming. *Annales de Zootechnie* Vol. 49: 231-253.

Netzband, K., (1991): Breeding of tetraploid *Festulolium* fodder grasses with different maturity. In: Proceedings of the 16th EUCARPIA, Fodder Crops Section Meeting, Wageningen, the Netherlands, November 1990, 47—48.

Newbold, C. J. & Rode, L. M. (2006). Dietary additives to control methanogenesis in the rumen. In: *International Congress Series Greenhouse Gases and Animal Agriculture :An Update. Soliva RC, Takahashi J and Krezer M (eds) Proceedings of the 2nd International conference on Greenhouse Gases and Animal Agriculture Zurich, Switzerland.* 20th-24th September 2005.Vol.: 138-147.

Nolan, J.V. & Dobos R.C. (2005) . Nitrogen transactions in ruminants. In: Dijkstra, J., Forbes, J.M., France, J. (eds) *Quantitative Aspects of Ruminant Digestion and Metabolism, 2nd Edn.* CABI (pub), Cambridge (ISBN 0-85199-814-3)

Nocek, J., & Russell J. B. (1988). Protein and carbohydrate as an integrated system. Relationship of ruminal availability to microbial contribution and milk production. *Journal of Dairy Science*.71:2070

Nordlee, J., S. Taylor, et al. (1996). "Identification of a Brazil-nut allergen in transgenic soybeans." *New England Journal of Medicine* 334: 688.

Oweis, T. & Peden, D. (2008). Water and livestock. In: Rowlinson, P., Steele, M., Nefzaoui, A. (eds) *Livestock and Global Climate Change 2008*. Cambridge University Press, pp 19-20 (ISBN 978-0-906562-62-8).

Palmer, R., Croston, D., Garvey, A. & Mead, S. (2006). Marketing of beef and lamb in England: the role of EBLEX. *British Food Journal*, 108: 808-823.

Päsakinskienė, I., Griffiths, C., Bettany, A., Paplauskiene, V. & Humphreys, M.W. (2000). Anchored simple-sequence repeats as primers to generate species-specific DNA markers in *Lolium* and *Festuca* grasses. *Theoretical and Applied Genetics.* 100: 384-390.

Pearson K. (1990). On the criterion that a given system of deviation from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. *The London, Edinburgh and Dublin Philosophical Magazine and Journal of Science*, 50:157-175.

Pennell, I.R. & Lamb, C. (1997). Programmed Cell Death in Plants. *The Plant Cell*. 9:1157-1168

Pichard,G.R., Tesser, B.R., Vives, C., Solari,C., Hott, A., & Larraín, R.E (2006). Proteolysis and characterization of peptidases in forage plants. *Journal of Agronomy,* 98:1392-1399

Powell, J., C. Gourley, C.J.P., Rotz,C.A. and Weaver, D.M. . (2010). "Nitrogen use efficiency: A potential performance indicator and policy tool for dairy farms." *Environmental Science & Policy*. 13:217-228

Putterill, J., Robson, F., Lee, K., Simon, R., & Coupland, G. (1995). The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell,* 80:847-857.

Queitsch, C., Hong, S.W., Vierling, E., & Lindquist, S (2000). Heatshock protein 101 plays a crucial role in thermotolerance in Arabadopsis. *The Plant Cell*, 12: 479-492.

Reed, J.D. (1995). Nutritional toxicology of tannins and related polyphenols in forage legumes. *Journal of Animal Science*, 73: 1516-1528.

Reynolds, M.P., Balota, M., Delgado, M.I.B., Amani, I. & Fischer, R.A. (1994). Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. *Australian Journal of Plant Physioogy*, 21: 717-30.

Recommended Grass and Clover Lists (2012). In: *Recommended List of Hybrid Ryegrass Varieties 2012/2013*. Pub: British Grassland Society pp. 20.

Ristic, Z., Bukovnik, U., & Prasad, P.V.V. (2007). Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under heat stress. *Crop Science*, 47: 2067–2073.

Rodríguez, G. (2010) Sustainable Production as a Branded-Product Attribute in the UK Food Market: The Case of Lakeland Herdwick Lamb In: Rosa, F., & Nassivera, F., *Assessment of Customer Satisifaction at Farmgate markets (2013). Journal of International Food and Agri-business*, 25 : 146-170.

Ross G.J.S. (1987). *Maximum likelihood program, version 3.08.* Oxford: Numerical Algorithms Group.

Ross G.J.S. (1990). *Nonlinear estimation.* New York: Springer

Russell, J., O'Connor, J., Fox, D., Van Soest, P. & Sniffen, C. (1992). A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Jorunal of Animal Science*, 70 : 3551.

Russell, J.B., and Rychlik, J.L., (2000). The isolation, characterisation and enumeration of hyper-ammonia producing ruminal bacteria. *Asian-Australasian Journal of Animal Sciences* 13, 121–127.

Sakuraba, Y., Schelbert, S., Park, S-Y., Han, S-H., Lee, B-D., Andrès, C.B., Kessler, F., Hörtensteiner,S & Paek, N-C. (2012).Stay-green and chlorophyll, catabolic enzymes interact at light-harvesting Complex II for chlorophyll detoxification during leaf senescence in *Arabidopsis. The Plant Cell.* 24: 508-518.

Sapan, C.V., Lundblad, R.L. & Price ,N.C. (1999) Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry*, 29: 99–108.

Semagn K., Bjornstad A., & Ndjiondjop M.N. (2006). An overview of molecular marker methods for plants. *African Journal of Biotechnology* 5:2540-2568

Stebbins G.L. (1950) Variation and evolution in plants. Columbia University Press, New York

Shaw, R. K. (2006). Effects of gene transfer from *Festuca* to *Lolium* on plant-mediated proteolysis. In *IBERS:Animal and Microbial Sciences*, PhD Thesis: Aberystwyth University.

Sim, S., Chang, T., Curley, J., Warnke, S., Barker, R. & Jung, G. (2005). Chromosomal rearrangements differentiating the ryegrass genome from the Triticeae, oat, and rice genomes using common heterologous RFLP probes. *Theoretical and Applied Genetics*, 110: 1011-1019.

Sinclair, L., Garnsworth, P., Newbold, J. & Buttery, P. (1993). Effect of synchronizing the rate of dietary energy and nitrogen release on rumen fermentation and microbial protein synthesis in sheep. *Journal of Agricultural Science,* 120: 251-263.

Skøt, L,; Humphreys, J.; Humphreys, M.O.; Thorogood, D.; Gallagher, J.; Sanderson, R.; Armstead, I.P.; Thomas, I.D. (2007). Association of candidate genes with flowering time and water-soluble carbohydrate content in *Lolium perenne* (L.). *Genetics*, 2007, 177(1): 535-547

Skøt, L., Sanderson, R., Thomas, A., Skøt, K., Thorogood, D., Latypova, G., Asp, T. & Armstead, I. (2010). Allelic variation in the *Lolium perenne* L. (perennial ryegrass) FLOWERING LOCUS T (*LpFT3*) gene is associated with changes in flowering time across a range of populations. *Plant Physiology,* DOI:. 110.169870 v169871.

Smart, C.M. (1994). Gene expression during leaf senescence. *New Phytologist*. 126:419-448.

Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Mara, F. & Rice, C. (2008). Greenhouse gas mitigation in agriculture. *Phil. Philosophical Transactions of the Royal Society London. B, Biological Science,* 363 : 789.

Sullivan, M.L. & Hatfield, R.D. (2006). Polyphenol oxidase and *o*-diphenols inhibit post-harvest proteolysis in red clover and alfalfa'. *Crop Science, 46, 662-670.*

Sung ,D.Y., Kaplan, F., Lee,K.J. &Guy, C.L. (2003). Acquired tolerance to temperature extremes. *Trends in in Plant Science*. 8:179-187.

Theodorou, M.K., Kingston-Smith, A., & Abberton, M. (2008). Improvement of forages to increase the efficiency of nutrient and energy use in temperate pastoral livestock systems. *Archiva Zootechnia*, 11, 5-20.

Theodorou, M.K., Merry, R.J. & Thomas, H. (1996). Is proteolysis in the rumen of grazing animals mediated by plant enzymes? *British Journal of Nutrition*. 75:507-510.

Thomas, H. (1978). Enzymes of nitrogen mobilisation in detached leaves of *Lolium temulentum* during senescence. *Planta.* 142:161-169.

Thomas, H.M., Morgan, W.G. & Humphreys, M.W. (2003). Designing grasses with a future–combining the attributes of *Lolium* and *Festuca*. *Euphytica,* 133: 19-26.

Thomas, H. & Humphreys, M.O. (1991). Progress and potential of interspecific hybrids of *Lolium* and *Festuca*. *Journal of Agricultural Science*, 117: 1-8.

Thomas, H. & Stoddart, J.L. (1980). Leaf senescence. *Annual Review of Plant Physiology and Plant Molecular Biology.* 31: 83-111.

Thomas, H. & Stoddart, J.L. (1982). Leaf senescence. In: *Encyclopedia of Plant Physiology 14A:Nucleic Acids and Proteins in Plants* (eds D. Boulter & B. Parthier),pp.592-636. Springer Verlag, Berlin, Germany.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R. & Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature*, 418: 671-677.

Turner, L.B., Cairns, A., Armstead, I.P., Thomas, H., Humphreys, M.W. & Humphreys, M.O. (2008). Does fructan have a functional role in physiological traits? Investigation by quantitative trait locus mapping. *New Phytologist*, 179: 765-775.

Van Soest, P. J. (1963) Use of detergents in the analysis of fibrous feeds. I. Preparation of fiber residues of low nitrogen content. *Journal of Analytycal Chemistry*.46: 825-835.

Wang, J.P., & Bughrara, S.S. 2008. Evaluation of drought tolerance for Atlas fescue, perennial ryegrass, and their progeny. *Euphytica* 164: 113-122.

Webb, J.K., Cookson A., Allison G., Sullivan M.L. & Winters A.L. (2013). Gene expression patterns, localization, and substrates of polyphenol oxidase in red clover (*Trifolium pratense* L.) *Journal of Agricultural and Food Chemistry*, 2013, 61 , pp 7421–7430

Weekes, R., Allnutt,T., Boffey, C., Morgan, S., Bilton, M., Daniels, R., Henry,C.(2007). A study of crop-to-crop gene flow using farm scale sites of fodder maize (Zea mays L.) in the UK. *Transgenic research* 16: 203-211.

Weinberg, Z., Muck, R. & Weimer, P. (2003). The survival of silage inoculant lactic acid bacteria in rumen fluid. *Journal of Applied Microbiology*, 94(6): 1066-1071.

Wheeler,T. & von Braun, J. (2013). Climate change impacts on global food security.*Science* 341: 508-513.

WHO, (2000). Safety aspects of genetically modified foods of plant origin.
Report of a joint FAO/WHO Expert Consultation. World Health
Organisation, Geneva.

Wilkins, P. & Lovatt, A. (2004). Recent gains from forage grass breeding. *IGER Innovations, Aberystwyth University* 8: 18-21

Wilkins, R. & Jones, R. (2000). Alternative home-grown protein sources for ruminants in the United Kingdom. *Animal Feed Science and Technology*, 85: 23-32.

Wilson,J.R. & Mertens, D.R.(1995). Cell-wall accessibility and cell structure limitations to microbial digestion of forage. *Crop Science*. 35:251-259.

Winters, A.L., Minchin, F.R., Michaelson-Yeates, T.P.T., Lee, M.R.F. & Morris, P. (2008). Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. *Journal of Agricultural and Food Chemistry*, 56: 2817– 2824

Wodnar-Filipowicz, A., C. H. Heusser, *et al*. (1989). Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. *Nature*, 339: 150-152.

Xu,Y., Zhan,C & Huang, B. (2011). Heatshock proteins in association with heat tolerance in grasses. (review article). *International Journal of Proteomics.* Volume 2011: 1-11. doi.10.1155/2011/529648

Yamada, T., Forster, J., Humphreys, M.W., & Takamizo, T. (2005). Genetics and molecular breeding in *Lolium/Festuca* grass species complex. *Grass Science* 51: 89-106.

Zohary, D., & Feldman, M. (1962) Hybridization between amphiploids and the evolution of polyploids in the wheat (*Aegilops-Triticum*) group. *Evolution* 16: 44-61.

Zwierzykowski, Z., Tayyar, R., Brunell, M., & Lukaszewski, A.J. (1998). Genome recombination in intergeneric hybrids between tetraploid *Festuca pratensis* and *Lolium multiflorum*. *Journal of Heredity* 89: 324-328.

Zwierzykowski, Z., Lukaszewski, A.J., Naganowska, B., & Leśniewska, A. (1999). The pattern of homoeologous recombination in triploid hybrids of *Lolium multiflorum* with *Festuca pratensis*. *Genome* 42: 720-726.

Zwierzykowski Z, Kosmala A, Zwierzykowska E, Jones N, Jokœ W, Bocianowski J, 2006. Genome balance in six successive generations of the *allotetraploid Festuca pratensis* × *Lolium perenne*. *Theoretical Applied Genetics*, 113: 539–547.