Differential responses of tillers to floral induction in perennial ryegrass (*Lolium perenne* L.): implications for perenniality.

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ABSTRACT

Development of sustainable practices is an important goal in agriculture. One possibility involves the development of perennial cereal crops, but the mechanisms of perenniality first need to be understood. While in annual cereals flowering structures die following seed production, in perennial grasses, perenniality is achieved by maintaining at least one shoot in a vegetative state.

There are two views on perennating tiller origin in perennial grasses: some authors suggest that all over-wintering tillers flower in spring and summer, leaving spring-initiated tillers to perennate, while others indicate that spring-initiated tillers are too immature to survive summer conditions, thereby implying that flowering must be prevented in some over-wintering tillers. An understanding of perenniality will therefore require an understanding of flowering control in these species. Temperate perennial grasses have dual induction requirements for flowering, where plants become competent to perceive inductive signals following vernalisation, and flowering is initiated by inductive photoperiods. Two hypotheses were formulated to test these models. The 'environmental control hypothesis' stated that all adequately vernalised perennial ryegrass tillers would flower on sufficient exposure to inductive photoperiods. Alternatively, the 'spatial control hypothesis' stated that in addition to the environmental mechanisms, a spatial control mechanism acts to regulate flowering. Two experiments were conducted to test these hypotheses.

Perennial ryegrass and Italian (annual) ryegrass were induced to flower and differences between the annual and perennial habits at flowering time were observed. However neither hypothesis was proven. In the second experiment, flowering was studied in detail in individual tillers of perennial ryegrass. The eldest tiller flowered in all flowering plants. The second eldest tiller did not flower in 72% of plants with more than one reproductive tiller, while the third eldest tiller flowered in 94% of these plants. These data favour the spatial control hypothesis which suggests that a spatial regulatory mechanism might act to repress flowering in some competent perennial ryegrass tillers. These results were supported by studies of meristem morphology and by a preliminary gene expression study. Maintenance of older, established tillers in a vegetative state might allow the perennial plant a greater chance of survival during summer.

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And thanks to all of my family and friends, of which, my nephew Matthew was clearly the most supportive. When Matthew was about 9 years old, the following conversation took place:

MATTHEW: Aunty Mush is going to be famous. She's writing a book.

MATTHEW'S GRANDMA: Do you know what the book is about?

MATTHEW: No.

MATTHEW'S GRANDMA: Grass. How many people are going to want to read that?

MATTHEW: Oh. (Somewhat disappointed)

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ABBREVIATIONS

AP1	*APETALA 1*
CO	*CONSTANS*
FLC	*FLOWERING LOCUS C*
FT	*FLOWERING LOCUS T*
LD	long day
LpFT3	*Lolium perenne FLOWERING LOCUS T 3*
LpGAPDH	*Lolium perenne GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*
LpMADS3	*Lolium perenne MADS 3*
NISD	non-inductive short day
PCR	Polymerase chain reaction
PPF	photosynthetic photon flux
PRC2	Polycomb group Repressor Complex 2
RT-PCR	Reverse transcription-polymerase chain reaction
SD	short day
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
VIN3	*VERNALIZATION INSENSITIVE 3*
VRN1	*VERNALIZATION 1*
VRN2	*VERNALIZATION 2*
VRN3	*VERNALIZATION 3*

Chapter 1. Introduction

1.1. Sustainable agriculture

Current agricultural productivity is based on high input-high output methodologies, with impacts of climate change, and cost limitations of fossil fuels increasingly recognised (Humphreys et al., 2006; Glover et al., 2007). Development of more sustainable agricultural practices is an important goal which will need to include development of new and improved food crops that will withstand adverse environmental conditions and meet production demands. Future crops need to tolerate changing weather patterns (e.g. water stress, temperature extremes, increased ultra-violet light), pests and pollutants, scarce or expensive fossil fuels (limiting tillage and application of agrochemicals) and complement natural ecosystems (Humphreys et al., 2006; Glover et al., 2007).

The top ten harvested crops in the world by land use are all annual species which require intensive management (Glover et al., 2007). Annual crops need to be re-established each year, irrigated, fertilised, and treated with pesticides. These processes lead to loss of soil structure, erosion, leaching of nutrients, and contamination and silting of waterways (Langer, 1979; Wagoner, 1990; Cox et al., 2002). Several articles have been published promoting the potential value of perennial crops (Wagoner, 1990; Cox et al., 2002; Glover et al., 2007). A major advantage associated with perennial crops is improved soil structure due to increased rooting and less tillage which reduces soil erosion and water run-off (Langer, 1979; Wagoner, 1990; Cox et al., 2002). Annual soil losses are estimated at 39.6 and 15 tonnes ha^{-1} in annual maize and wheat crops, respectively, but only 0.7 tonnes ha^{-1} for perennial bluegrass (in Langer, 1979). Water run-off is estimated at around 25% for annual species and 11.6% in perennial bluegrass (in Langer, 1979). Improved soil stability may allow increased use of marginal environments such as hillsides. Additional advantages of perennials include tolerance to pests and water stress, potential for companion or complementary crops (e.g. nitrogen fixing legumes), improved soil nutrition, opportunities for off-season grazing, and reduced production costs.

There are two strategies for the development of perennial crops: transforming perennial species into crops and converting annual crops into perennials (Wagoner, 1990; Cox et al., 2002). Transforming perennials into crops would involve selection for genotypes with high seed yields and large grain size. While these characteristics have been valued in annuals for up to 10 000 years, most perennials have been subject to selection for other traits, such as vegetative growth in the perennial forage grasses. Suitable genotypes are probably present in the perennial grasses but breeding goals may be achieved earlier by interspecific hybridisation with closely related annual crops and introgression of desirable yield traits. Converting annuals into perennials would benefit from interspecific hybridisation with closely related perennials, with introgression of genes for the perennial habit and retention of the economic traits of the annual species. The latter strategy is being used in perennial wheat and perennial rice breeding programmes (Cai et al., 2001; Sacks et al., 2003b; Sacks et al., 2003a; Sacks et al., 2006; Sacks et al., 2007).

Since annual cereals make up more than half of the world's crops (Glover et al., 2007), research into regulation of perenniality in perennial grasses, for example perennial ryegrass (*Lolium perenne*), might provide information valuable to future cereal crop breeding programmes. In grasses, flowers are terminal structures which die following seed production. Therefore, in perennial plants, at least one shoot must be maintained in a vegetative state enabling survival in the next season. In annuals and biennials, vegetative growth ceases when shoots either switch to flowering or are repressed by flowering shoots (e.g. apical dominance). Development of perennial crops from annual species will require an understanding of flowering mechanisms, and of the genes controlling them.

1.2. Floral induction in grasses

Control of flowering time is critical for survival of a plant species. Flowering and seed production should occur during favourable weather conditions and coincide with availability of water and pollinators as well as flowering within the population allowing cross-pollination. The fine balance between environmental and endogenous cues ensures flowering occurs in the appropriate season.

Floral induction has been extensively studied in the model species *Arabidopsis thaliana*, and inroads have been made in recent years into elucidation of floral control in the grasses (Cockram et al., 2007; Trevaskis et al., 2007). Genetic mechanisms appear somewhat conserved between dicots and monocots (e.g. Tadege et al., 2003; Winichayakul et al., 2005; Dennis and Peacock, 2007; Alexandre and Hennig, 2008). There are four well-known genetically controlled flowering pathways in *Arabidopsis*: vernalisation, photoperiod, autonomous, gibberellin (GA) pathways, in addition to more recently identified light quality, ambient temperature and carbohydrate pathways (Figure 1.1; Mouradov et al., 2002; Simpson and Dean, 2002; Boss et al., 2004; Putterill et al., 2004; Ausin et al., 2005; Bernier and Perilleux, 2005). In temperate grasses, vernalisation, photoperiod, gibberellin and intrinsic earliness pathways have been identified (Cockram et al., 2007). Whereas flowering pathways appear to act redundantly in *Arabidopsis*, many temperate grasses have obligate requirements for vernalisation followed by inductive photoperiods before flowering can occur (Cooper, 1951; Cooper, 1960; Heide, 1994; Aamlid et al., 2000).

1.2.1 Vernalisation

Vernalisation in many temperate perennial grasses is achieved following exposure to several weeks of low temperatures and/or short photoperiods, and requirements appear to be specific to ecotype (Cooper, 1951; Cooper, 1960; Heide, 1994; Aamlid et al., 2000). The vernalisation requirement prevents plants from flowering in late summer when seed production would be unsuccessful with the onset of autumn and winter. In nature, vernalisation conditions are met by winter, and winter conditions vary with latitude, altitude and location (e.g. inland versus maritime). For example, nine to twelve weeks at temperatures below 6°C is typically sufficient to meet the vernalisation requirements of most central European varieties of perennial ryegrass (Cooper, 1960; Aamlid et al., 2000). However, Scandinavian ecotypes require longer exposure (twelve or more weeks), while some Mediterranean varieties require little or no vernalisation (Aamlid et al., 2000). Vernalisation is not required for flowering in the closely related annual Italian ryegrass (*L. multiflorum*), but flowering is advanced when plants are vernalised (Cooper, 1951). Vernalisation does not initiate any morphological changes in the shoot apical meristem, but as a result of vernalisation, plants become competent to

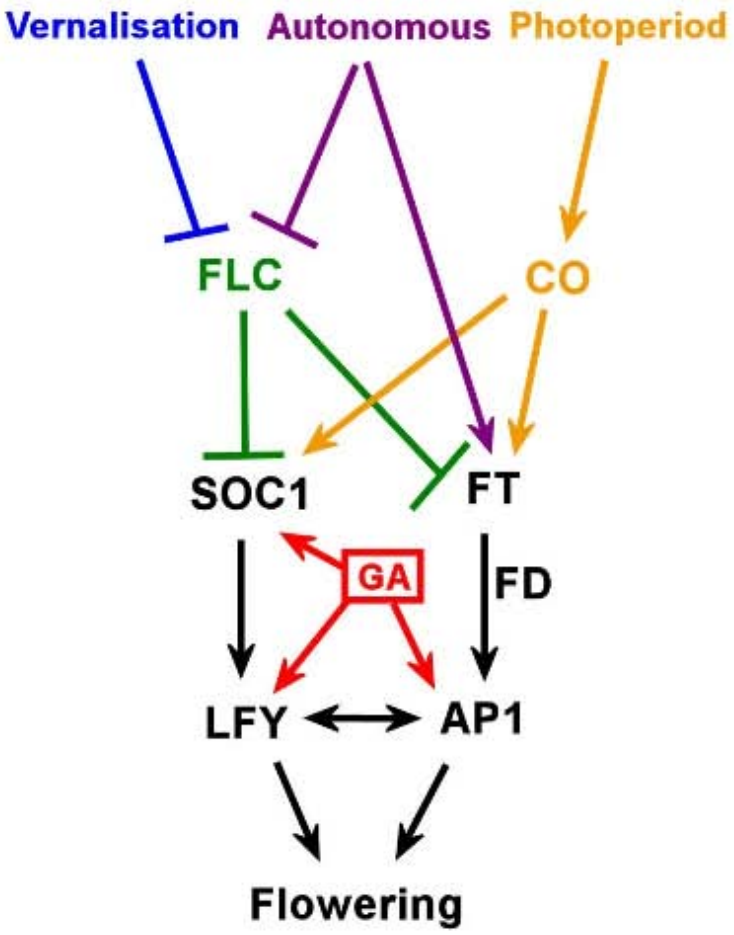


Figure 1.1 Four main flowering pathways in *Arabidopsis*.

Vernalisation and autonomous pathways repress *FLC*, a potent repressor of flowering. The autonomous, photoperiod and gibberellin (GA) pathways promote flowering via the floral integrators *SOC1* and *FT* which, along with GAs, promote expression of the meristem identity genes *LFY* and *AP1*. Abbreviations: *AP1 - APETALA 1*, *CO - CONSTANS*, *FD - FLOWERING TIME GENE D*, *FLC - FLOWERING LOCUS C*, *FT - FLOWERING LOCUS T*, *LFY - LEAFY*, *SOC1 - SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*. [Adapted from Ausin et al., 2005; Bernier and Perilleux, 2005.]

perceive floral inductive signals such as long photoperiods and/or warm temperatures (Chouard, 1960).

According to models elucidated in *Arabidopsis* (Figure 1.2), *FLOWERING LOCUS C* (*FLC*) encodes a potent repressor of flowering that represses transcription of the floral integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*). In plants with recessive *flc* alleles, *SOC1* and *FT* are not repressed and plants flower early. Vernalisation promotes flowering by repressing *FLC* expression (Michaels and Amasino, 1999; Sheldon et al., 1999; Helliwell et al., 2006; Searle et al., 2006).

Three complexes are known to maintain *FLC* chromatin in an actively transcribed state during vegetative growth (reviewed in Dennis and Peacock, 2007; Schmitz and Amasino, 2007). The FRIGIDA (FRI) complex is thought to associate with *FLC* chromatin and recruit a second complex, PAF1. PAF1 shares homology with the yeast RNA polymerase II associated factor which recruits methyltransferase activity, enabling transcription. A third complex, SWR1, shares homology to a yeast ATP-dependent chromatin remodelling complex involving histone replacement, giving actively transcribed chromatin.

Vernalisation in plants is mediated through changes to the chromatin structure (Bastow et al., 2004; Sung and Amasino, 2004). *Arabidopsis* plants treated with the demethylating agent 5-azacytidine showed advanced flowering (Burn et al., 1993; Finnegan et al., 1998). In addition, *Arabidopsis* plants carrying a *decreased demethylation 1* (*ddm1*) mutation, and transgenic plants with an antisense *METHYL TRANSFERASE 1* (*MET1*) transgene showed reduced DNA methylation, and advanced flowering (Burn et al., 1993; Finnegan et al., 1998). Winter wheat plants treated with 5-azacytidine had reduced methylation in their DNA, but demethylation alone was not sufficient to fully substitute for cold exposure (Brock and Davidson, 1994; Horvath et al., 2003). This may represent a difference between the facultative cold requirement in *Arabidopsis* and an obligate requirement in winter wheat.

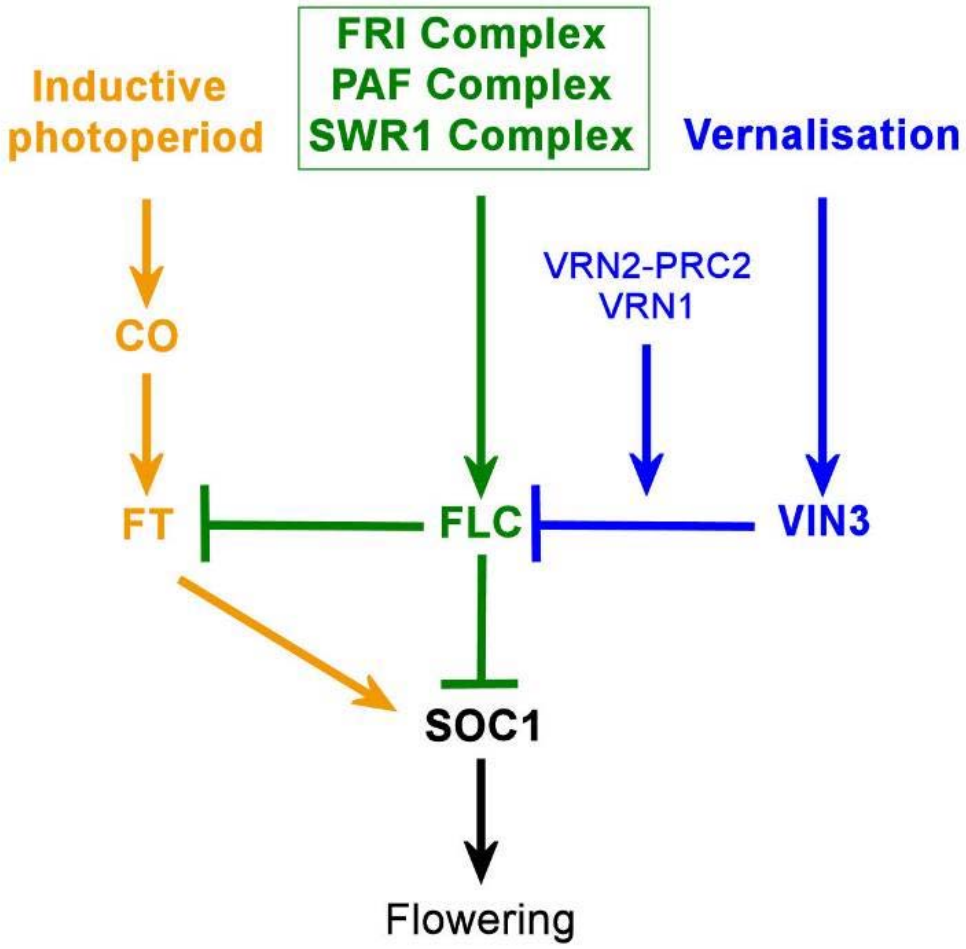


Figure 1.2 Regulation of *FLC* in *Arabidopsis*.

FLC expression is maintained by FRI, PAF and SWR1 complexes during vegetative growth. Vernalisation represses *FLC* expression by inducing inactivating chromatin modifications in the *FLC* regulatory region, thereby allowing expression of the floral integrators *SOC1* and *FT*. [Adapted from Cockram et al., 2007; Alexandre and Hennig, 2008.] Abbreviations: *CO* - *CONSTANS*, *FLC* - *FLOWERING LOCUS C*, FRI - FRIGIDA, *FT* - *FLOWERING LOCUS T*, PAF - RNA polymerase II associated factor, PCR2 - Polycomb repressor complex 2, *SOC1* - *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, SWR1 - SWI/SNF related protein, *VIN3* - *VERNALIZATION INSENSITIVE 3*, *VRN1* - *VERNALIZATION 1*, *VRN2* - *VERNALIZATION 2*.

VERNALIZATION INSENSITIVE 3 (VIN3) is required for initiation of the vernalisation response (Sung and Amasino, 2004). During vernalisation, *VIN3* expression levels are quantitatively upregulated by prolonged cold, and correlate with down-regulation of *FLC*. When plants are returned to warm temperatures, *VIN3* expression declines rapidly, and if the duration of cold exposure was insufficient, *FLC* expression levels return. *vin3* mutants subjected to prolonged cold have normal *FLC* expression levels and no histone modifications. VIN3 associates with cold-induced histone deacetylation in *FLC* chromatin, which may allow access to further chromatin modifiers. VIN3 is implicated in establishment of the vernalisation response, although other factors must be involved as overexpression of *VIN3* is not sufficient to substitute for cold treatment (Sung and Amasino, 2004).

VERNALIZATION 2 (VRN2) forms part of a Polycomb Repressor Complex 2 (PRC2) which shares homology with the PRC2 complex from the fruit fly, *Drosophila melanogaster*. VIN3 is thought to recruit a histone deacetylase to the *FLC* chromatin, enabling subsequent access by VRN2-PRC2. *vrn2* mutants are unable to maintain *FLC* repression, and flower late. VRN2 activity is believed to alter methylation of *FLC* chromatin (Gendall et al., 2001; Bastow et al., 2004; Wood et al., 2006).

VRN1 is also required for the vernalisation response. VRN1 is recruited to the *FLC* chromatin in association with VRN2-PRC2, and is thought to alter methylation state further. It is believed that VRN1 recruits LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) which is involved in epigenetic silencing of *FLC* (Levy et al., 2002; Bastow et al., 2004; Nakahigashi et al., 2005; Mylne et al., 2006; Sung et al., 2006).

Epigenetic silencing of *FLC* is mitotically stable and inherited within cell lines through mitosis. There is speculation that the actively transcribed state of *FLC* is reset in the next generation during meiosis or gametogenesis although the exact mechanisms are unclear (Mylne et al., 2006).

Other factors are involved in regulation of *FLC* (e.g. the autonomous pathway; Simpson, 2004), and there are *FLC*-independent pathways (e.g. *AGAMOUS-LIKE 24*; *AGL24*; reviewed in Alexandre and Hennig, 2008), but the mechanisms outlined above have the most significance to this study.

It has been known for more than a century that some grasses, such as winter wheat, have

a vernalisation requirement (Chouard, 1960). Vernalisation in temperate grasses is achieved by prolonged exposure to low temperatures and/or short photoperiods. It is believed that grasses originated from tropical regions, and that the vernalisation requirement evolved with species radiation into temperate climates (in Cockram et al., 2007). However, vernalisation models similar to those in *Arabidopsis* have been assembled for the grasses based mainly on research in barley and wheat (Figure 1.3; Table 1.1; reviewed in Cockram et al., 2007; Trevaskis et al., 2007; Alexandre and Hennig, 2008). New techniques and comparative genomics have advanced the understanding of the molecular genetics involved. Several vernalisation responsive loci have been identified in the grasses including *VRN1*[a], *VRN2*[a], *VRN3* and *VERNALIZATION TO REPRODUCTIVE TRANSITION 2* (*VRT2*).

VRN1 loci from diploid wheat (*Triticum monococcum*), hexaploid wheat (*T. aestivum*) and barley (*Hordeum vulgare*) were characterised in 2003 (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003), and three putative *VRN1* loci, *LpMADS1*, *LpMADS2* and *LpMADS3*, have been identified in perennial ryegrass (Petersen et al., 2004; Jensen et al., 2005; Petersen et al., 2006). Prior to vernalisation, *VRN1* is not expressed in vernalisation-sensitive winter wheat, winter barley or perennial ryegrass, and *VRN1* expression levels increase with vernalisation and long photoperiods. *VRN1* expression is constitutive in vernalisation-insensitive spring wheat, spring barley, and annual ryegrasses. Allelic variation at *VRN1* has been observed between winter and spring varieties (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Fu et al., 2005; von Zitzewitz et al., 2005; Petersen et al., 2006). *VRN1* is a member of the *A. thaliana APETALA 1* (*AP1*) clade of MADS box genes, but it appears to have functional similarities to *Arabidopsis* SOC1 (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Yan et al., 2004a; Ciannamea et al., 2006b). In perennial ryegrass, *LpMADS1* and *LpMADS2* are upregulated by vernalisation and appear to have a SOC1-like function while *LpMADS3* is upregulated by inductive photoperiods following vernalisation. LpMADS3 may have retained an AP1-like function (N. T. Forester, I. Kardailsky and B. Veit, AgResearch Ltd., unpublished).

[a] Grass *VRN1* and *VRN2* genes are different to *Arabidopsis VRN1* and *VRN2* genes.

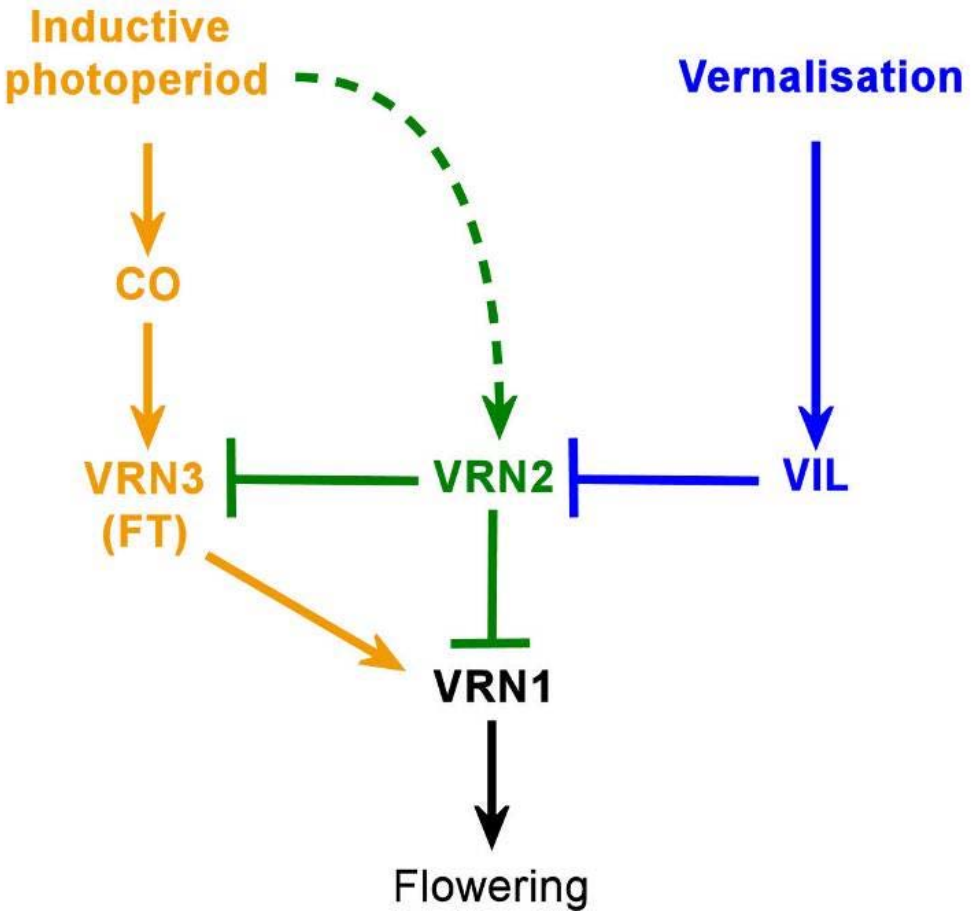


Figure 1.3 Vernalisation response in temperate grasses.

VRN2 acts as a potent repressor of flowering. Vernalisation represses *VRN2* expression, allowing expression of the floral integrators *VRN1* and *VRN3*. Abbreviations: *CO - CONSTANS*, *VIL - VERNALIZATION INSENSITIVE 3-LIKE*, *VRN1 - VERNALIZATION 1*, *VRN2 - VERNALIZATION 2*, *VRN3 - VERNALIZATION 3*. [Adapted from Cockram et al., 2007; Trevaskis et al., 2007; Alexandre and Hennig, 2008]

VRN2 was characterised as a repressor of flowering which is down regulated by vernalisation, correlating with upregulation of *VRN1* (Yan et al., 2004b). *VRN2* encodes a ZCCT transcription factor with Zinc finger (Z) and CO, CO-like and TOC1 (CCT) domains. CCT domains are often found in genes regulated by photoperiod (e.g. *CONSTANS*), and the presence of the CCT domain may suggest a mechanism enabling vernalisation by short photoperiods (Cooper, 1951; Cooper, 1960; Yan et al., 2004b; Dubcovsky et al., 2006; Trevaskis et al., 2006). VRN2 appears to act as a functional orthologue of *Arabidopsis* FLC in the grasses (Yan et al., 2004b).

Table 1.1 *Arabidopsis* vernalisation genes and encoded proteins, and their putative counterparts in the grasses.

Arabidopsis		**Grasses**	
VIN3	PHD finger domain	*VIL*	PHD finger domain
VRN1	DNA binding		
VRN2	Polycomb group		
LHP1	heterochromatin		
REF6	Jumonji	*JMJC*	Jumonji
FLC	MADS	*VRN2*	ZCCT
SOC1	MADS	*VRN1*	MADS
FT	Raf kinase inhibitor	*VRN3*	Raf kinase inhibitor
AGL24/SVP	MADS	*VRT2*	MADS

VRN3 is a homologue of the *Arabidopsis FT* gene which encodes a Raf kinase inhibitor. *VRN3* is upregulated in inductive photoperiods (as in *Arabidopsis*) and repressed by VRN2 (Yan et al., 2006). LpFT3 is a putative orthologue of FT in perennial ryegrass (Gagic, 2007).

VRT2 (*LpMADS10* in perennial ryegrass) encodes a SHORT VEGETATIVE PHASE (SVP) or AGL24-like MADS protein, and is thought to have a repressive effect on flowering at the *VRN1* promoter (Petersen et al., 2004; Ciannamea et al., 2006b; Petersen et al., 2006; Kane et al., 2007).

In addition, *VIN3-LIKE* (*VIL*) genes have been identified in wheat, barley and rice. Expression increased quantitatively during vernalisation (low temperature and short photoperiods) and declined when plants were returned to warm temperatures, similar to *Arabidopsis VIN3* expression (Fu et al., 2007). This suggests that prolonged cold might

be perceived in a similar way in dicots and monocots. A vernalisation-responsive *LpJMJC* gene, which encodes a Jumonji-C (jmj-C) domain protein has been identified in perennial ryegrass which may play a role in vernalisation-induced DNA demethylation (Ciannamea et al., 2006a). *REF6* encodes a jmj-C protein in *Arabidopsis* implicated in demethylation of *FLC* chromatin (Noh et al., 2004).

1.2.2 Photoperiodic induction of flowering

Once plants have been vernalised, they are competent to perceive floral induction signals such as long photoperiods and/or warm temperatures. Both perennial and Italian ryegrass varieties have obligate long photoperiod requirements (Cooper, 1951; Cooper, 1960; Aamlid et al., 2000). The photoperiod response has been studied in the long day (LD) plant *Arabidopsis*, and in short day (SD) rice (*Oryza sativa*), in which flowering is promoted by short photoperiods rather than long photoperiods. Many *Arabidopsis* genes have been identified in rice suggesting a high degree of conservation between dicots and monocots. The LD-SD difference between *Arabidopsis* and rice appears to involve a single gene interaction which is switched from promotion in *Arabidopsis*, to repression in rice (Izawa et al., 2003; Izawa, 2007).

In *Arabidopsis*, CONSTANS (CO) is the key regulator linking detection of photoperiod by the circadian clock to floral induction (Figure 1.4). CO up-regulates expression of the floral integrators *FT* and *SOC1* (Putterill et al., 2004). Over-expression of *CO* leads to early flowering in long and short photoperiods, and *co* mutants flower late in long days (Putterill et al., 1995; Onouchi et al., 2000; Robson et al., 2001; Suarez-Lopez et al., 2001).

Aspects of photoperiodic induction of *CONSTANS* (*CO*) expression have been reviewed thoroughly by Jarillo et al. (2008). Briefly, florally inductive far-red and blue light are perceived by PHYTOCHROME-CRYPTOCHROME (PHY/CRY) complexes, which promote expression of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*). The CCA1-LHY complex auto-regulates by feedback loop involving repression of *TIMING OF CAB EXPRESSION 1* (*TOC1*), *GIGANTEA* (*GI*), and others genes, which promote *CCA1* and *LHY* expression. The CCA1-LHY complex, and light, promote expression of *PSEUDO-RESPONSE*

REGULATORS (*PRRs*), which promote *CO* by repressing *CYCLING DOF FACTOR 1* (*CDF1*). Several clock proteins, including CO are expressed during daylight, but are degraded in the dark by protease activity. Degradation does not happen during daylight as the proteases are inactivated by light. The main output of the circadian clock, with respect to floral induction, is synchronisation of *CO* expression with light (Jarillo et al., 2008). In wild-type *Arabidopsis* in short photoperiods, *CO* mRNA levels are high overnight, but CO protein is degraded by proteases during the dark. In long photoperiods, *CO* mRNA levels peak just before dusk allowing synthesis of CO protein. Clock gene mutants affect the normal coincidence of *CO* expression with light resulting in plants that flower early in short photoperiods and late in long photoperiods (e.g. Roden et al., 2002; Yanovsky and Kay, 2002).

The floral stimulus is produced in leaves in response to inductive photoperiods, and then transported to the shoot apical meristem via the phloem (Corbesier et al., 2007). Several experiments have shown that CO acts in vascular tissue to promote *FT* expression. Plants expressing the *CO::GUS* transgene showed strong GUS expression in the phloem of leaves and stems. *CO* driven by a phloem specific promoter complemented a *co* mutant, while *CO* driven by a shoot apex promoter could not complement the *co* mutant (Takada and Goto, 2003; An et al., 2004; Ayre and Turgeon, 2004). *FT* is expressed in the leaves, but FT protein acts in the meristem to induce flowering. Expression of *FT* mRNA is only transiently required in the leaves to induce flowering. FT fusion proteins expressed in the phloem moved to the shoot apex, and were shown to cross graft junctions (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). *SOC1* expression in the shoot apex is dependent on FT (Searle et al., 2006). FT and FD (encoded by the flowering time gene *FD*) interact to upregulate the floral meristem identity genes *AP1* and *LEAFY* (*LFY*), which are involved in upregulation of the floral organ identity genes (Abe et al., 2005; Wigge et al., 2005; Liu et al., 2007).

Although extensive research into photoperiod induction of flowering has been carried out in rice, little information is available for other monocots. Several *Photoperiod* (*Ppd*) loci have been identified in wheat and barley (Cockram et al., 2007). *CO*-like genes have been identified in wheat, barley and ryegrass (Griffiths et al., 2003; Nemoto et al., 2003; Martin et al., 2004). Perennial ryegrass *LpCO* showed similar expression patterns

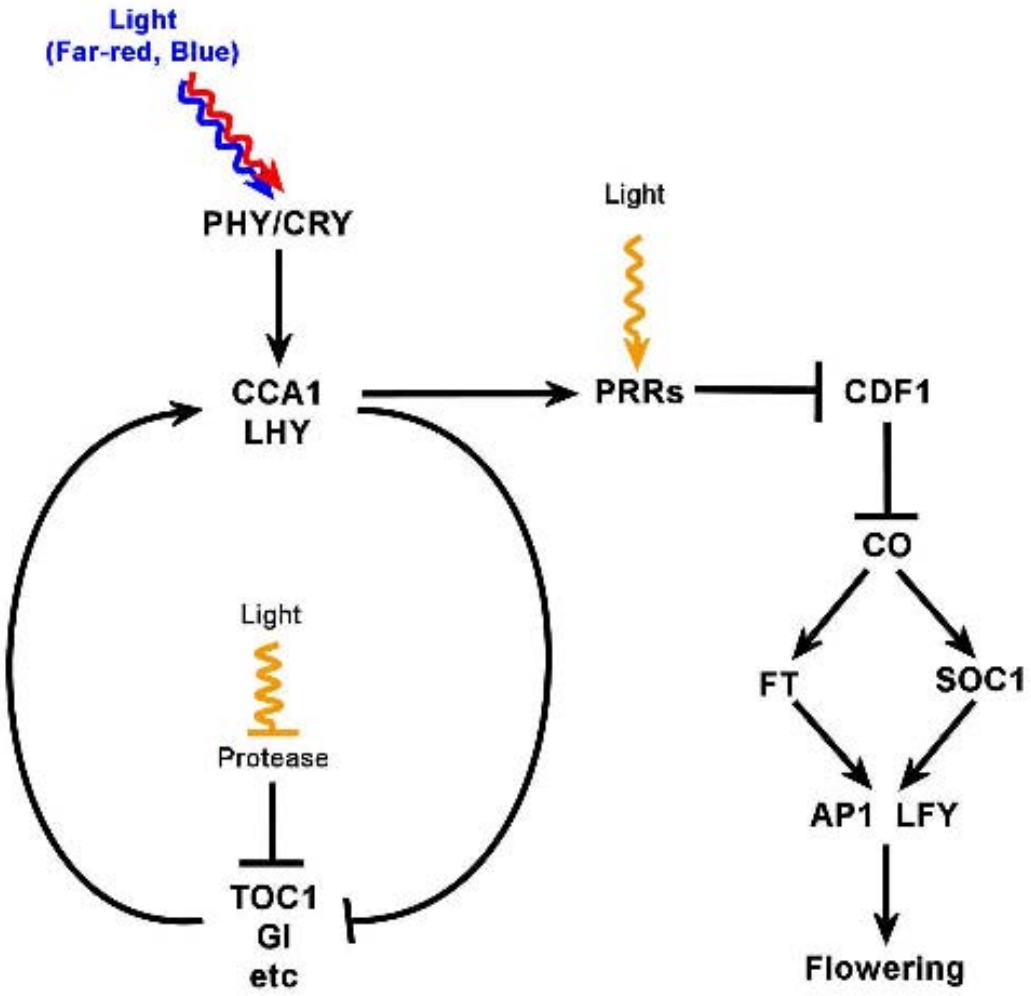


Figure 1.4 Simplified model of photoperiodic induction of *CO* by the circadian clock.

Light is perceived by PHY/CRY complexes resulting in upregulation of *CCA1* and *LHY*. CCA1 and LHY autoregulate by repressing *TOC1*, *GI* and other genes which promote *CCA1* and *LHY* expression. CCA1 and LHY promote expression of *PRRs*, which promote *CO* expression by repressing *CDF1*. When *CO* mRNA coincides with light, CO protein accumulates, promoting expression of the floral integrator *FT*. Abbreviations: *AP1 - APETALA 1*, *CCA1 - CIRCADIAN CLOCK ASSOCIATED 1*, *CDF1 - CYCLING DOF FACTOR 1*, *CO - CONSTANS*, CRY - Cryptochrome, *FT - FLOWERING LOCUS T*, *GI - GIGANTEA*, *LFY - LEAFY*, *LHY - LATE ELONGATED HYPOCOTYL*, PHY - Phytochrome, *PRR - PSEUDO RESPONSE REGULATOR*, *SOC1 - SUPPRESOR OF OVEREXPRESSION OF CONSTANS 1*, *TOC1 - TIMING OF CAB EXPRESSION 1*. [Adapted from Jarillo et al., 2008.]

to *Arabidopsis CO* in both long and short photoperiods, and *LpCO* complemented an *Arabidopsis co* mutant (Martin et al., 2004). *VRN3*, an *FT* homologue has been identified in wheat, and a family of *FT*-like genes has been identified in barley (Yan et al., 2006; Faure et al., 2007). Gagic (2007) recently characterised putative *GI*, *CO* and *FT* homologues from perennial ryegrass.

1.3. Perennial ryegrass growth and development

Perennial ryegrass is one of the most highly utilised forage grasses in temperate regions (Jung et al., 1996; Gilliland et al., 2007). Its value comes from its palatability, high nutritional value, capacity to withstand grazing and treading, and tolerance to a wide range of environmental stresses (Jung et al., 1996; Moser and Hoveland, 1996; Gilliland et al., 2007).

Growth and development in grasses has been described by Langer (1979; Figure 1.5). Briefly, perennial ryegrass plants consist of numerous basal shoots or tillers. During vegetative growth, each tiller is made up of several leaves on a short stem (less than 1 cm in length). Leaves can be divided into lamina (distal part) and leaf sheath (basal part), with a ligule and auricles at the axis in between (Figure 1.5). The lamina is the leafy part of the grass plant, while the sheath is enclosed within the leaf sheath of older leaves, and enfolds younger leaves, giving a stem-like appearance. An axillary shoot meristem is present in the axil of each leaf, and its outgrowth results in formation of a new daughter tiller. Daughter tillers develop their own root systems and become independent from their mother tiller allowing clonal growth as a means of vegetative reproduction. Leaf and tiller production continues until plants switch to reproductive development in response to specific environmental cues.

Vernalisation is achieved by exposure to several weeks of low temperatures, short photoperiods (in temperatures below 17°C), or a combination of both, although plants become photoperiod insensitive at temperatures below 6°C (Cooper, 1951; Cooper, 1960). Both seeds and seedlings can be vernalised, although vernalisation is more effective in seedlings (Chouard, 1960; Gangi et al., 1983). Short photoperiods and warm temperatures (e.g. 9 h at 20°C) are neither vernalising nor capable of inducing floral

initiation. Plants continue to grow vegetatively until secondary induction signals (lengthening photoperiods in spring) are perceived and changes occur in the shoot meristems resulting in inflorescence initiation. Flowering usually occurs in late spring and early summer ahead of the summer dry period.

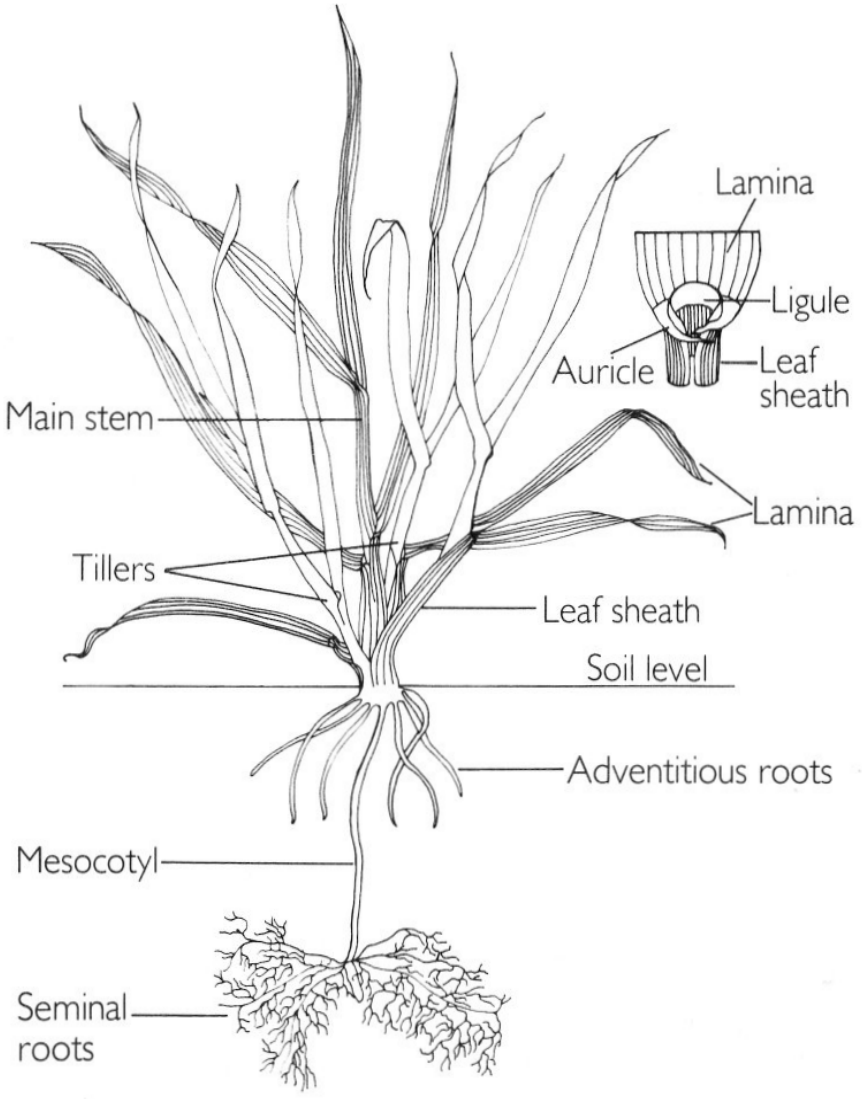


Figure 1.5 Structure of a grass plant. [Taken from Langer, 1979.]

Perennial forages have opposing selective pressures placed upon them during plant breeding (Kalton et al., 1996). Seed production practices favour genotypes with high numbers of reproductive tillers, and good seed yields. However, nutritional quality is reduced during flowering (Jung et al., 1996), so the end-user, the farmer, requires varieties with fewer reproductive tillers. Effective regulation of resource partitioning between vegetative and reproductive growth is important in perennial forage grasses.

1.4. Perennial mechanisms in the grasses

Grasses have several mechanisms allowing the perennial growth habit including stolons (horizontal creeping shoot or stem), rhizomes (horizontal underground stem) and underground bulbs (Volaire, 1995; Nelson, 1996; Volaire and Norton, 2006). These are vegetative organs which store carbohydrates, allowing plant dormancy during drought or cold, and producing shoots when conditions become favourable. Perennial rice breeding programmes are focussing on interspecific hybridisation between annual cultivated rice (*O. sativa*) and perennial rice species with rhizomes (*O. longistaminata*) and stolons (*O. rufipogon*; Sacks et al., 2003b; Sacks et al., 2003a; Sacks et al., 2006; Sacks et al., 2007; Zhao et al., 2008a; Zhao et al., 2008b). Perennial ryegrass does not have a dormant phase and does not appear to use these mechanisms. Its continued survival is linked to maintenance of non-flowering tillers (Langer, 1963; Jung et al., 1996).

Chouard (1960) commented that the association with perenniality and a vernalisation requirement was contradictory. All meristems in the perennial plant exposed to vernalisation should be competent to flower, as should all meristems arising from those meristems due to mitotic inheritance of the vernalisation response. On exposure to floral induction (e.g. inductive photoperiods), all shoots should flower, resulting in monocarpic flowering and the annual habit. Chouard (1960) suggested that there must be alterations to the normal vernalisation process in perennial plants, and proposed three potential mechanisms: some buds are devernalised over summer; some buds are not vernalised during winter; and vernalisation is not permanently fixed in indirectly vernalised daughter shoots. Almost fifty years later, the mechanisms of vernalisation, and devernalisation, in perennials remain unresolved. Several authors have investigated

whether vernalisation is permanently fixed in temperate grasses, with opposing conclusions (e.g. Kleinendorst, 1974; Havstad et al., 2004).

There are differing opinions regarding the origin of perennating tillers in temperate grasses. Some authors imply that all over-wintering tillers flower on exposure to inductive photoperiods, leaving spring-initiated tillers to perennate (e.g. Langer, 1963). Other authors suggest that spring-initiated tillers are too immature to survive a typical summer drought (e.g. Davies, 1988), implying that some over-wintering tillers must remain vegetative. Other authors offer no opinion (e.g. Aamlid et al., 2000).

Havstad et al. (2004) studied floral induction in four temperate perennial grasses, and indicated the number of tillers present at the onset of vernalisation, and the number of those tillers that flowered following exposure to inductive photoperiods. These authors showed that not all tillers that had emerged prior to vernalisation flowered, while some tillers emerging later, during and after vernalisation, did flower.

Matthew et al. (1993) studied the tiller contribution to grass swards following flowering and subsequent grazing or cutting. They observed tillers of three origins: older vegetative tillers, daughter tillers of those older vegetative tillers, and daughter tillers of reproductive tillers. All three tiller-types were present in different species and cultivars, but the proportions of each varied. It was inferred that plants were not subjected to summer drought (Matthew et al., 1993; Bahmani et al., 2002), and that conditions might have been favourable for survival of small spring and summer-emerging tillers.

1.5. Aims of this thesis

The overall aim of this thesis was to investigate how perennial ryegrass maintains its perennial growth habit, with particular emphasis on whether all fully vernalised tillers are induced to flower on exposure to a sufficient number of long day cycles, or whether some vernalised tillers remain in a vegetative state.

To represent these two alternative models, two hypotheses were formulated. The first stated that all sufficiently vernalised perennial ryegrass tillers would flower after exposure to a critical number of long days, hereafter referred to as the ‘environmental

control hypothesis'. The alternative hypothesis is that, in addition to the environmental mechanisms, a spatial control mechanism also acts to restrict flowering in some tillers in perennial ryegrass. This will be referred to as the 'spatial control hypothesis'. Two experiments were conducted to test these hypotheses. The first experiment was conducted to observe the differences between annual (Italian) and perennial ryegrasses at flowering. In the second experiment, flowering of individual tillers was studied in vernalised perennial ryegrass exposed to inductive photoperiods.

In both cases, the outcome would be controlled by regulation of some or all of the genes involved in the flowering process. If a spatial control mechanism exists, then differential expression of these genes among tillers exposed to vernalisation would be expected to occur. Here, a preliminary reverse transcription-polymerase chain reaction (RT-PCR) analysis of two candidate genes was carried out.

Chapter 2. Materials and Methods

2.1. Experiment I

2.1.1 Ryegrass varieties

Seeds of the perennial ryegrass (*Lolium perenne*) line GA66/542 x A10654/1, an F_1 hybrid family between 'Samson' and 'Impact' plants, were provided by I. Kardailsky, (AgResearch Ltd.). The annual Italian ryegrass (*L. multiflorum*) cultivar Grasslands Tama (Margot Forde Forage Germplasm Centre, accession number MT404) was used as a control for floral induction (Table 2.1).

Table 2.1 Ryegrass lines used in Experiments I and II.

Species	Line	Experiment	Source
Lolium perenne Perennial ryegrass	GA66/542 x A10654/1 (F_1 Samson x Impact)	Experiment I	I. Kardailsky, AgResearch
Lolium multiflorum Italian ryegrass	Grasslands Tama MT404	Experiment I	Margot Forde Forage Germplasm Centre
Lolium perenne Perennial ryegrass	Samson A13900	Experiment II	Margot Forde Forage Germplasm Centre
Lolium perenne Perennial ryegrass	Impact A8393	Experiment II	Margot Forde Forage Germplasm Centre

2.1.2 Conditions used for plant growth

Germination and establishment: Seeds were germinated on moist filter paper in Petri dishes in the dark at room temperature. After five days, germinated seedlings with 1 cm long coleoptiles were planted in media in cell trays (50 mm diameter, 100 mm depth) and established in non-inductive short days at 20°C. Growth media consisted of peat (60% v/v) and coarse sand (40% v/v) to which 3-month Osmocote (600 g m^{-3}), 9-month Osmocote (2.4 kg m^{-3}), dolomite lime (3 kg m^{-3}) and super phosphate (1 kg m^{-3}) were added. Plants were watered throughout the experiment as required. Cell trays were rotated and shifted within the growth rooms weekly to minimise position effects.

Non-inductive short days: Non-inductive short day (NISD) exposure was carried out at 20°C with a 9 h photoperiod (0800 - 1700 h). Lighting (PPF 400 µmol m^{-2} s^{-1}, light intensity 6400 lx) was supplied by fluorescent tubes (12× Phillips Alto TLD 865, 58 W) and mercury lamps (4× Sylvania HSL MBF-U, 400 W). NISDs were neither vernalising nor inductive.

Vernalisation: Vernalisation was carried out at 6°C with a 9 h photoperiod (0800 - 1700). Lighting (PPF 200 µmol m^{-2} s^{-1}, light intensity 3100 lx) was supplied by fluorescent tubes (12× Phillips Alto TLD 865, 58 W).

Inductive photoperiod - Natural long days: Plants were put outside in Palmerston North (40.383°S, 175.617°E) and exposed to natural long days (LD; Appendix A) under prevailing environmental conditions (Appendix B). Early in 2004, there was more rain, temperatures were lower and there were fewer sunshine hours than the 30 year average (Table 2.2).

Inductive photoperiod - Greenhouse with daylength extension and heating: A Duralite Tedlar greenhouse maintained at 25°C with supplementary lighting was used for inductive photoperiods. PPF varied with natural light but was approximately 40% of natural daylight. Three mercury lamps (Sylvania HSL MBF-U, 400 W) were used to supplement and extend the daylength to a 14 h photoperiod (0600 - 0900 and 1700 - 2000).

Table 2.2 Palmerston North climate daily means.

Means of daily rainfall, temperature maxima and minima, and sunshine hours for January 13 to March 11, 2004 (Grasslands Weather Station, New Zealand) and 30-year averages of daily means for January to March, 1971-2000 (www.niwa.co.nz, 2008).

Month	Year	Rain (mm)	Max (°C)	Min (°C)	Sun (h)
January	2004[a]	3.8	23.5	13.5	5.1
	1971-2000	2.1	22.4	13.4	6.7
February	2004[a]	10.3	20.7	13.4	3.8
	1971-2000	2.2	22.9	13.5	6.8
March	2004[a]	2.2	20.4	10.6	6.8
	1971-2000	2.4	21.1	12.1	5.5

[a] Values adjusted to include dates plants were under natural LDs only (January 13 to March 11).

2.1.3 Vernalisation and photoperiod treatments

Five treatments were set up using two ryegrass species and three vernalisation periods. Approximately 250 seeds were set to germinate for each of the five treatments. Germination dates were staggered so that all plants were exposed to the same inductive photoperiods (Table 2.3).

Treatment 1: Perennial ryegrass (F_1 GA66/542 x A10654/1) seedlings were established in NISDs until they were 28 d old, followed by 52 d vernalisation and a further 14 NISDs before being transferred outside to natural LDs from mid January to early March 2004. Plants were then transferred to a heated greenhouse with daylight extension (Table 2.3). Initially, 168 seedlings were planted, of which 62 were culled, 58 were destructively harvested for analysis, and 48 were assessed for flowering (Table 2.4).

Treatment 2: Treatment 2 was similar to Treatment 1, except that the plants were vernalised for 33 d only (Table 2.3). Initially, 144 seedlings were planted, of which 41 were culled, 58 were destructively harvested, and 45 were allowed to flower (Table 2.4).

Table 2.3 Timeline for Experiment I.

	Treatments 1 and 4 **Perennial ryegrass** **Italian ryegrass** **52 d vernalisation**	**Treatment 2** **Perennial ryegrass** **33 d vernalisation**	**Treatments 3 and 5** **Perennial ryegrass** **Italian ryegrass** **No vernalisation**
10/10/03	Seeds set (5 d)		
15/10/03	Seedlings planted (23 d)		
29/10/03		Seeds set (5 d)	
03/11/03		Seedlings planted (23 d)	
07/11/03	Vernalisation (52 d)		
24/11/03			Seeds set (5 d)
26/11/03		Vernalisation (33 d)	
29/11/03			Seedlings planted (23 d)
22/12/03			Warm SD (7 d)
29/12/03	NISD (14 d)	NISD (14 d)	NISD (14 d)
13/01/04	LDs (outside, 58 d)	LDs (outside, 58 d)	LDs (outside, 58 d)
11/03/04	LDs (greenhouse, 46 d)	LDs (greenhouse, 46 d)	LDs (greenhouse, 46 d)

Seed germination was staggered to synchronise LD exposure across all five treatments. Shaded - non-inductive short days (NISD)

Treatment 3: Plants in Treatment 3 were not vernalised, but subjected to one extra week of NISDs (49 d in total) in place of the vernalisation treatment. This extra week was to compensate for the longer growing time of the vernalised treatments. Plants were then transferred to LDs as in Treatments 1 and 2 (Table 2.3). Initially, 216 seedlings were planted, of which 62 were culled, 53 were destructively harvested, and 101 were assessed for flowering (Table 2.4).

Treatments 4 and 5: Treatments 4 and 5 were similar to Treatments 1 and 3, respectively, except that Italian ryegrass (Grasslands Tama) was used in place of perennial ryegrass (Table 2.3). In Treatments 4 and 5, respectively, 120 and 144 seedlings were planted. In Treatment 4, 6 plants died, 30 were destructively harvested and 84 were allowed to flower. In Treatment 5, 2 plants died, 67 plants were destructively harvested, and 75 were assessed for flowering (Table 2.4).

Table 2.4 Fate of perennial and Italian ryegrass plants germinated in Experiment I.

Treatment[a]	Seedlings[b]	Dead[c]	Analysis[d]	Flowering[e]
1 P 52 d	168	62	58	48
2 P 33 d	144	41	58	45
3 P 0 d	216	62	53	101
4 I 52 d	120	6	30	84
5 I 0 d	144	2	67	75

[a] 1-5 - Treatment numbers, P - perennial ryegrass, I - Italian ryegrass, d - days vernalisation. [b] Number of germinated seeds planted. [c] Number of culled or dead plants. [d] Number of plants destructively harvested. [e] Number of plants assessed for flowering.

2.1.4 Sample collection and analysis

Plants were destructively harvested for fresh dissections, sectioning and staining at several time points throughout the experiment: immediately prior to vernalisation (28 d after germination), immediately following vernalisation, after exposure to 0, 5 and 15 LDs for histology, and after exposure to 0, 8 and 17 LDs for fresh dissections. In most cases, five plants were selected from a cell-tray chosen at random. Flowering was assessed in all remaining plants in March and April 2004.

Fresh dissections: The main tiller is the most florally advanced tiller (Langer, 1979), so its meristem state was indicative of whether a plant had begun flowering. Shoot meristems from the main tiller were dissected by hand to examine the stage of tiller development, and record floral progression. Dissections were carried out on perennial and Italian ryegrass vernalised for 52 d immediately prior to vernalisation (28 d after germination), immediately after vernalisation, and after exposure to 0, 8 and 17 LDs. Remaining treatments (vernalised for 33 d and 0 d) were not examined at 28 d old, but were dissected at the later time-points.

Sectioning and staining: Basal segments of tillers containing the shoot apical meristem were fixed, sectioned, and stained with Johansen's Safranin-O and Fast Green method to aid visualisation (Table 2.5; Table 2.6; Ruzin, 1999). The numbers of tillers and the physical origin of each tiller was recorded. Vernalised (52 d and 33 d) plants were harvested at all five time-points while non-vernalised plants were not sampled prior to vernalisation.

Flowering: The number of plants that flowered after 58 LDs outside was recorded (March 11, 2004). Plants were moved into a heated greenhouse with supplementary lighting and flowering was recorded again after a further 46 LDs on April 26.

Table 2.5 Protocol for wax-embedding of meristem shoots (Ruzin, 1999).

FAA[a]	15 min (vacuum), RT
FAA	15 min (vacuum), RT
FAA	2 h, RT
50% (v/v) ethanol	rinse, RT
50% (v/v) ethanol	90 min, RT
70% (v/v) ethanol	90 min, RT
85% (v/v) ethanol	90 min, RT
95% ethanol: 0.1% (w/v) eosin	90 min, RT
95% ethanol	5 min, RT
95% ethanol	5 min, RT
Absolute ethanol	90 min, RT
Absolute ethanol	overnight, RT
Absolute ethanol	2 h, RT
1:1 Histoclear (limonene):ethanol	1 h, RT
100% Histoclear	1 h, RT
100% Histoclear	1 h, RT
100% Histoclear	1 h, RT
Wax chips	overnight, RT
Fresh wax at 60°C, 6-8 changes of wax over 2-3 d	

[a] FAA - 3.7% (v/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol. RT- room temperature.

Table 2.6 Protocol for Johansen's safranin-O and fast green stain (Ruzin, 1999).

100% Histoclear	10 min, RT
1:1 Histoclear:ethanol	5 min, RT
Absolute ethanol	5 min, RT
Absolute ethanol	5 min
95% ethanol	5 min
85% (v/v) ethanol	5 min
70% (v/v) ethanol	5 min
Safranin[a]	overnight, RT
Water	rinse, RT
70% (v/v) ethanol	5 min, RT
95% ethanol + picric acid (0.5% w/v)	10 s, RT
95% ethanol + NH_4OH (4 drops)	1 min, RT
95% ethanol	10 s, RT
Fast Green[b]	20 s, RT
Clove oil	5 s, RT
2:1:1 Clove oil:ethanol:histoclear	5 s, RT
1:1 Histoclear:ethanol	5 min, RT
100% Histoclear	5 min, RT
100% Histoclear	5 min, RT

[a] 50% (v/v) methyl cellosolve, 25% (v/v) ethanol, 2% (v/v) formalin, 1% (w/v) sodium acetate, 1% (w/v) safranin. [b] 33% (v/v) methyl cellosolve, 33% (v/v) ethanol, 33% (v/v) clove oil, 0.05% (w/v) Fast Green. RT- room temperature.

2.1.5 Statistical analysis

Mean ($\bar{x}$) and standard error of the mean (SEM) values were calculated for tiller data using the following formulae:

$$\bar{x} = \frac{\sum x}{n} \qquad \text{SD} = \sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}} \qquad \text{SEM} = \frac{SD}{\sqrt{n}}$$

Where x represented the number of tillers per plant, n represented the number of plants. SD - standard deviation.

2.2. Experiment II

2.2.1 Ryegrass varieties

Perennial ryegrass cultivars 'Samson' and 'Impact' (Margot Forde Forage Germplasm Centre, accession numbers A13900 and A8393, respectively) were used in Experiment II (Table 2.1).

2.2.2 Conditions used for plant growth

Germination: Seeds were germinated directly into media in cell trays (30 mm diameter, 100 mm depth) and established in NISDs at 20°C. Growth media consisted of peat (60% v/v) and coarse sand (40% v/v) to which 3-month Osmocote (600 g m^{-3}), 9-month Osmocote (2.4 kg m^{-3}), dolomite lime (3 kg m^{-3}) and super phosphate (1 kg m^{-3}) were added. Plants were watered throughout the experiment as required.

Non-inductive short days: NISD exposure was carried out at 20°C with a 9 h photoperiod (0800 - 1700). Lighting (PPF 200 µmol m^{-2} s^{-1}, light intensity 3100 lx) was supplied by fluorescent tubes (12× Phillips Alto TLD 865, 58 W). NISDs were neither vernalising nor inductive.

Vernalisation: Vernalisation was carried out at 6°C with a 9 h photoperiod (0800 - 1700). Lighting (PPF 29 µmol m^{-2} s^{-1}, light intensity 1760 lx) was supplied by cool white lamps (64× Ecolife MD8B, 27W).

Inductive photoperiods: A Duralite Tedlar greenhouse maintained at 25°C with supplementary lighting was used for inductive photoperiods. PPF varied with natural light but was approximately 40% of natural daylight. A mercury lamp (Sylvania HSL MBF-U, 400 W) was used to supplement and extend the daylength to a 14 h photoperiod (0600 - 2000).

2.2.3 Vernalisation and photoperiod treatments

Three treatments with varying vernalisation and photoperiod regimes were replicated using ‘Samson’ and ‘Impact’ perennial ryegrass (Table 2.7).

Treatment 1: Perennial ryegrass seedlings were established in NISDs for 14 d, followed by exposure to 64 d vernalisation, and then transferred to a heated greenhouse with additional lighting to supplement and extend natural day length to a 14 h photoperiod (Table 2.7). Of 324 seedlings each, 84 ‘Samson’ and 83 ‘Impact’ plants were destructively harvested, 121 ‘Samson’ and 167 ‘Impact’ plants were allowed to flower, while the balance did not germinate, were thinned, or died (Table 2.8).

Treatment 2: Plants in Treatment 2 were vernalised as in Treatment 1, and then grown in NISDs for the remainder of the experiment (Table 2.7). Of 162 seedlings of each cultivar, 34 of each were destructively harvested. At least 50 plants of each cultivar were monitored for flowering (Table 2.8).

Treatment 3: Plants in Treatment 3 were not vernalised, but remained in NISDs for 78 d (Table 2.7). Of 162 seedlings of each cultivar, 6 of each were collected for analysis. Remaining plants died or were very unhealthy after 78 NISDs, most likely due to low lighting in the NISD room (Table 2.8).

Table 2.7 Timeline for Experiment II.

Date	Treatment 1 64 d vernalisation LD	Treatment 2 64 d vernalisation NISD	Treatment 3 No vernalisation LD
31/07/07	Seeds sown NISD (14 d)	Seeds sown NISD (14 d)	Seeds sown NISD (14 d)
14/08/07	Vernalisation (64 d)	Vernalisation (64 d)	NISD (64 d)
17/10/07	LD (62-65 days)	NISD (60 days)	LD
18-21/10/07	Flowering recorded	Flowering recorded	-

Shaded - phases of the experiment that were planned but not carried out.

2.2.4 Sample collections and analysis

Plants were destructively harvested for fresh dissections and mRNA expression analysis throughout the experiment, including before and during vernalisation, and on alternate days during LD exposure (Appendix C to F). Flowering was recorded in December 2007.

Fresh dissections: One plant of each cultivar was destructively harvested and the apical meristem of every tiller examined to determine whether it was vegetative or floral. Representative meristems were photographed and a scale of floral development was constructed. The physical origin of every tiller was recorded so that floral development could be compared across individual plants.

Table 2.8 Fate of perennial ryegrass plants germinated in Experiment II.

Treatment[a]			Seedlings[b]	Dead[c]	Analysis[d]	Flowering[e]
Samson	1	vern → LD	324	119	84	121
	2	vern → SD	162	ND	34	ND (>50)
	3	SD → LD	162	156	6	0
Impact	1	vern → LD	324	74	83	167
	2	vern → SD	162	ND	34	ND (>50)
	3	SD → LD	162	156	6	0

[a] three treatments for each cultiver: 64 d vernalisation (vern) followed by LDs, 64 d vern followed by NISDs (non-inductive SDs), 78 NISDs followed by LDs. [b] Number of seeds planted. [c] Number of culled or dead plants. [d] Number of plants destructively harvested. [e] Number of plants assessed for flowering. ND - not determined.

Flowering: Reproductive tillers were identified in 121 'Samson' and 167 'Impact' plants after two months of LDs on December 18, 20 and 21, 2007.

mRNA expression: Reverse transcription-polymerase chain reaction (RT-PCR) was used to assess gene expression of *LpFT3* and *LpMADS3* (putative orthologues of *Arabidopsis* flowering time genes *FT* and *AP1*), relative to the house-keeping gene *LpGAPDH*. Plants were harvested early in the afternoon and within an hour of dusk to coincide with expected gene expression patterns (N. T. Forester, M. Gagic, I. Kardailsky, B. Veit, AgResearch Ltd., unpublished). Tillers were isolated and stored individually at -80°C for future use. The physical origin of every tiller was recorded.

Selected 'Samson' tillers (Table 2.9) collected at dusk were ground in liquid nitrogen using a Retch MM301 mixer mill. RNA was isolated using TRIzol® Reagent (Invitrogen 15596-018) according to the manufacturer's instructions. Briefly, 100 µL of ground tissue (measured by eye) was homogenised in 1 mL of TRIzol and incubated at room temperature for 5 min. Chloroform (200 µL) was added and the samples were incubated at room temperature for a further 3 min, followed by centrifugation at 12000g at 4°C for 15 min. The aqueous supernatant was transferred to a clean tube and 500 µL of propan-2-ol was added to precipitate the RNA. Samples were incubated at room temperature for 10 min, followed by centrifugation at 12000g at 4°C for 15 min. mRNA pellets were washed with 75% (v/v) ethanol, then air-dried and resuspended in RNase-free water. RNA was quantified using A_{260}/A_{280} ratios, and 50 ng μL^{-1} stocks were used in cDNA synthesis reactions. RNA samples were stored at -20°C in the short term and at -80°C for the long term.

Table 2.9 Tillers of 'Samson' plants used in determination of mRNA expression.

LD cycles	Main tiller	Primary tillers		
		First	Second	Third
2	X			
6	X			
8	X	X	X	
12	X	X	X	X
16	X	X	X	X
20	X	X	X	X
25	X	X	X	X

cDNA was synthesised from the extracted mRNA using the ThermoScript™ RT-PCR System (Invitrogen 11146-016) according to the manufacturer's instructions. Briefly, primers (random hexamers, supplied), mRNA and dNTPs (final concentrations of 2.5 ng μL^{-1}, 10 ng μL^{-1} and 0.4 mM, respectively) were first denatured at 65°C for 5 min. The cDNA synthesis cocktail consisting of cDNA synthesis buffer (1x), DTT (5 mM), RNaseOUT™ (2 U μL^{-1}) and ThermoScript™ Reverse Transcriptase (0.75 U μL^{-1}), was added and the reactions were incubated at 20°C for 10 min, followed by 50°C for 50 min. The reaction was terminated by incubation at 85°C for 5 min. RNase H (2 U; incubated at 37°C for 20 min) was added to prevent amplification of products from RNA in subsequent PCR reactions.

Amplification of specific cDNAs were carried out using primers specific for *LpFT3*, *LpMADS3*, and *LpGAPDH* (provided by M. Gagic and N. T. Forester, AgResearch Ltd.; Table 2.10). PCR conditions including magnesium concentration, annealing temperatures and extension times had been optimised by Gagic (2007). A PCR cocktail was assembled which contained PCR buffer (1x), $MgCl_2$ (15 mM), dNTPs (0.2 mM), primer pairs (0.2 µM) and Platinum® *Taq* DNA polymerase (0.025 U μL^{-1}, Invitrogen 10966-034). Reactions were carried out in 20 µL volumes which included 1 µL of cDNA (unquantified). Amplification of *LpFT3* and *LpMADS3* was carried out at 94°C for 5 min, followed by 35 cycles with denaturing at 94°C (30 s), annealing at 63°C (30 s), extension at 72°C (25 s). A final extension was carried out at 72°C for 5 min, followed by incubation at 4°C. Amplification of *LpGAPDH* was similar but with an annealing temperature of 60°C.

Table 2.10 Primers used in for RT-PCR.

Gene	Primer	Primer sequence	Expected size (bp)	Annealing temperature (°C)
LpFT3	GIK19 GIK20	CGCCGGAGCCKGCCTCGCG GACAACTGGTGCTTCCTTCGGGCA	229	63
LpMADS3	GTF130 GTF131	GAAGCAGCAGGAGAGGAGCCA TCGCCTTCCAGTGGAGTCACA	148	63
GAPDH	GIK55 GIK56	CTCAAGGGCATTTTGGGTTA GCTGTATCCCCACTCGTTGT	155	60

PCR products were expected to be less than 300 bp, and were resolved on 2% (w/v) agarose gels in 1x TAE (40 mM Tris-base, 1.1% (v/v) acetic acid, 1 mM EDTA) with 0.5 μg mL^{-1} ethidium bromide (Sambrook and Russell, 2001). Bands were visualised using an ultra-violet trans-illuminator and images were recorded digitally.

2.2.5 Statistical analysis

Mean and SEM values were calculated for tiller data using the formulae in §2.1.5.

Chapter 3. Results

3.1. Experiment I

3.1.1 Introduction

The first experiment was conducted to compare growth and development between perennial and Italian (annual) ryegrass. Vernalised and non-vernalised perennial and Italian ryegrass plants were exposed to LDs to induce flowering. Meristem morphology and flowering were recorded.

3.1.2 Fresh dissections of main tillers of perennial and Italian ryegrass

The main tillers from up to five plants in each of the five treatments at each of the five time-points, were dissected fresh in order to view morphology of the apical meristem (Figure 3.1; Figure 3.2). The numbers of plants dissected at each time-point are listed in Table 3.1.

Meristem morphology in perennial ryegrass: At 28 d old (prior to vernalisation), and immediately following vernalisation, the apical meristems of the main tillers were vegetative with two or three leaf primordia encircling the meristem flanks (termed 'short'; Figure 3.1 a). Vernalised (52 d and 33 d) perennial ryegrass plants developed 'stacked' apical meristems with four to six leaf primordia after 14 NISDs (at 0 LDs; Figure 3.1 b). One plant, vernalised for 52 d and exposed to 8 LDs, showed further stacking (Figure 3.1 c). A second plant, vernalised for 52 d and exposed to 17 LDs, had an inflorescence meristem (Figure 3.1 d). In plants vernalised for 33 d and exposed to 8 LDs and 17 LDs, meristem development had not visibly progressed beyond the 'stacked' stage. The apical meristem in non-vernalised plants remained in the 'short' stage with two or three leaf primordia throughout the experiment.

Meristem morphology in Italian ryegrass: Similar observations were made in Italian ryegrass (Figure 3.2), although the progression to flowering was advanced compared to perennial ryegrass (Table 3.1). Signs of flowering were evident after only 8 LDs, even in unvernalised plants. Vernalisation appeared to advance flowering in Italian ryegrass.

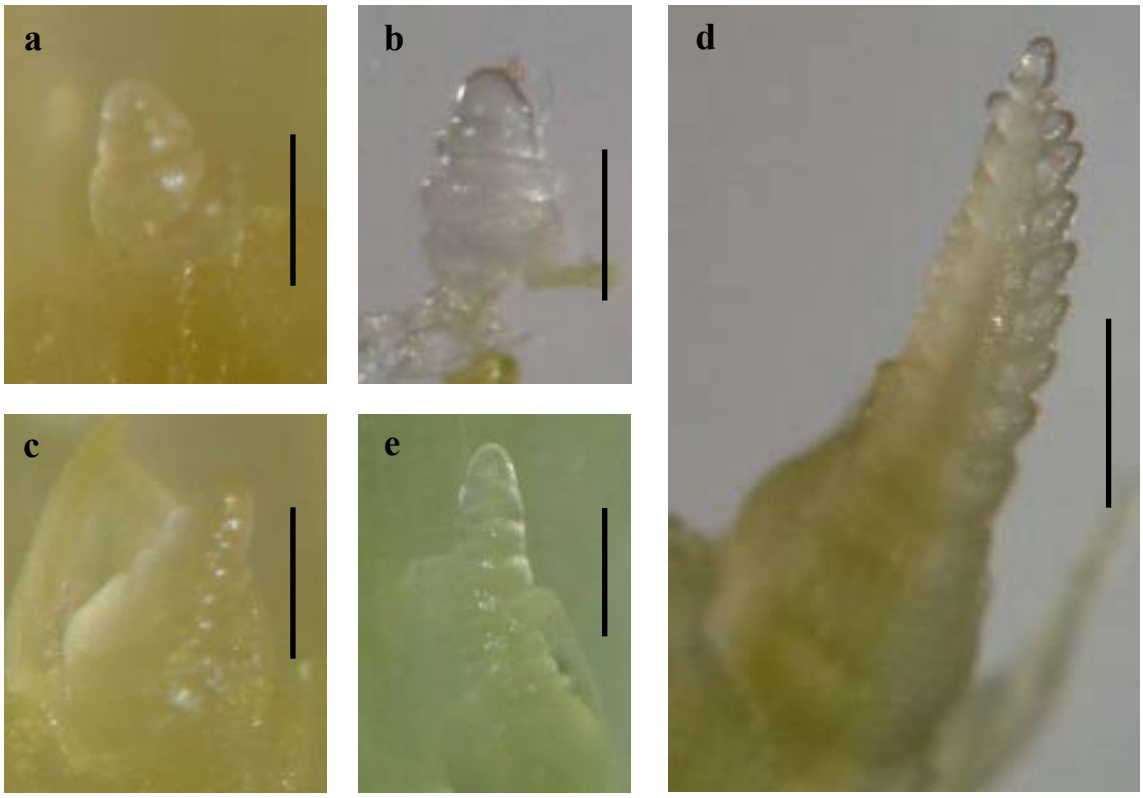


Figure 3.1 Floral progression in the apical meristem of perennial ryegrass.

Perennial ryegrass plants were vernalised for 52 d (a) and exposed to 0 LD (b), 8 LDs (c) and 17 LDs (d and e). Apical meristems were dissected to observe meristem morphology. (a) Vegetative meristem with two or three leaf primordia encircling the meristem flanks. (b) Stacked meristem with five or six leaf primordia encircling the meristem flanks. (c) Stacked meristem with seven or eight leaf primordia on the meristem flanks. (d) Inflorescence meristem with developing spikelet meristems. (e) Elongated meristem from a primary tiller. Scale: a-c, e: bars = 0.2 mm; d: bar = 0.5 mm

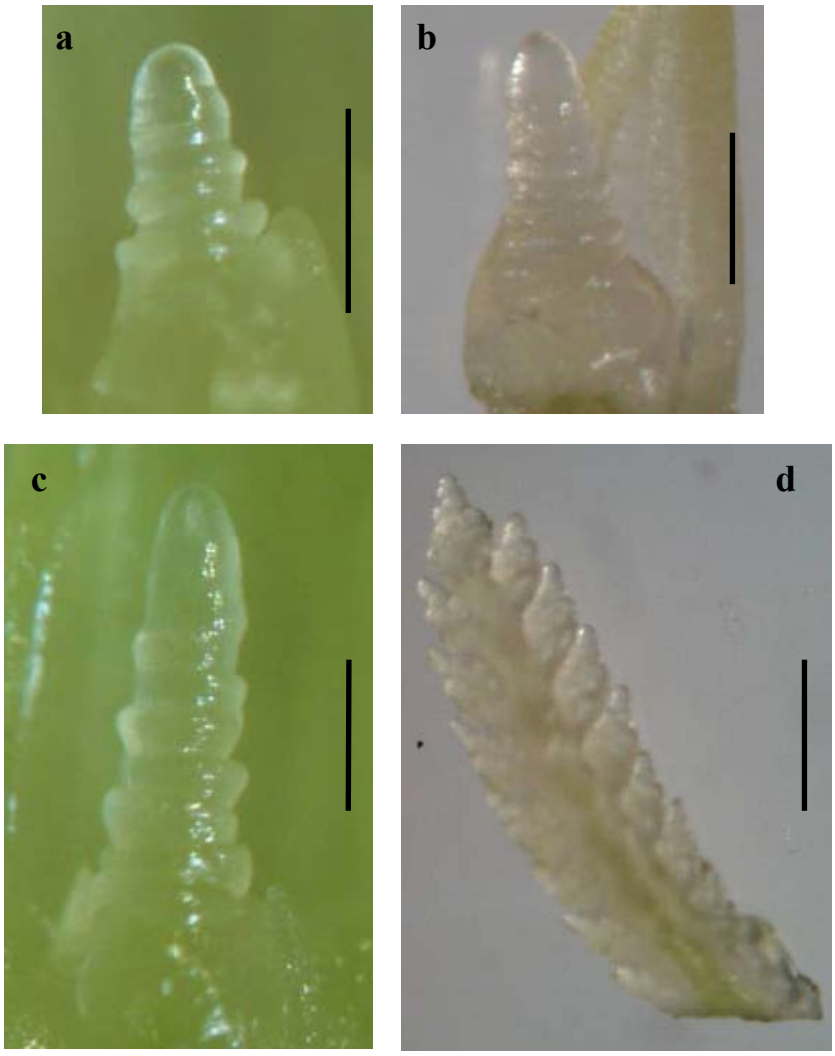


Figure 3.2 Floral progression in the apical meristem of Italian ryegrass.

Italian ryegrass plants were vernalised for 0 d or 52 d and exposed to 0 LD (a and b), 8 LDs (c) and 17 LDs (d). Apical meristems were dissected to observe meristem morphology. (a) Stacked meristem in with seven or eight leaf primordia encircling the meristem flanks. (b) Stacked meristem with ten to twelve leaf primordia were stacked on the flanks of the meristem. (c) Elongated inflorescence meristem. (d) Inflorescence meristem. Scale: a-c: bars = 0.2 mm; d: bar = 0.5 mm

Table 3.1 Meristem morphology in perennial and Italian ryegrass

Perennial and Italian ryegrass plants, vernalised for 52 d, 33 d and 0 d, were dissected fresh at key time points throughout the experiment to observe meristem development.

Time point	Perennial ryegrass			Italian ryegrass	
	52 d vern.	33 d vern.	0 d vern.	52 d vern.	0 d vern.
Prior to vern.	Short 2 or 3 lp (5)	-	-	Short 2 or 3 lp (5)	-
Following vern.	Short 2 or 3 lp (5)	Short 2 or 3 lp (5)	Short 2 or 3 lp (5)	Stacked 5 or 6 lp (5)	Stacked 5 or 6 lp (5)
0 LD	Stacked 5 or 6 lp (5)	Stacked 4 or 6 lp (5)	Short 2 or 3 lp (5)	Stacked 10 to 12 lp (5)	Stacked 7 or 8 lp (5)
8 LD	Stacked 7 or 8 lp (2)	Stacked 4 or 6 lp (2)	Short 2 or 3 lp (2)	Elongated (2)	Stacked 10 to 12 lp (2)
17 LD	Inflorescence (1)	Stacked 4 or 6 lp (1)	Short 2 or 3 lp (1)	Inflorescence (1)	Inflorescence (1)

Vern. - vernalisation. lp - number of leaf primordia visible on the meristem flanks. Short and stacked meristems were considered vegetative, and elongated and inflorescence meristems were considered reproductive. A typical 'short' meristem is shown in Figure 3.1a, and 'stacked' meristems are shown in Figure 3.1b, 3.1c, 3.2a and 3.2b. Elongated meristems are shown in Figure 3.1e and 3.2c, and inflorescence meristems are shown in Figure 3.1d and 3.2d. Numbers of plants dissected are indicated in brackets. No SEMs available for this data.

3.1.3 Tillering in perennial and Italian ryegrass

Tillering was determined retrospectively in plants harvested for sectioning (Table 3.2). In perennial ryegrass, after exposure to 15 LDs, plants from Treatments 1 and 2 (vernalised for 52 d and 33 d, respectively) had more tillers than plants from Treatment 3 (not vernalised; $P<0.05$; Table 3.2). Numbers of primary tillers were comparable for the three treatments (6.2, 6.0 and 5.2 in Treatments 1, 2 and 3, respectively, data not shown), but plants from Treatment 1 had more secondary tillers than plants in Treatment 2, which had more secondary tillers than plants from Treatment 3 (6.4, 4.6 and 2.0 secondary tillers per plant respectively). A similar pattern was observed between Italian ryegrass treatments. Vernalised perennial ryegrass plants had more primary and secondary tillers than vernalised Italian ryegrass plants ($P<0.05$), and non-vernalised perennial ryegrass had more tillers than non-vernalised Italian ryegrass ($P<0.05$; Table 3.2). Tertiary tillers were observed in perennial ryegrass, but not in Italian ryegrass.

Table 3.2 Tillers per plant in perennial and Italian ryegrass.

Average numbers of tillers per plant at key time-points were determined from plants harvested for histology.

Time point	Perennial ryegrass			Italian ryegrass	
	52 d vern.	33 d vern.	0 d vern.	52 d vern.	0 d vern.
Prior to vern.	2.4 (0.40)	2.4 (0.24)	-	1.4 (0.24)	-
Following vern.	3.0 (0.00)	2.8 (0.20)	1.0 (0.0)	1.6 (0.40)	1.0 (0.00)
0 LD	5.6 (1.17)	6.4 (0.24)	4.0 (0.32)	4.4 (0.93)	5.4[a] (0.75)
5 LD	9.8 (2.48)	7.0 (1.48)	4.0 (0.84)	3.8 (1.16)	6.8 (0.97)
15 LD	14.6 (1.89)	11.8 (1.16)	8.2 (0.58)	7.8 (1.50)	6.6 (0.51)

Values represent average number of tillers for 5 plants, except [a] where 10 plants were averaged. SEMs are given in brackets.

3.1.4 Meristem morphology in fixed and stained sections

To investigate what happens to individual tillers, or tiller fate, in perennial and Italian ryegrass at flowering, every meristem on a plant should be examined. The morphological state (vegetative or reproductive) of freshly dissected meristems was relatively easy to determine (Figure 3.1; Figure 3.2), but the process of dissecting out every meristem from five representative plants across five treatments was time-consuming. Therefore, additional material was prepared for light microscopy.

The apical meristem from each tiller of a single perennial ryegrass plant vernalised for 52 d and exposed to 15 LDs were examined. The vegetative or reproductive state (according to Cooper, 1951) of each tiller was recorded, and a schematic map of tiller fate for this plant was constructed (Figure 3.3 a). The main tiller was reproductive (Figure 3.3 a M; Figure 3.3 b), as were the first, second and third primary tillers, and the third secondary tiller of the first primary tiller (Figure 3.3 a, orange). Sixty tillers were identified as vegetative (Figure 3.3 a, green; Figure 3.3 d). Meristem states in many tillers were not determined (Figure 3.3 a, blue; Figure 3.3 c) due to difficulties in ensuring the tissue samples (generally 1 mm in diameter) were in the optimal plane for sectioning, and the two-dimensional nature of the sections. Other ambiguities resulted from distortions created during slide preparation.

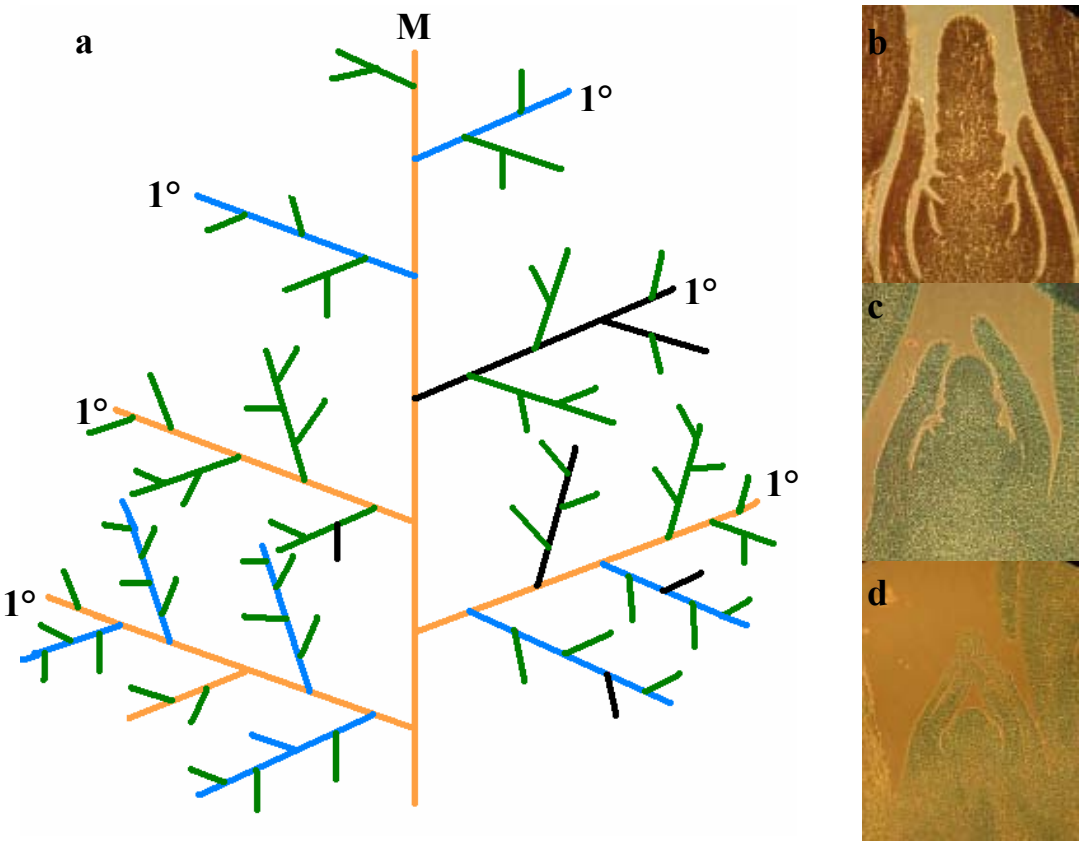


Figure 3.3 Tiller fate in a vernalised perennial ryegrass plant exposed to 15 LDs.

(a) Schematic diagram of tiller fate derived from meristem morphology in a perennial ryegrass plant vernalised for 52 d and exposed to 15 LDs. The apical meristem of the main tiller (M) was reproductive (orange). Primary tillers (1°) branched from the main tiller, secondary tillers branched from the primary tillers, and tertiary tillers branched from the secondary tillers. Meristem states are indicated by tiller colour, and examples of each are shown (b, c and d): meristems of orange tillers were reproductive (b), and meristems of green tillers were vegetative (d). The meristem state of blue tillers could not be determined from sections (c). Black tillers were damaged during preparation.

3.1.5 Flowering in perennial and Italian ryegrass

Following vernalisation, perennial and Italian ryegrass have an obligate LD requirement for floral induction (Cooper, 1951; Cooper, 1960; Aamlid et al., 2000). All plants were moved outside into natural LDs, under prevailing environmental conditions (Table 2.2)

on January 13, 2004 (Table 2.3), and into a heated greenhouse with daylength extension on March 11. Flowering was recorded on March 11 (58 LDs) and April 26, (104 LDs; Table 3.3).

In perennial ryegrass, longer vernalisation resulted in a higher proportion of flowering plants (61% and 23% for plants vernalised for 52 d and 33 d, respectively). Non-vernalised perennial ryegrass plants did not flower during LD exposure (Table 3.3), and had not flowered after a further six months outside in Palmerston North. After 58 LDs all of the vernalised Italian ryegrass had flowered, although only 11% of non-vernalised Italian ryegrass had flowered at this time. All but two Italian ryegrass plants had flowered after a further 46 LDs under artificial induction in a heated greenhouse with supplementary lighting (Table 3.3). At flowering, perennial ryegrass plants consisted of extensive basal foliage with a few reproductive tillers, while Italian ryegrass plants had little or no basal foliage (Figure 3.4).

Figure 3.4 Flowering in perennial and Italian ryegrass after exposure to 58 LDs. *l-r*: Perennial ryegrass vernalised for 52 d, 33 d and 0 d, and Italian ryegrass vernalised for 52 d and 0 d.

Table 3.3 Flowering in perennial and Italian ryegrass exposed to LDs.

Treatment		Vernalisation	Plants exposed to LDs	Plants flowering 58 LD		104 LD	
Perennial ryegrass	1	52 d	48	21	(44%)	29	(61%)
	2	33 d	45	3	(7%)	10	(23%)
	3	0 d	101	0	(0%)	0	(0%)
Italian ryegrass	4	52 d	84	84	(100%)	84	(100%)
	5	0 d	75	8	(11%)	73	(97%)

3.2. Experiment II

3.2.1 Introduction

The second experiment was carried out to investigate the behaviour of individual perennial ryegrass tillers under floral induction. Two perennial ryegrass cultivars, 'Samson' and 'Impact', were vernalised and then exposed to LDs to induce flowering. Meristem morphology, flowering, and expression of flowering time genes were assessed.

3.2.2 Meristem morphology in perennial ryegrass

Fresh dissections were carried out on all tillers of whole plants during NISDs, vernalisation, and on alternate LDs to assess the progression of floral development (Appendix C to F). A photographic series of floral progression in the perennial ryegrass meristem was developed (Figure 3.5). Stages were similar to those shown in wheat by Trevaskis et al. (2007). 'Short' and 'stacked' vegetative meristems were observed, and were renamed Stage I (Figure 3.5 a) and Stage II (Figure 3.5 b), respectively. Both produced leaf primordia, but the axis in a Stage II meristem had elongated, and extra leaf primordia accumulated on the meristem flanks. All meristems in plants prior to vernalisation, and during exposure to vernalisation were at Stage I. Stage I meristems were observed in all plants throughout the experiment. Stage II meristems were only observed in vernalised perennial ryegrass plants which had been growing in warm conditions (LD or NISD) for at least 12 d (Table 3.5).

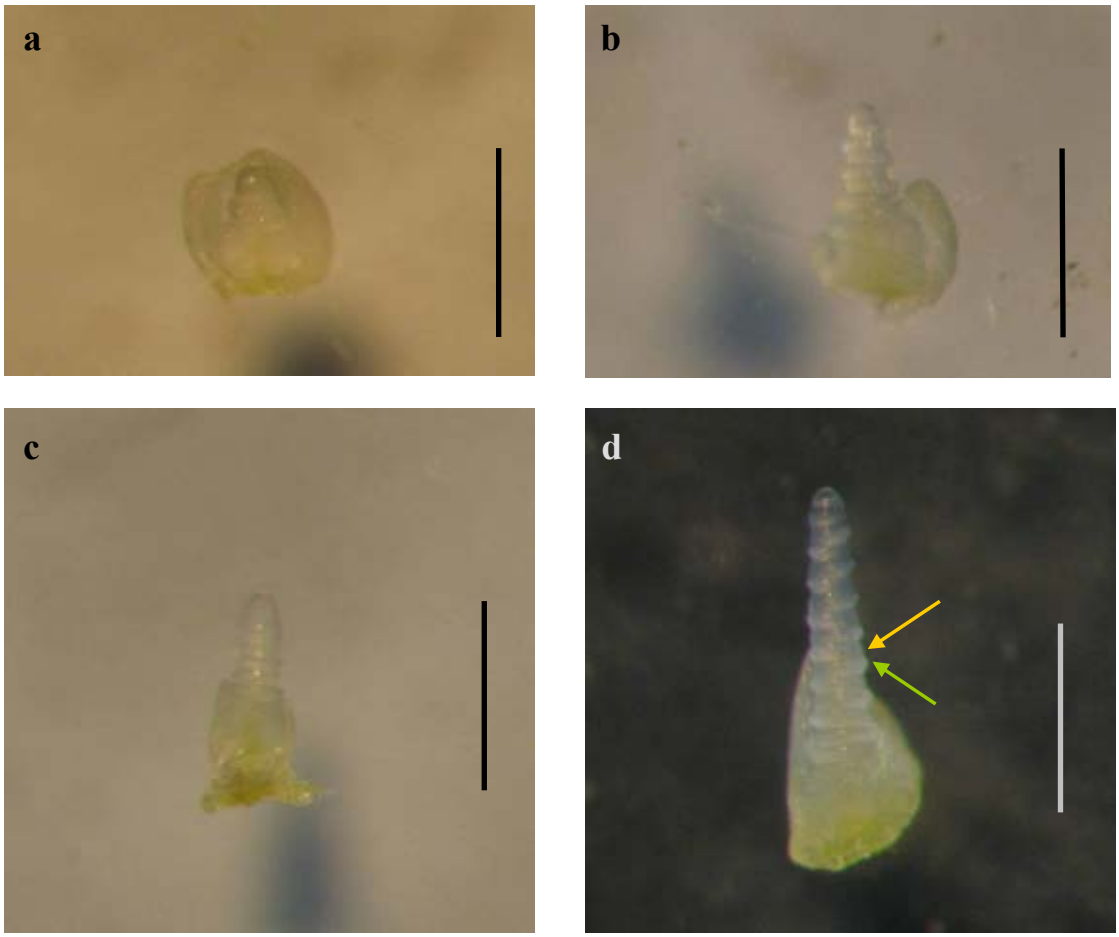
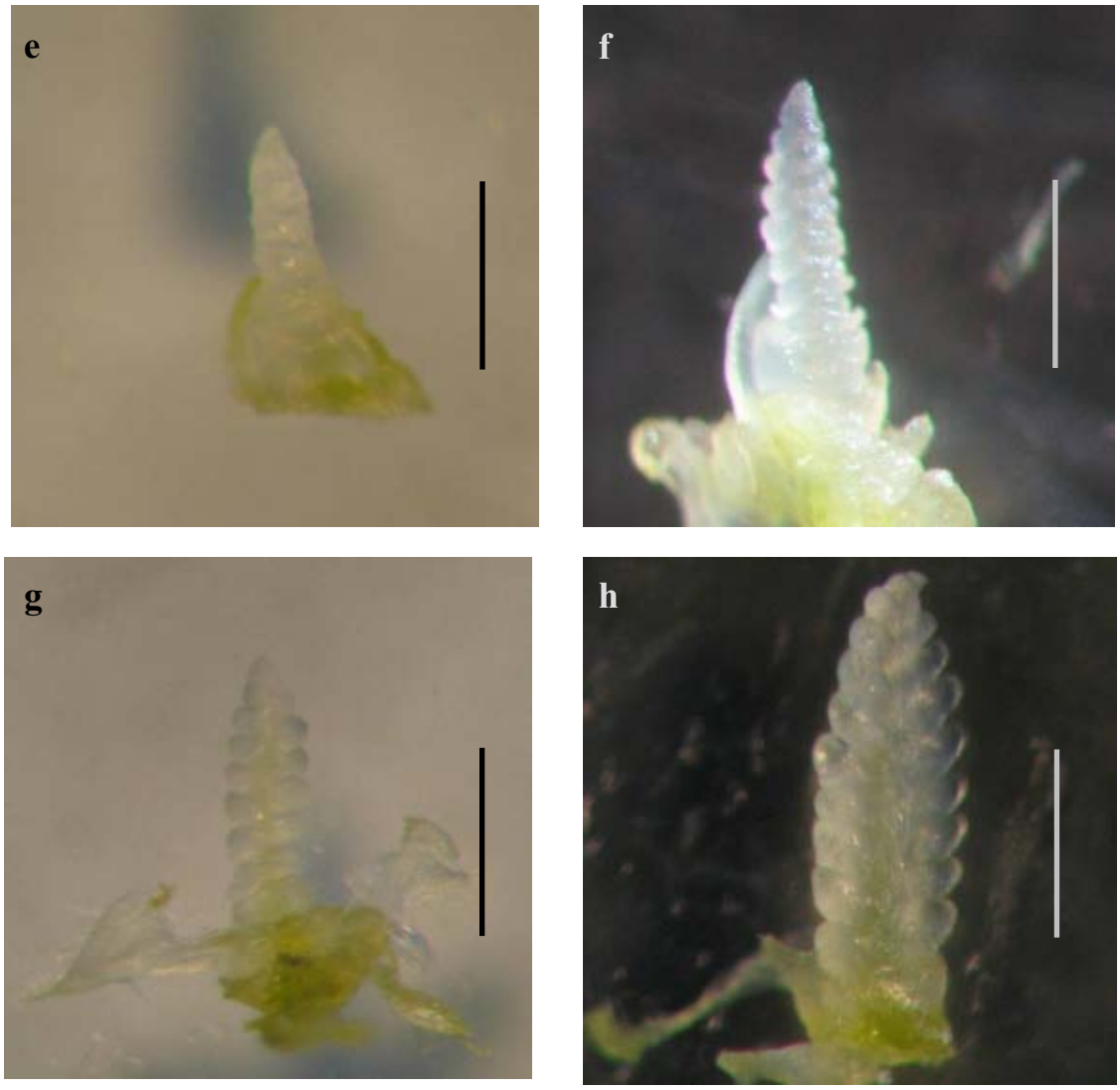
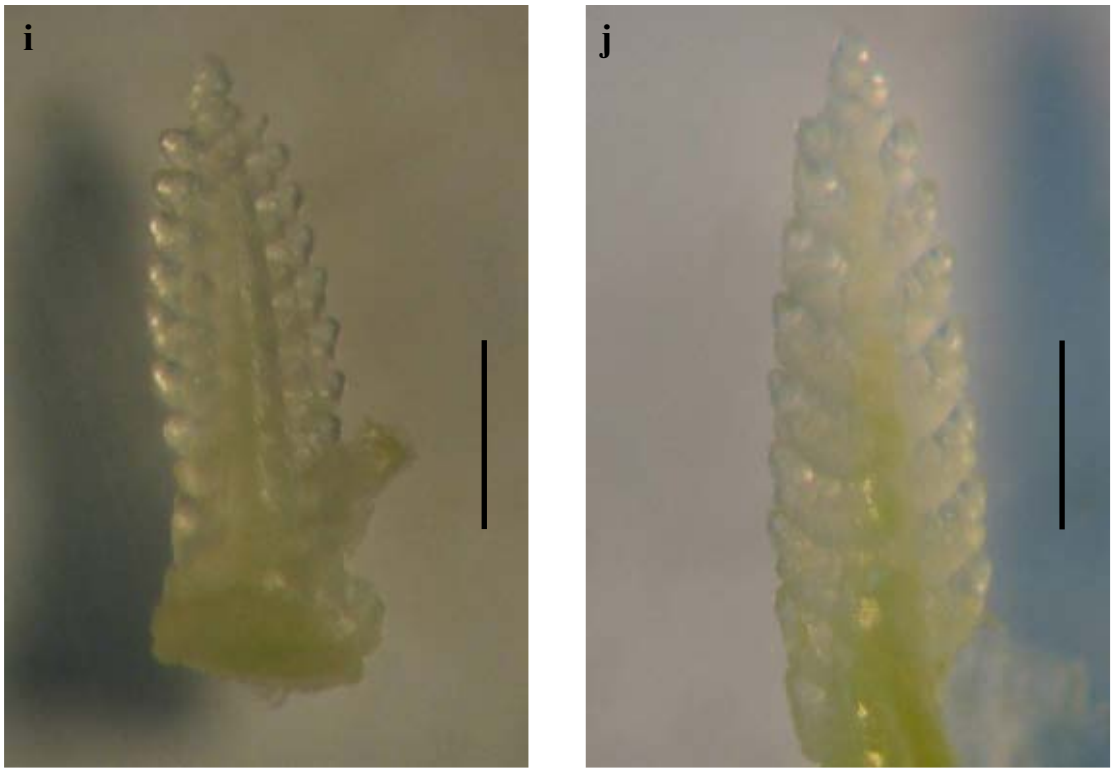


Figure 3.5 Stages of meristem development in perennial ryegrass.

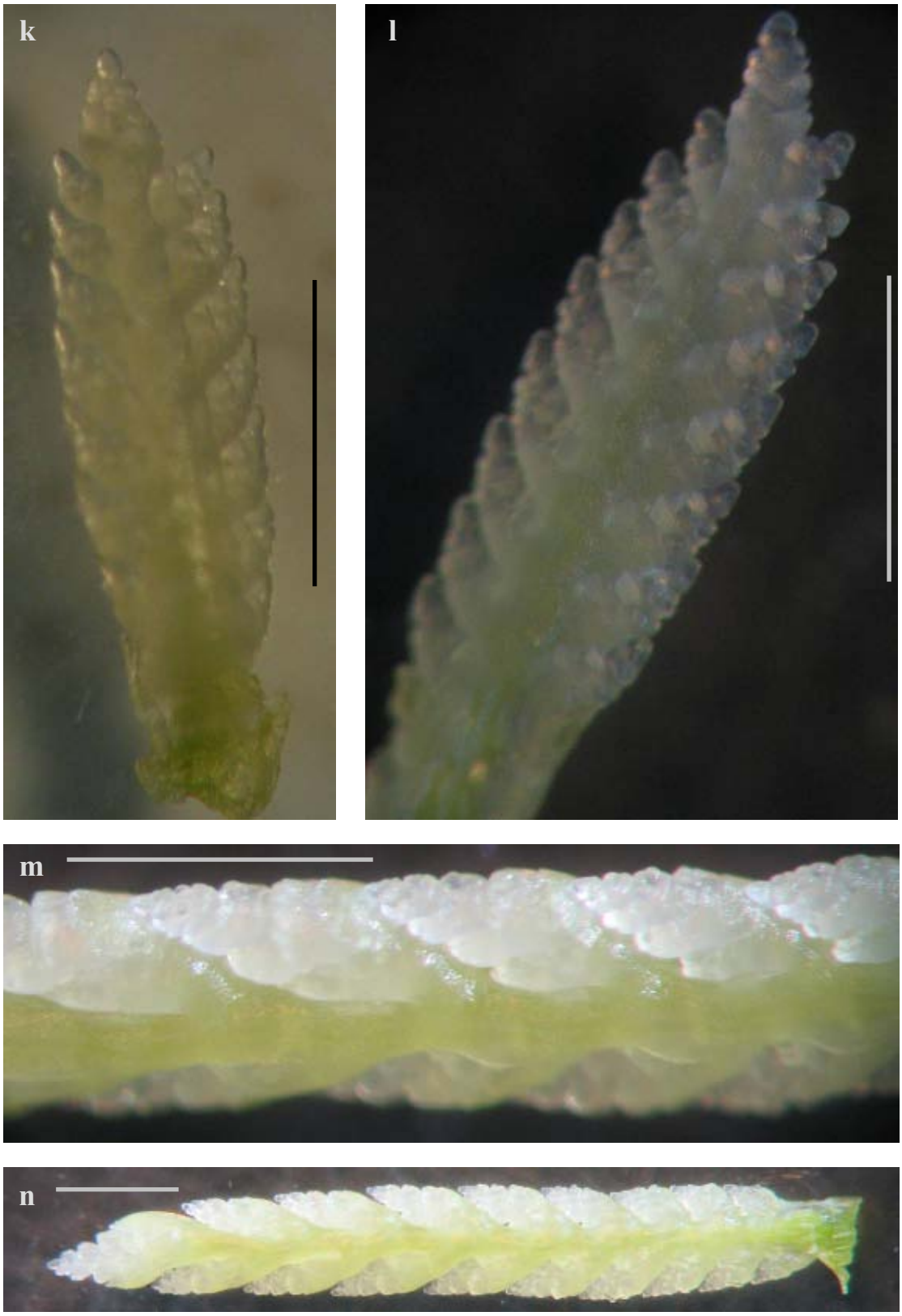
Perennial ryegrass plants vernalised for 64 d were exposed to LDs to induce flowering. Tillers were dissected to observe meristem morphology. Vegetative and reproductive phases were distinct, with the vegetative phase divided into two stages (Stage I and II; a and b), and reproductive development divided into 6 stages (Stages III to VIII; c to t). (a) Stage I vegetative meristems were typically short (up to 0.25 mm) with two or three leaf primordia encircling the meristem flanks. (b) Stage II vegetative meristems were elongated (ranging from 0.2 mm to 0.5 mm), with up to ten leaf primordia encircling the flanks of the meristem. (c, d) Stage III reproductive meristems ranged from 0.4 to 0.8 mm in length. Outgrowth of leaf primordia (green arrow) was delayed or repressed and axillary meristems began to differentiate (orange arrow), giving a double-ridge structure. Scale: bars = 0.5 mm.



(e - h) Stage IV inflorescence meristem leaf primordia remained repressed, while axillary buds began to develop into secondary inflorescence meristems (spikelets), which is in contrast to the order of development in vegetative meristems. The main axis of the inflorescence meristem (rachis) appeared to flatten, while continuing to elongate ranging from 0.5 to 1.2 mm in length. Scale: bars = 0.5 mm.

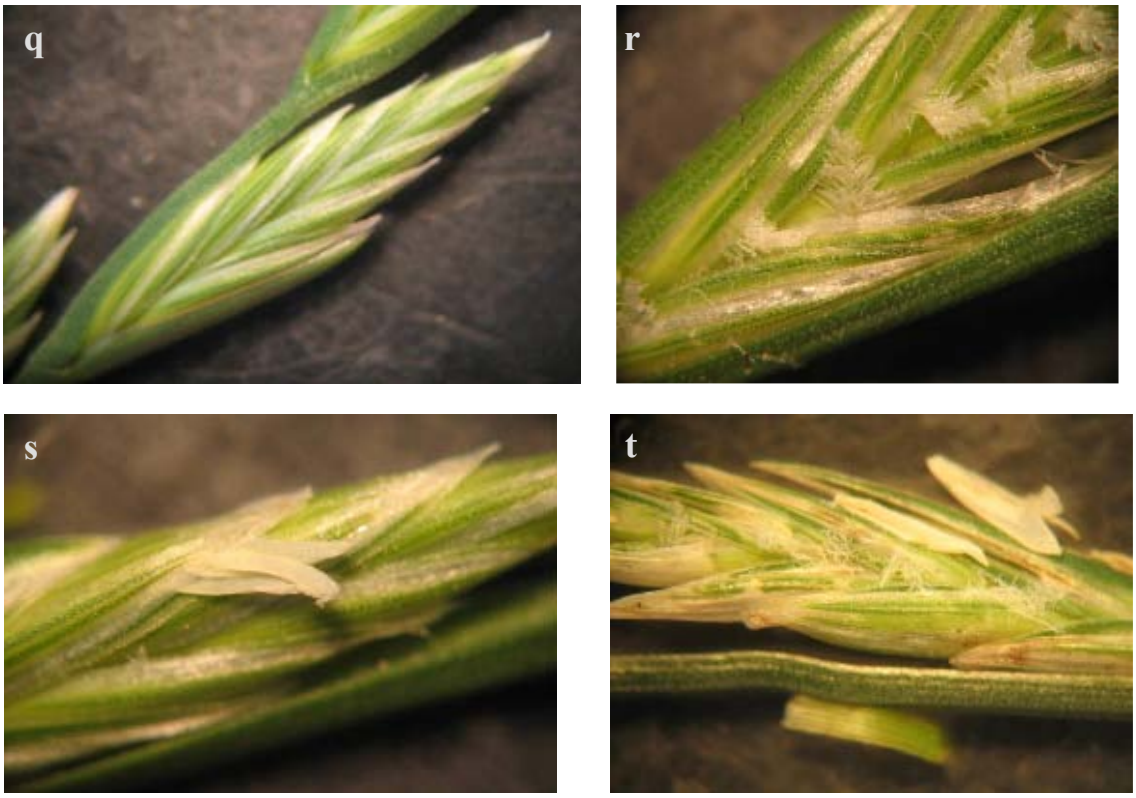


(i, j) Stage V inflorescence meristems continued to elongate (up to 2 mm) while the axillary meristems on the spikelets began to differentiate into florets. Scale: bars = 0.5 mm.



(k - n) Stage VI inflorescences were visible by eye. Well-developed floral meristems were observed on spikelet flanks. Scale: bars = 1 mm.

(o, p) Stage VII inflorescences were about to head with floral development almost complete. Individual spikelets were visible by eye, and the differentiated leaf primordia finally began to emerge as glumes wrapped around the developing spikelets. Scale: bars = 1 mm.



(q - t) Spikelets from Stage VIII inflorescences. Stage VIII was defined by heading, the emergence of the inflorescence from the flag (last) leaf. Protrusion of feathery stigma (r) and anthers (s) followed. Heads ranged in length from about 10 cm in length to more than 20 cm. Magnification: a-m, o = 50 ×; n, p, r and s = 20 ×; t = 16×; and q = 8×.

Reproductive development was divided into six stages (Stages III to VIII). The first physical indication of floral induction was the presence of double-ridges on the meristem flanks (Stage III; Figure 3.5 c). In contrast to vegetative meristems where leaf primordia develop on the meristem flanks after floral transition has occurred, outgrowth of leaf primordia was repressed and axillary meristems were visible (Figure 3.5 d, arrows). During Stage IV, axillary buds differentiated into spikelet meristems (Figure 3.5 h), and spikelet meristems differentiated into floral meristems during Stage V (Figure 3.5 j). By Stage VI, inflorescence meristems were visible by eye, and individual flower buds were distinguishable (Figure 3.5 m). At Stage VII, the inflorescence continued to expand (Figure 3.5 p), which was accompanied by rapid elongation of the culm or flowering stem. Stage VIII was defined by heading, or emergence of the inflorescence from the sheath of the flag (last) leaf. Protrusion of stigma and anthers from the florets followed (Figure 3.5 r; Figure 3.5 s).

The earliest signs of flowering in ‘Samson’ were seen after exposure to 12 LDs. The main tiller was at Stage IV and two secondary tillers (second and third) were at Stage III (Table 3.4). The earliest sign of flowering in ‘Impact’ was after exposure to 18 LDs. The main tiller was at Stage IV, and the second and third primary tillers were stage III (Table 3.5). Of the 18 ‘Samson’ and 15 ‘Impact’ plants dissected after the first sign of flowering, eight plants showed no signs of flowering, and a further six plants showed reproductive development in the main tiller only (Table 3.4; Table 3.5).

3.2.3 Flowering in vernalised ‘Samson’ and ‘Impact’ perennial ryegrass

After 62 to 65 LDs, flowering was assessed in 121 ‘Samson’ and 167 ‘Impact’ perennial ryegrass plants. Both the number of reproductive tillers per plant and the physical location of each reproductive tiller on each plant were recorded (Table 3.6; Table 3.7). Eighty-two ‘Samson’ (68%) and 93 ‘Impact’ (56%) plants had one or more reproductive tillers and were considered to have flowered (Cooper, 1951). The main tiller flowered in all 175 flowering plants without exception. In 59 flowering plants (30 ‘Samson’ and 29 ‘Impact’ plants), the main tiller was the only reproductive tiller. In 38 plants with two reproductive tillers, in addition to the main tiller the other reproductive tiller was either the second (34 plants), third (3 plants) or fourth primary tiller (1 plant). The first primary tiller did not flower in any plants with only two reproductive tillers (Table 3.6). The remaining 78 flowering plants had between three and thirteen reproductive tillers per plant. The first primary tiller flowered in only 33 of these plants while the second and third primary tillers flowered in 75 and 69 plants, respectively. Prolifically flowering plants with six or more reproductive tillers (21 plants) were more likely to have a flowering first primary tiller (16 plants) than those plants with fewer flowers (e.g. of 66 plants with 2 or 3 reproductive tillers, only 3 plants had flowering first primary tillers; Figure 3.6). Fourteen plants had flowering secondary tillers.

Table 3.4 Meristem morphology in 'Samson' perennial ryegrass.

Meristems were dissected from individual 'Samson' plants vernalised for 64 d and exposed to increasing numbers of LD cycles.

Tiller	LD	0	2	6	8	10	12	14	16	18	20	22	27	29	31	33	35	37	39	43	43	43	47	55
Main tiller[a]		I	I	I	I	I	IV	VI	III	VI	II	IV	III	III	VII	VII	VIII	II	II	VIII	VIII	VIII	IV	VIII
1° tillers[b]	T	0	0	1	1	3	4	4	5	4	4	4	4	5	6	5	5	7	5	6	5	5	7	6
	V			1		3	2	0	5	3	4	4	3	5	6	2	2	7	5	0	1	3	4	6
	R						2	4	0	1	0	0		0	0	3	3	0	0	6	4	2	3	0
	1st			I	*	I	I	III	I	I	I	I	I	I	I	I	VIII	I	II	III	VIII	I	III	II
	2nd					I	III	IV	II	I	I	II	*	I	II	VII	VIII	I	II	VIII	VIII	VIII	III	II
	3rd					I	III	IV	I	III	I	I	I	I	I	VI	VIII	II	II	V	III	VII	III	II
	4th						I	IV	I	I	I	I	I	I	I	IV	II	II	I	IV	III	II	II	I
	5th								I					I	I	II	I	I	I	III	II	I	I	I
	6th														I			I		III			I	II
	7th																	I					I	
2° tillers[c]	T							4	3	3	2	5	6	5	4	9	6	4	8	16	8	12	11	12
	V							4	3	3	2	5	6	5	4	9	6	4	8	14	3	9	11	12
	R							0	0	0	0	0	0	0	0	0	0	0	0	2	5	3	0	0
3° tillers[d]	T											1	1	0	0	3	0	0	0	6	10	6	2	2
4° tillers[e]	T																				1			

[a] Meristem state of main tiller: Stage I and II meristems were vegetative, while Stage III to Stage VIII meristems were considered to be reproductive (Figure 3.5). [b] Total (T) number of primary tillers per plant, and number of vegetative (V) and reproductive (R) tillers. Meristem states of individual primary tillers are listed. [c] Total (T) number of secondary tillers per plant, and number of vegetative (V) and reproductive (R) tillers. [d] and [e] Total number of tertiary and fourth order tillers per plant (all vegetative).
* Meristem was destroyed during dissection. Shading: white - non-flowering plants; light grey - main tiller flowering only, dark grey - main and primary tillers flowering. One plant was analysed at each time-point (except 43 LDs, when three plants were dissected).

Table 3.5 Meristem morphology in 'Impact' perennial ryegrass

Meristems were dissected from individual 'Impact' plants vernalised for 64 d and exposed to increasing numbers of LD cycles.

Tiller	LD	0	2	6	8	10	12	14	16	18	20	22	27	29	31	33	35	37	39	43	43	43	47	55
Main tiller[a]		I	I	I	I	I	II	I	I	IV	I	V	VII	V	I	I	I	I	II	VIII	VIII	II	VIII	VIII
1° tillers[b]	T	0	0	1	1	3	3	3	4	4	4	5	4	4	6	5	6	5	5	6	5	5	6	5
	V			1	1	3	3	3	4	2	4	1	3	2	6	5	6	5	6	3	2	5	4	4
	R			0	0	0	0	0	0	2	0	4	1	2	0	0	0	0	0	3	3	0	2	1
	1st			I	I	I	I	I	I	II	I	III	II	II	I	I	I	I	I	I	II	II	II	II
	2nd					I	I	I	I	III	I	IV	III	III	I	I	I	I	II	VIII	III	II	VIII	VII
	3rd					I	I	I	I	III	I	IV	II	III	I	I	I	I	II	VII	III	II	III	II
	4th								I	II	I	III	II	I	I	I	I	I	I	VII	III	I	I	I
	5th											II			I	I	I	I	I	I	I	I	I	I
	6th														I		I			I			I	
2° tillers[c]	T								1	5	5	7	5	7	11	9	13	8	5	9	8	8	15	7
	V								1	5	5	7	5	7	11	9	13	8	5	8	8	8	15	7
	R								0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
3° tillers[d]	T									1	0	2	0	0	5	4	9	0	0	6	1	3	3	2

[a] Meristem state of main tiller: Stage I and II meristems were vegetative, while Stage III to Stage VIII meristems were considered to be reproductive (Figure 3.5). [b] Total (T) number of primary tillers per plant, and number of vegetative (V) and reproductive (R) tillers. Meristem states of individual primary tillers are listed. [c] Total (T) number of secondary tillers per plant, and number of vegetative (V) and reproductive (R) tillers. [d] Total number of tertiary tillers per plant (all vegetative). Shading: white - non-flowering plants; grey - main and primary tillers flowering. One plant was analysed at each time-point (except 43 LDs when 3 plants were dissected).

Table 3.6 Pattern of flowering in 'Samson' and 'Impact' perennial ryegrass.

Plants were vernalised for 64 d and exposed to 62-65 LDs. The numbers of reproductive tillers per plant and the origin of each reproductive tiller on each plant was recorded.

Reproductive tiller configuration[a]	Total	Samson	Impact
Total number of plants	**288**	121	167
Plants not flowering	**113**	39	74
Plants flowering	**175**	82	93
One reproductive tiller			
M	**59**	30	29
Two reproductive tillers	**38**		
M + 1	0	0	0
M + 2	34	15	19
M + 3	3	2	1
M + 4	1	1	0
Three reproductive tillers	**28**		
M + 1 + 2	3	2	1
M + 2 + 3	23	10	13
M + 2 + (1)	1	1	0
M + 3 + 4	1	1	0
Four reproductive tillers	**29**		
M + 1+ 2 + 3	12	5	7
M + 1 + 2 + 6	1	1	0
M + 1 + 2 + (1)	1	1	0
M + 2 + 3 + 4	12	3	9
M + 2 + 4 + 5	1	0	1
M + 3 + 4 + 5	2	1	1
Five reproductive tillers	**7**		
M + 1 + 2 + 3 + 4	4	2	2
M + 1 + 2 + 3 + (1)	1	0	1
M + 2 + 3 + 4 + 5	1	0	1
M + 2 + 4 + 5 + 6	1	1	0
Six reproductive tillers	**5**		
M + 1 + 2 + 3 + 4 + (1)	2	1	1
M + 1 + 2 + (3)	1	1	0
M + 2 + 3 + 4 + 5 + 6	2	0	2
Seven reproductive tillers	**1**		
M + 1 + 2 + 3 + 4 + (2)	1	0	1
Eight reproductive tillers	**5**		
M + 1 + 2 + 3 + 4 + 5 + 6 + 7	1	1	0
M + 1 + 2 + 3 + 4 + 5 + (2)	3	0	3
M + 2 + 3 + 5 + 8 + 9 + (2)	1	1	0
Ten reproductive tillers	**1**		
M + 1 + 2 + 3 + 4 + (5)	1	1	0
Eleven reproductive tillers	**1**		
M + 1 + 2 + 3 + 4 + 5 + 6 + (4)	1	0	1
Thirteen reproductive tillers	**1**		
M + 1 + 2 + 3 + 4 + 5 + 6 + (6)	1	1	0

[a] M - flowering main tiller, 1 - flowering first primary tiller, 2 - flowering second primary tiller etc. Numbers in brackets refer to the numbers of flowering secondary tillers. Numbers in bold refer to total numbers of plants in each class.

Table 3.7 Distribution of reproductive tillers in perennial ryegrass.

Reproductive tiller	Number of plants	
Main	116	(100%)
1st primary	33	(28%)
2nd primary	109	(94%)
3rd primary	72	(62%)
4th primary	35	(30%)
5th primary	14	(12%)
6th primary	7	(6%)
7th primary	1	(1%)
8th primary	1	(1%)
9th primary	1	(1%)
Secondary tiller(s)	14	(12%)

Plants were vernalised for 64 d and exposed to 62 to 65 LDs to induce flowering. The identities of individual reproductive tillers were determined in plants with two or more reproductive tillers.

3.2.4 Vegetative development of primary tillers

The numbers of daughter (secondary and tertiary) tillers on each of the first three primary tillers in 'Samson' and 'Impact' vernalised for 64 d and exposed to increasing numbers of LD cycles were determined retrospectively (Table 3.8). After exposure to thirty or more long days, 'Samson' plants had fewer total tillers than 'Impact' plants ($P<0.05$). 'Samson' and 'Impact' plants had similar numbers of primary tillers per plant, but 'Impact' plants had more daughter tillers branching from their primary tillers than 'Samson' plants. The numbers of daughter tillers branching from first and second primary tillers were comparable within each cultivar (Table 3.8).

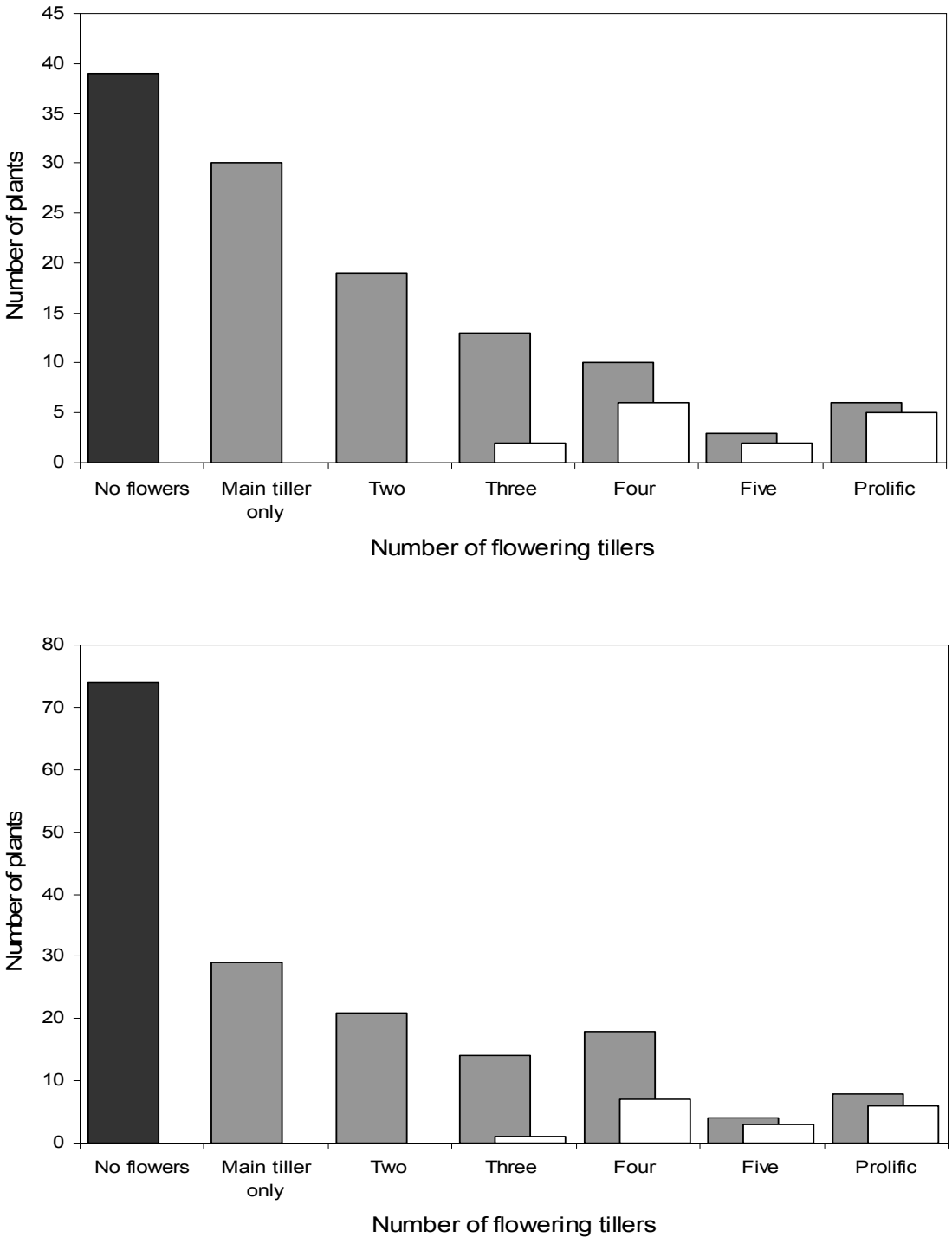


Figure 3.6 Number of reproductive tillers per plant in 'Samson' (above) and 'Impact' (below) perennial ryegrass.

Plants were vernalised for 64 d and exposed to 62 to 65 LDs. Black - non-flowering plants; grey - flowering plants. Plants with flowering first primary tillers are shown (white). Plants with six or more flowers were considered prolific-flowering.

Table 3.8 Tillering in 'Samson' and 'Impact' perennial ryegrass.

	LD	0	2	6	8	10	12	14	16	18	20	22	25	27	29	31	33	35	37	39
Samson	n	3	2	4	4	3	5	4	4	3	4	4	2	4	4	3	4	3	4	3
	T	1.0 (0.00)	1.0 (0.00)	2.5 (0.29)	3.5 (0.65)	4.7 (0.67)	4.6 (0.24)	7.0 (1.22)	8.0 (0.41)	6.7 (0.88)	9.3 (1.31)	9.8 (1.03)	15.0 (1.00)	9.5 (1.04)	9.0 (0.91)	9.3 (1.20)	11.3 (2.56)	11.7 (1.20)	15.0 (2.12)	10.7 (1.76)
	1st				0.3 (0.25)	0.3 (0.33)	0.0 (0.00)	1.3 (0.48)	1.0 (0.41)	0.7 (0.33)	1.3 (0.95)	2.3 (0.25)	3.0 (0.00)	1.5 (0.50)	1.3 (0.25)	2.0 (0.58)	1.8 (0.85)	2.3 (0.88)	1.3 (0.75)	2.0 (0.58)
	2nd						0.2 (0.20)	1.3 (0.48)	1.3 (0.25)	1.0 (0.58)	1.8 (0.25)	1.8 (0.48)	2.5 (0.50)	2.0 (0.41)	1.8 (0.48)	1.7 (0.33)	2.3 (0.63)	2.3 (0.33)	3.0 (0.71)	1.7 (0.67)
	3rd								0.5 (0.29)	0.0 (0.00)	0.8 (0.25)	0.5 (0.29)	2.0 (0.00)	0.8 (0.48)	0.3 (0.25)	0.0 (0.00)	1.3 (0.75)	0.7 (0.33)	2.3 (0.25)	1.0 (0.58)
Impact	n	3	2	4	4	3	5	4	4	3	4	4	2	4	4	3	4	2	4	3
	T	1.0 (0.00)	1.0 (0.00)	2.5 (0.29)	3.0 (0.41)	5.0 (0.58)	4.6 (0.24)	6.5 (1.04)	7.5 (0.87)	10.7 (0.67)	9.0 (0.58)	12.0 (1.41)	18.5 (3.50)	14.3 (1.55)	14.8 (0.95)	19.3 (1.86)	17.0 (0.87)	28.5 (0.50)	16.0 (1.08)	14.3 (1.76)
	1st					0.7 (0.67)	0.2 (0.20)	0.5 (0.29)	1.8 (0.25)	2.0 (0.58)	1.3 (0.25)	2.0 (0.41)	4.5 (2.50)	2.5 (0.65)	2.3 (0.25)	3.0 (1.00)	4.7 (0.58)	8.0 (1.00)	2.8 (0.48)	1.7 (0.33)
	2nd						0.2 (0.18)	0.8 (0.48)	0.8 (0.25)	2.3 (0.33)	1.8 (0.25)	2.8 (0.75)	4.5 (1.50)	3.3 (0.48)	4.8 (0.48)	3.7 (1.20)	4.3 (0.76)	7.0 (0.00)	3.8 (0.48)	3.3 (0.33)
	3rd							0.3 (0.25)	0.3 (0.25)	1.0 (0.00)	0.8 (0.48)	1.3 (0.25)	2.5 (0.50)	2.0 (0.41)	1.8 (0.48)	2.7 (0.33)	2.3 (0.29)	4.5 (0.50)	2.5 (0.87)	2.3 (0.88)

Plants were vernalised for 64 d and exposed to increasing numbers of LD cycles. LD - date tillers were dissected, n - number of plants sampled on each date, T - average number of tillers per plant (average of n plants), 1st - average (n plants) number of daughter tillers on the first primary tiller, 2nd - average (n plants) number of daughter tillers on the second primary tiller, 3rd - average (n plants) number of daughter tillers on the third primary tiller. SEM are given in brackets.

3.2.5 *LpFT3* and *LpMADS3* gene expression in 'Samson' perennial ryegrass

Expression of *LpFT3* and *LpMADS3*, putative orthologues of the *Arabidopsis* floral regulators *FT* and *AP1* (Gagic, 2007; N. T. Forester, I. Kardailsky, B. Veit, AgResearch Ltd., unpublished), were determined using RT-PCR on selected main and primary tillers harvested at dusk from 'Samson' plants vernalised for 64 d, exposed to increasing LD cycles (Table 2.9). A minimum of three replicate cDNA synthesis reactions were carried out on each RNA sample, and a minimum of two PCR amplifications were carried out on each cDNA sample for each gene to ensure repeatability. Representative results are shown in Figure 3.7. The house-keeping gene *LpGAPDH* (Gagic, 2007) was used as a positive control to ensure the mRNA extraction, cDNA synthesis and PCR had worked successfully.

In 'Samson' perennial ryegrass vernalised for 64 d *LpFT3* expression was upregulated after exposure to 12 LDs, and *LpMADS3* expression was strongly upregulated in plants exposed to 20 and 25 LDs, although expression of both genes was lower in the main tillers on these dates.

RT-PCR should be used with care when assessing quantitative gene expression as different sequences and samples will behave differently during amplification. *LpFT3* expression levels were similar in most tillers from within plants (Figure 3.7; e.g. 12 LDs, tillers M, 1 and 2), but different between plants (Figure 3.7; e.g. compare expression between the plants harvested at 12 LDs and 16 LDs).

LpMADS3 was strongly expressed in samples exposed to 20 and 25 LDs, but PCR products were also detected in samples exposed to 8 LDs, 12 LDs and 16 LDs (Figure 3.7). *LpMADS3* expression in the main tillers (Figure 3.7, 'M') showed a gradual increase with increasing LD exposure, up until 25 LDs (although *LpFT3* and *LpGAPDH* expression were also low in the main tillers exposed to 20 and 25 LDs, and the main tiller in the plant exposed to 25 LDs was almost flowering; data not shown). Although the bands might be PCR artefacts, in plants exposed to 12 and 16 LDs, *LpMADS3* expression was higher in the main (Figure 3.7, 'M') and second primary (Figure 3.7, '2') tillers than in the first primary tiller (Figure 3.7, '1').

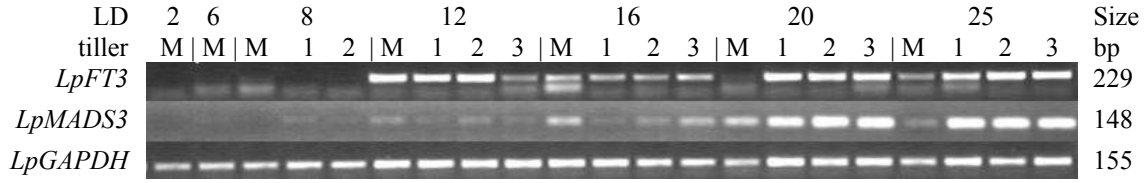


Figure 3.7 mRNA expression of *LpFT3* and *LpMADS3* in 'Samson' perennial ryegrass.

Plants were vernalised for 64 d, and then exposed to increasing LD cycles. Tillers were harvested at dusk. M - main tiller, 1 - first primary tiller, 2 - second primary tiller, 3 - third primary tiller. PCR products for *LpFT3*, *LpMADS3* and *LpGAPDH* were expected to be 229 bp, 148 bp and 155 bp, respectively (N. T. Forester and M. Gagic, AgResearch Ltd., personal communication). The house-keeping gene *LpGAPDH* was used as a loading control. PCR products were resolved on a 2% agarose gel. 1 kb Plus DNA Ladder (Invitrogen 10787-026) which had relevant bands at 100 bp, 200 bp and 300 bp, was used to check product size. *LpFT3* ran between the 200 bp and 300 bp bands, while both *LpMADS3* and *LpGAPDH* ran between the 100 bp and 200 bp bands (ladder not shown).

Chapter 4. Discussion

The main aim of this project was to investigate how perennial ryegrass maintains its perennial habit. Two experiments were carried out to test the environmental control and spatial control hypotheses. The environmental control hypothesis stated that all sufficiently vernalised tillers would flower after exposure to a critical number of LD cycles. Alternatively, the spatial control hypothesis stated that, in addition to environmental control, a spatial control mechanism regulates flowering in perennial ryegrass. The first experiment was conducted to investigate the differences in growth and development between annual (Italian) and perennial ryegrass species. The second experiment was carried out to investigate the behaviour of individual tillers of perennial ryegrass at flowering. It was anticipated that along with several reproductive tillers per plant, one or more contemporary tillers (i.e. tillers of the same age) would remain vegetative.

4.1.1 Experimental design

In Experiment I, the perennial ryegrass seed line (F_1, GA66/542 x A10654/1) was selected as it was used in mapping programmes at AgResearch Ltd. It was envisaged that genetic information from the mapping programme would be useful for this project, and any useful information generated from this project could be mapped. Grasslands Tama was selected as it is a Westerwolds type Italian ryegrass, and known to be extremely annual in habit (W. M. Williams, AgResearch Ltd., personal communication). Seedlings were grown in non-inductive conditions until they were 28 days old, by which time most plants had four leaves and the first primary tiller was beginning to emerge from the leaf axil of the first leaf on the main tiller. The Italian ryegrass line grew relatively uniformly, but 31% of perennial ryegrass seedlings failed to produce a third leaf by the end of four weeks (data not shown). Successful establishment of surviving perennial ryegrass seedlings and Italian ryegrass suggested that growth conditions were satisfactory. The Italian ryegrass line was from a commercially produced cultivar, and seed had been stored in the Margot Forde Forage Germplasm Centre, at low temperature and humidity. The perennial ryegrass seed had

been stored in a laboratory cupboard. It is possible that seed quality was reduced due to conditions at seed harvesting, or storage at room temperature. An alternative possibility is that parents were heterozygous for a recessive lethal allele, leading to a 3:1 ratio of viable to non-viable seedlings. It was thought that the years spent under selection during cultivar development and seed production had led to uniform growth in the Italian ryegrass cultivar, and for this reason, commercial perennial ryegrass cultivars were used in Experiment II.

'Samson' perennial ryegrass was selected as it was low tillering, making it more manageable during the experiment. 'Impact' perennial ryegrass was selected for replication to validate results, and in case of a cultivar effect affecting the outcome of the experiment, (e.g. intolerance to growth conditions, incompatible primers for PCR, etc). 'Samson' and 'Impact' perennial ryegrass cultivars did not appear to grow any more uniformly than the F_1 hybrid line used in Experiment I: vernalisation requirement, number of tillers per plant, number of reproductive tillers per plant, onset of floral initiation and heading date were all variable.

Part way through Experiment II, it was noticed that the light intensity in NISDs was very low (not recorded), and most control plants had died during NISDs (Table 2.7; Treatment 3). Lighting was increased slightly (PPF = 200 μmol m^{-2} s^{-1}) and plant growth in Treatment 2 was improved. It is interesting that lighting during vernalisation was much lower (PPF = 29 μmol m^{-2} s^{-1}) than in NISDs, but here temperature was presumably the growth limiting factor rather than light.

In both experiments, plants were destructively harvested to examine meristem morphology at different stages and for histology and molecular analyses. In Experiment I, samples were collected at five key time-points, including after exposure to 5LDs (for histology; 8 LDs for dissections) and after 15 LDs (for histology; 17 LDs for dissections). The critical number of LD cycles for floral induction in the parent plants of the F_1 perennial ryegrass line used was previously shown to be between 5 and 8 LDs, and it was expected that morphological changes in the apical meristem would be observed 10 d after induction (I. Kardailsky, AgResearch Ltd., personal communication). In Experiment II, floral progression in individual meristems across entire plants was examined during exposure to increasing numbers of LD cycles. Plants were harvested on most alternate days during LD exposure.

Tillering was assessed retrospectively in destructively harvested perennial and Italian ryegrass plants in Experiment I (Table 3.2). Generally, older plants of each species had more tillers than younger plants. Secondary and tertiary tillers made up a significant proportion of the total number of tillers in older plants. In Experiment II, the total number of tillers per plant was fewer in 'Samson' than in 'Impact' (Table 3.8), although both cultivars had similar numbers of primary tillers (data not shown). 'Impact' had more daughter (secondary and tertiary) tillers branching from its primary tillers than 'Samson'. Within cultivars, the numbers of daughter tillers on the first and secondary primary tillers were comparable (Table 3.8).

Perennial ryegrass has obligate requirements for vernalisation and long photoperiods before flowering can occur, while Italian ryegrass has a facultative requirement for vernalisation and an obligate requirement for long photoperiods (Cooper, 1951; Cooper, 1960; Heide, 1994; Aamlid et al., 2000). Studies on the parent plants of the F_1 perennial ryegrass line used had previously indicated that between 28 and 35 d at 5°C in a 9 h photoperiod were sufficient for vernalisation (N. T. Forester, I. Kardailsky, B. Veit, AgResearch Ltd., personal communication), although Aamlid et al. (2000) had determined that 63 d of vernalisation was required for most European varieties of perennial ryegrass.

In Experiment I, perennial and Italian ryegrass plants were vernalised for 55 d, 33 d (perennial ryegrass only) and 0 d, and plants were then induced to flower in LDs. It was expected that all Italian ryegrass plants from both treatments would flower, and that vernalised Italian ryegrass would flower earlier than non-vernalised plants. All but two Italian ryegrass plants flowered after 104 LDs. The two non-flowering plants were not investigated further, but it is possible that they were perennial ryegrass contaminants or damaged.

Only 61% of the F_1 perennial ryegrass vernalised for 52 d, and 23% of plants vernalised for 33 d flowered in Experiment I (Table 3.3). It was concluded that vernalisation requirement is subject to genetic variation (previously concluded by Cooper, 1952; Cooper, 1954; Cooper, 1960; Gangi et al., 1983), and that the vernalisation requirement had been met in flowering plants, but not in non-flowering plants. While some plants (23%) became competent to flower after exposure to only 33 d vernalisation, others (39%) apparently required longer than 52 d.

In Experiment II, plants were vernalised for 64 d, and 68% of 'Samson' plants and 55% of 'Impact' plants flowered (Table 3.6). It was concluded that 'Samson' required less vernalisation to achieve 50% flowering than 'Impact'. Conclusions could not be drawn on the length of vernalisation required to achieve 100% flowering as this would depend on the degree of genetic variation within the populations. As expected, non-vernalised perennial ryegrass plants did not flower as the obligatory vernalisation requirement had not been met.

In Experiment I, all plants were initially put outside into natural LDs, under prevailing environmental conditions. At this time (mid to late summer, 2004) there was more rain, temperatures were lower and there were fewer sunshine hours than the 30 year average (Table 2.2). After 58 LDs in these conditions, all of the vernalised Italian ryegrass plants had flowered, but only 11% of non-vernalised Italian ryegrass had done so (Table 3.3). This was unexpected as the obligatory LD requirement had been satisfied, as indicated by 100% flowering in vernalised Italian ryegrass. Flowering was also lower than expected in vernalised perennial ryegrass (Table 3.3). Plants were then transferred to a heated glasshouse with daylength extension, and flowering commenced (Table 3.3), suggesting that the natural long days were insufficient for flowering. There are a number of possible explanations for the low flowering observed during the first 58 LDs, including low light, low temperature and changing photoperiod. Low light intensity and shading are known to inhibit tillering in grasses, a mechanism that prevents overcrowding in grass swards (Mitchell, 1953a; Mitchell, 1953b; Gautier et al., 1999). Axillary meristems develop into tillers during vegetative growth, and differentiate into spikelet meristems during reproductive growth. It is possible that the same mechanism controls development of axillary meristems into new tillers during vegetative growth, and differentiation into spikelet meristems during reproductive growth. Low temperatures during floral induction might have also delayed flowering. 'Veyo', a perennial ryegrass variety from Italy, flowers during vernalisation, but other central and Northern European cultivars do not flower until they are exposed to LDs and warmer temperatures (Aamlid et al., 2000). Cooper (1954) showed that flowering was delayed at lower temperatures. Light quality and ambient temperature flowering pathways have been identified in *Arabidopsis* (Blazquez et al., 2003; Cerdan and Chory, 2003; Samach and Wigge, 2005), and it would be interesting to investigate whether these pathways have been conserved in the grasses. Another possibility is that during late summer,

photoperiods were getting shorter (Appendix A), which would limit the amount of time that *CO* is expressed before dusk (Jarillo et al., 2008), and it is possible that, in addition to detecting photoperiod length, the circadian clock also detected that the photoperiod was getting shorter. In Experiment II, inductive photoperiods were provided in a heated greenhouse, with additional lighting and daylength extension, to avoid confounding effects of the weather encountered in Experiment I.

4.1.2 Flowering

There was a difference in plant morphology between perennial and Italian ryegrass at flowering in that perennial ryegrass had a high proportion of vegetative tillers, while Italian ryegrass had a higher proportion of reproductive tillers (Figure 3.4). Vernalised Italian ryegrass plants had an average of 7.8 tillers in total per plant after exposure to 15 LDs (Table 3.2) and an average of three reproductive tillers per plant after exposure to 58 LDs (data not shown). Perennial ryegrass vernalised under the same conditions had 14.6 tillers in total per plant (Table 3.2). The final number of reproductive tillers (after 104 LDs) in Experiment I was not recorded, but the average number of reproductive tillers per plant for vernalised perennial ryegrass in Experiment II (under different conditions) was 2.7 in 'Samson' and 2.8 in 'Impact' (Table 3.6). These data suggest that flowering in Italian ryegrass, an annual species, is controlled by exposure to inductive LDs, in accordance with the environmental control hypothesis. Effective resource partitioning between vegetative and reproductive tillers in perennial ryegrass allows both seed production and the perennial habit. In contrast, endogenous resources appear to be completely consumed by flowering in Italian ryegrass plants (Figure 3.4).

There is debate about whether the vernalisation response can be transferred between tillers in the temperate grasses (Kleinendorst, 1973; Heide, 1994; Havstad et al., 2004), but whatever the case, according to the environmental control hypothesis, the minimum number of reproductive tillers should equate to the minimum number of tillers present at the onset of vernalisation. At least three meristems are present in seeds (Kleinendorst, 1973; Langer, 1979; and this study). Therefore, even assuming no new tillers developed prior to onset of vernalisation (which was unlikely), the minimum number of reproductive tillers should be three. However, 60% of 'Samson' and 53% of 'Impact'

flowering plants had only one or two reproductive tillers (Table 3.6; Figure 3.6). Perennial ryegrass appears to have a regulatory mechanism which prevents flowering in some apparently competent tillers, therefore favouring the spatial control hypothesis. These data support the rejection of the environmental control hypothesis in favour of the spatial control hypothesis for control of flowering in perennial ryegrass. Restricting flowering to fewer tillers than are competent would confer a selective advantage in a perennial species.

In perennial ryegrass, if the environmental control hypothesis is valid, then if any tiller flowers, all contemporary tillers, i.e. tillers that emerged at the same time or earlier than the youngest reproductive tiller, would be exposed to the same duration of vernalisation and the same inductive LDs, and would be expected to flower. In this study, the main tiller flowered in every flowering plant. For the purposes of this discussion, only plants that had two or more reproductive tillers will be considered. If all contemporary tillers flower, then in plants with two reproductive tillers, only the main tiller and the first primary tiller would be expected to flower as they were the first two tillers to emerge and had been exposed to the longest vernalisation and the same inductive photoperiods. However, this combination of reproductive tillers did not occur in any of the 38 plants with only two reproductive tillers. The main tiller flowered in all 38 plants, along with either the second (34 plants), third (3 plants) or fourth (1 plant) primary tiller (Table 3.6). The oldest, first primary tiller flowered in only 28% of all plants with two or more reproductive tillers, while the younger, second primary tiller flowered in 94% of these plants (Table 3.7). The third and fourth primary tillers flowered in 62% and 30% of flowering plants, respectively (Table 3.7). This is a key observation, and there was no obvious explanation for the lack of flowering in the first primary tiller. The first primary tiller was present in all plants, did not appear diseased or damaged, and was actively producing daughter tillers at a similar rate to the second primary tiller (Table 3.8). These observations support the existence of a spatial control mechanism which regulates flowering in perennial ryegrass. Similar events may have been observed by Aamlid et al. (2000) in perennial ryegrass and by Havstad et al. (2004) in four temperate perennial grasses. These authors noted that flowering did not occur in all tillers which had been subjected to the entire vernalisation treatment, while younger tillers receiving a shorter period of vernalisation did flower. The specific identity of non-flowering tillers was not indicated.

According to the environmental control hypothesis, in a perennial grass species, all over-wintering tillers would be vernalised, and competent, and would flower on exposure to LDs in spring and early summer. Reproductive tillers die following flowering and seed production. Perennating tillers would emerge in spring, giving rise to daughter tillers in summer and autumn. All of these tillers would be vernalised over winter and repeat the cycle. However, it has been reported that there is a high mortality rate for tillers emerging in spring because they are too small to survive summer stressors (e.g. Davies, 1996). Although it is not clear from the literature, some over-wintering tillers must remain vegetative to allow perennation. Maintaining an older tiller in a vegetative state, in accordance with the spatial control hypothesis, would confer a selective advantage. Older tillers would have significant resources including established root systems, a larger biomass, and many daughter tillers. These resources would increase the chances of the plant surviving harsh summer conditions, and the presence of daughter tillers would give the plant a competitive advantage over annual seedlings when conditions became more favourable (e.g. when the summer drought ended).

4.1.3 Meristem morphology

In Experiment I, the apical meristems of main tillers were dissected at five key time-points. In Experiment II, all tillers were dissected on alternate days during LD exposure to observe changes in meristem morphology during floral induction in greater detail. Meristem morphology was divided into vegetative and reproductive development. Two forms of vegetative meristems were observed, and were initially termed ‘short’ (Stage I, Figure 3.1 a; Figure 3.5 a) and ‘stacked’ (Stage II; Figure 3.1 b and c; Figure 3.2 a and b; Figure 3.5 b). Both forms had leaf primordia on the meristem flanks, but Stage II meristems had an elongated meristem axis and more leaf primordia on their flanks. Stage I meristems were observed in all plants analysed, and most of the younger meristems were Stage I. In perennial ryegrass, Stage II meristems were only observed in plants that had been vernalised and then exposed to warm temperatures (e.g. 14 NISDs, Table 3.1; or 12 LDs, Table 3.5). Stage II meristems were also observed in non-vernalised Italian ryegrass at just five weeks old, and in vernalised Italian ryegrass immediately following vernalisation. Stage II morphology is regarded by Langer (1979)

as a prelude to flowering. Its structure is formed when leaf initiation at the meristem occurs faster than leaf elongation and emergence. Leaf emergence rates in perennial ryegrass are relatively constant over a wide range of temperatures and conditions, but leaf initiation rates vary with temperature and other factors (Cooper, 1951; Langer, 1963; Langer, 1979). In perennial ryegrass, during low temperature vernalisation, leaf emergence rates and leaf initiation rates are similar, so stacking does not occur. However, when plants are returned to warm temperatures, leaf initiation rates increase, and stacking does occur.

This does not explain why non-vernalised perennial ryegrass meristems remained at Stage I during prolonged exposure to warm temperatures (NISDs), or why Italian ryegrass had Stage II meristems during low temperature vernalisation. It is possible that stacking is controlled by the vernalisation pathway, and that Stage II morphology is an indicator of competence to flower. This would explain why Stage II meristems were not seen in non-vernalised plants, or prior to vernalisation. Italian ryegrass plants do not require vernalisation to become competent to flower, and could therefore stack without prior vernalisation. It is unclear why Italian ryegrass plants developed Stage II meristems in the cold, but it is possible that leaf emergence rates were slowed or that leaf initiation rates were not reduced.

Development of reproductive meristems was divided into six stages (Stages III to VIII; Figure 3.5 c to t). Although Stage III meristems looked similar to Stage II meristems, lateral organs differed between the two. Leaf primordia were slightly flattened and appeared to encircle the meristem flanks during vegetative development (Figure 3.5 a and b). At Stage III, outgrowth of leaf primordia was repressed, while axillary meristems in the axils of leaf primordia began to differentiate, giving rise to the double ridge morphology (Figure 3.5 d; Cooper, 1951; Langer, 1979). Later stages were easier to identify as reproductive as the meristem was larger and appeared to flatten slightly as lateral organs emerged.

There are three tiers of meristem activity in the developing grass inflorescence. The apical meristem produces spikelet meristems on its flanks (Stage IV; Figure 3.5 g), and eventually the axis of the apical meristem becomes the rachis, which is the main stem of the grass inflorescence. Spikelet meristems differentiate and produce floral meristems on their flanks (Stage V; Figure 3.5 j), and the axis of the spikelet meristem becomes

the rachilla, the stem that individual florets branch from. Florets develop from floral meristems (Stage VI; Figure 3.5 m). Stage VII and VIII involve rapid elongation of the culm, or floral stem, and emergence of the inflorescence above the sward canopy.

The first signs of reproductive development were seen in 'Samson' and 'Impact' after 12 and 18 LDs, respectively. Generally, plants that had been exposed to more LDs had more advanced inflorescence meristems, but this was not always the case. Although environmental effects cannot be ruled out, genetic variation in flowering time genes were probably responsible for the variation observed. Dissected plants provided similar data to flowering plants (Table 3.5; Table 3.6; Table 3.7). Of 33 plants dissected, about 70% had reproductive meristems, similar to flowering plants (61%), and the first primary tiller only flowered in 35% of plants with two or more reproductive tillers (28% in flowering plants). Reproductive secondary tillers were observed in 24% of dissected plants and 14% of flowering plants.

4.1.4 Molecular analyses

At the outset of this project, *in situ* hybridisation of meristem sections with floral indicator genes was planned. It was envisaged that gene expression patterns would indicate which tillers had remained vegetative and which had adopted a floral fate. From this information, the spatial control hypothesis could be tested. Samples were collected, fixed and embedded to observe meristem morphology, and to look at gene expression patterns. Unfortunately, insufficient plant material was collected and technical difficulties with the methodology prevented this plan progressing further. Histological studies were not straight-forward either, with technical difficulties in aligning apices for sectioning, and damage to tissues during processing. Meristem states (vegetative or floral) were unable to be determined for many of the meristems that were of interest to this study (Figure 3.3). Neither method was used in the second experiment.

In the second experiment, RT-PCR was used to assess gene expression of floral integrator genes *LpFT3* and *LpMADS3*. In accordance with the spatial control hypothesis, and in view of the results from the flowering and dissection studies, a pilot study was conducted where expression of floral integrator genes was examined in the main tiller and the first, second and third primary tillers from selected 'Samson' plants

vernalised for 64 d and exposed to increasing LDs (Figure 3.7).

Perennial ryegrass *LpFT3* is a putative functional orthologue of the *Arabidopsis FT* gene (Gagic, 2007). According to the genetic models for flowering in the grasses (Figure 1.3; Figure 1.4; Cockram et al., 2007; Trevaskis et al., 2007; Jarillo et al., 2008), in vernalised plants, *LpFT3* would be expressed just before dusk after exposure to a critical number of LDs. *Arabidopsis* FT protein is a mobile signal which is synthesised in leaves and is transported to the apical meristem where it upregulates the floral meristem identity genes *AP1* and *LFY*, thereby initiating flowering (Abe et al., 2005; Wigge et al., 2005; Liu et al., 2007). It is assumed that a similar mechanism applies to the grasses. The second gene, *LpMADS3* is a putative functional orthologue of the *Arabidopsis* floral meristem identity gene *AP1* (N. T. Forester, I. Kardailsky, B. Veit, AgResearch Ltd., personal communication). Theoretically, *LpMADS3*, is regulated downstream of *LpFT3*. The house-keeping gene *LpGAPDH* was used as a positive control to ensure that mRNA had been extracted, that cDNA had been synthesised, and that PCR products had been amplified. In addition, *LpGAPDH* was used a loading control to check relative expression levels (Gagic, 2007).

It was expected that neither gene would be detected until plants had been exposed to a critical number of LDs, and that upregulation of *LpFT3* would be observed at the same time as, or before upregulation of *LpMADS3*. *LpFT3* was expressed strongly in tillers exposed to 12 LDs or more, and *LpMADS3* was strongly expressed in tillers exposed to 20 LDs or more (Figure 3.7).

Based on flowering and dissection data, it was expected that *LpFT3* and *LpMADS3* expression would be detected in the main tillers of all flowering plants, as the main tiller always flowered in flowering plants. It was also expected that the genes would be expressed in only a few of the first primary tillers, most of the second primary tillers, and a high proportion of the third primary tillers, reflecting the flowering results.

LpFT3 expression levels varied between plants, but were similar in most tillers from within plants (Figure 3.7), although expression levels were reduced in the main tillers from plants exposed to 20 and 25 LDs. Variation in *LpFT3* expression levels between plants might have been due to genotypic differences, and/or slight variations in harvesting times each day. Reduced *LpFT3* expression in the main tillers of plants

exposed to 20 and 25 LDs might have occurred because tillers were already induced to flower. Alternatively, the proportion of leaf material in the tiller sample might have been lower, and older senescing leaves might have had completely different transcriptomes to younger leaves. *LpFT3* expression levels were consistent between primary tillers, even the first primary tiller, where gene expression levels were expected to be reduced, based on flowering data. This might reflect the nature of LpFT3 activity, where the protein is synthesised in the leaves and is then transported to the meristem where further signalling and interactions occur. *LpFT3* gene expression in the leaves might not necessarily reflect whether the tiller will flower.

LpMADS3 PCR products were detected in samples exposed to 12 LDs, and more strongly in samples exposed to 20 and 25 LDs (Figure 3.7). *LpMADS3* expression in the main tillers (Figure 3.7, M) showed a gradual increase with increasing exposure to LD cycles, up until 25 LDs, although the main tiller in the plant exposed to 25 LDs was almost flowering (Stage VII; not shown) and both *LpFT3* and *LpGAPDH* expression levels were also low in this tiller (Figure 3.7).While it is possible that the PCR products observed in tillers exposed to 16 or fewer LDs might have been PCR artefacts, in plants exposed to 12 and 16 LDs, *LpMADS3* expression was higher in the main and second primary tillers than in the first primary tiller (Figure 3.7), which reflects the observations made in flowering plants.

Further analysis of gene expression levels are required to better understand floral induction in perennial ryegrass. Real-Time PCR should be used so that gene expression can be quantified and the inferences made above can be tested. Three plants of each cultivar were collected on most alternate LDs. A larger sample size should give a better picture of the relationship between gene expression patterns and flowering in perennial ryegrass.

4.1.5 Concluding remarks

The aim of this thesis was to investigate whether all competent perennial ryegrass tillers flowered in response to inductive photoperiods, in accordance with the environmental control hypothesis. In Experiment I, observations revealed that perennial ryegrass had a higher proportion of vegetative tillers at flowering than the annual, Italian ryegrass.

Although this indicated that there was a difference in flowering between the perennial and annual habits, neither of the hypotheses were substantiated. The second experiment was planned with observations of floral development in greater detail in two perennial ryegrass cultivars. The main tiller flowered in all flowering plants. It was expected that the oldest of the primary tillers, the first primary tiller, would flower in all flowering plants with two or more reproductive tillers. However, the first primary tiller flowered in only 28% of plants, favouring the spatial control hypothesis, which stated that a spatial regulatory mechanism might act to repress flowering in some competent tillers. These results were supported by morphology of meristems during floral initiation and development, and by a preliminary gene expression study. Maintenance of an older, established tiller in a vegetative state might give the perennial plant a greater chance of survival during summer.

4.1.6 Future work

Although it has been implied that maintaining the first primary tiller in a vegetative state would favour perenniality in perennial ryegrass, this has not been tested. The logical next step in this research area would be to test whether the trait does impact on perenniality. Some authors report that young, spring emerging tillers are too immature to survive summer in the field (e.g. Davies, 1996), thereby implying that perennation is achieved by maintaining some older tillers in a vegetative state, as observed here. Young tillers would probably survive summer in well-watered plant pots, so conditions of water stress should be applied to assess survival. Two perennial ryegrass selections could be used: plants with flowering first primary tillers and plants with non-flowering first primary tillers. Plants would be allowed to flower, and then subjected to different levels of water-stress over summer. Survival of the plants (and tillers) into autumn would give an indication of the importance of the vegetative first primary tiller.

It would be interesting to follow the fate of the first primary tiller into its second summer. Following flowering, the main tiller dies, and one possibility is that the first primary tiller becomes a ‘main’ tiller, and that secondary tillers become primary tillers. It would, therefore, be of interest to follow the fates of the new primary tillers (and other tillers) and see if they all flower in the second summer, or whether one or more are

maintained in a vegetative state. Another possibility is that the first primary tiller never flowers, perhaps due to epigenetic regulation.

Both experiments in this study were largely carried out under artificial conditions, typically involving low light levels and constant temperatures, while plants in the field would be subjected to daily light intensity, spectra and temperature fluctuations. Studies should be conducted to assess whether plants develop in the same way in the field, and to compare gene expression between field and laboratory grown plants.

Controlled cross-pollinations should be conducted to determine whether tiller fate (vegetative or floral) in the first primary tiller is subject to Mendelian inheritance, or more complex regulation. The following crosses should cover all combinations. Crosses giving segregating populations could be used for genetic analysis and trait mapping.

$T1^V \times T1^V$ $T1^F \times T1^F$ $T1^V \times T1^F$ $T1^F \times T1^V$

($T1^V$ refers to plants with a vegetative first tiller, and $T1^F$ refers to plants with a reproductive first tiller.)

A preliminary gene expression study was carried out here using the floral indicator genes *LpFT3* and *LpMADS3*. Gene expression was analysed in up to four tillers from eight 'Samson' plants exposed to a selected number of LD cycles. Further gene expression analysis is needed to validate the results of this experiment.

Three 'Samson' and three 'Impact' plants were collected on alternate LDs, one plant at midday and two plants in the evening. *LpFT3* expression had been shown to peak before dusk and was lowest at midday, while *LpMADS3* expression was highest at midday, but still showed reasonable expression in the evening (N. T. Forester, M. Gagic, I. Kardailsky, B. Veit, AgResearch Ltd., personal communication). *LpFT3* and *LpMADS3* expression in the main and first three primary tillers of remaining plants needs to be assessed. RT (reverse transcriptase)-PCR was used to give an indication of whether genes were expressed, but Real-Time PCR should be used for future analysis to give a more reliable indication of gene expression levels. Once analysis of *LpFT3* and *LpMADS3* expression in the available plant material is complete, expression patterns of other flowering time genes in the same tillers may be useful, including some or all of the following:

Based on the flowering pattern observed in this experiment (where the main and second primary tillers flowered in the majority of plants, and the first primary tiller flowered in only 28% of plants), it would be interesting to assess expression of vernalisation genes to determine whether the non-flowering first primary tiller actually became competent to flower. In *Arabidopsis*, plants are considered competent to flower following vernalisation-mediated repression of the potent floral repressor *FLC* (Cockram et al., 2007; Alexandre and Hennig, 2008). VRN2 is a putative functional orthologue of FLC in the grasses (Yan et al., 2004b). VRN1 is a putative functional orthologue of *Arabidopsis* SOC1 (Danyluk et al., 2003, Trevaskis et al., 2003; Yan et al., 2003; Yan et al., 2004a; Ciannamea et al., 2006b). In the grasses and *Arabidopsis*, *VRN1* and *SOC1* are repressed by VRN2 and FLC respectively, and upregulated by FT (known as VRN3 in the grasses, and LpFT3 in perennial ryegrass; Figure 1.2; Figure 1.3; Cockram et al., 2007; Trevaskis et al., 2007; Alexandre and Hennig, 2008). *VRN1* expression is upregulated by vernalisation, coinciding with down regulation of *VRN2* (Yan et al., 2004b). Together *VRN1* and *VRN2* expression patterns might give an indication of whether the non-flowering first primary tiller had become vernalised.

AP1 and *LFY* are floral meristem identity genes in *Arabidopsis* (Ausin et al., 2005; Bernier and Perilleux, 2005). Both genes are upregulated by (and downstream of) the floral integrators FT and SOC1 (Figure 1.1; Figure 1.2). LpMADS3 is a putative AP1 functional orthologue, and it is likely that *LFY* and *LpMADS3* would have similar expression patterns. The perennial ryegrass *LFY* sequence is GC-rich and achieving good PCR results has been difficult, although new generation DNA polymerase enzymes may alleviate this problem (N. T. Forester, AgResearch Ltd., personal communication).

TERMINAL FLOWER 1 (*TFL1*) encodes a Raf kinase inhibitor (*FT* gene family member) which was characterised in perennial ryegrass by Jensen et al. (2001). TFL1 and FT antagonistically regulate each other in *Arabidopsis*, where FT promotes and TFL1 represses flowering. In perennial ryegrass, TFL1 maintains indeterminate growth, and its expression is upregulated during inflorescence development, giving the indeterminate development seen in spikelet meristems (Jensen et al., 2001). *TFL1* expression patterns may be indicative of whether a tiller has adopted a floral fate, but variable expression levels during the different stages of inflorescence development may

make interpretation of gene expression difficult.

Floral organ identity genes have been identified in maize, and homologues could be identified in perennial ryegrass. Their expression would obviously indicate that floral initiation was underway. However, they might only be expressed in the later stages of inflorescence development (Figure 3.5). *In situ* hybridisation in meristem sections might be useful for determining where specific genes are expressed.

Results from this experiment, in two perennial ryegrass cultivars, indicate that maintenance of the first primary tiller in a vegetative state might be an important mechanism favouring perenniality in perennial ryegrass. Therefore, other perennial ryegrass cultivars, and other temperate perennial grass species, could be investigated. Physiological and genetic studies in a wide range of perennial ryegrass varieties from diverse ecological origins could indicate whether maintenance of the first primary tiller in a vegetative state is a species-wide trait, or signify that it is limited to a particular gene pool. In addition, wider studies, in other temperate perennial grasses might give some further insight into the mechanisms at work. Given the results of the study by Havstad et al. (2004), *Bromus inermis*, *Dactylis glomerata* and *Festuca pratensis* should be among those investigated. Results should indicate whether the trait is common in the temperate perennial grasses, or only in one or a few species.

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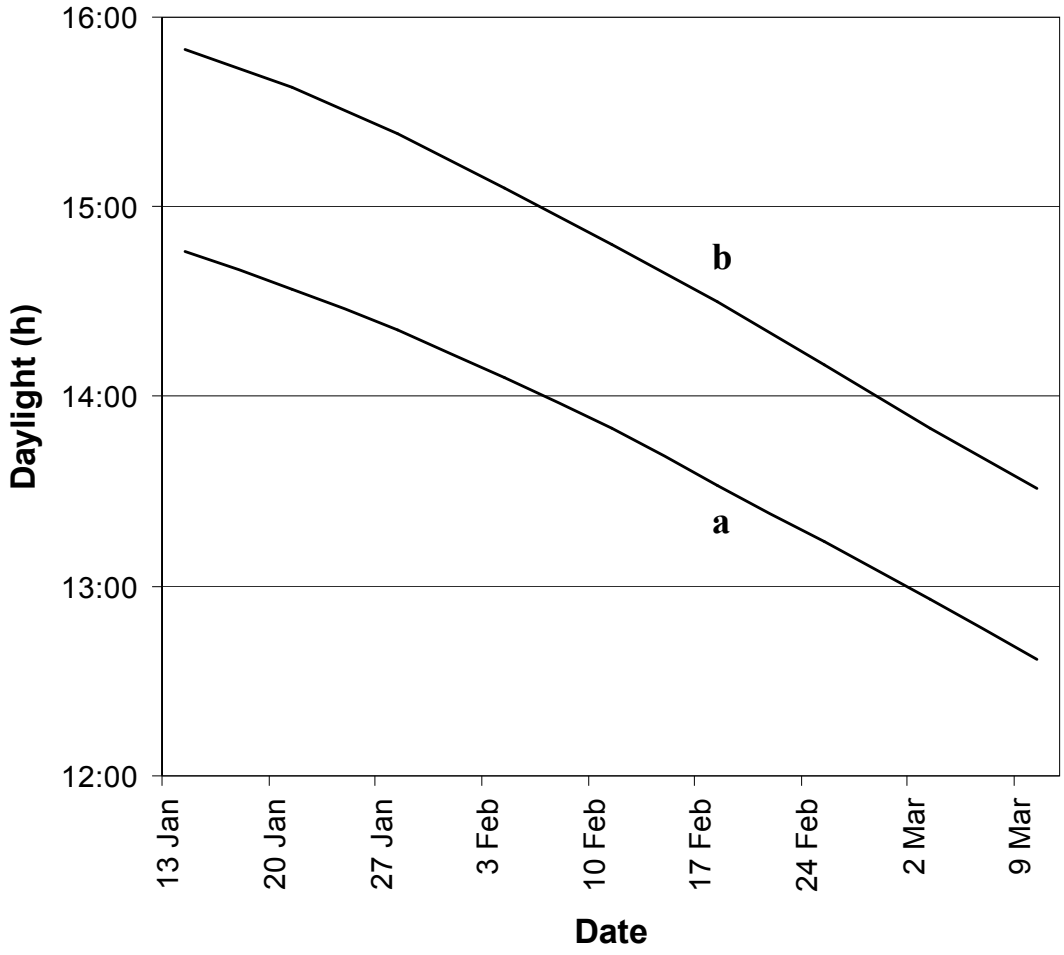
APPENDICES



Appendix A Daylength (a) and civil daylength (b) during natural LD exposure.

Daylength refers to time between sunrise and sunset. Civil daylength refers to time between civil twilights, when the sun is 6° below the horizon before sunrise and after sunset (LINZ).

Appendix B Daily rainfall, maximum and minimum temperatures, and hours of sunlight in Palmerston North.

January 13 to March 11, 2004 (AgResearch Weather Station, NIWA).

Date	Rain (mm)	Max (°C)	Min (°C)	Sun (h)
13/01/2004	2.6	20.2	15.9	0.0
14/01/2004	0.0	25.1	14.6	13.4
15/01/2004	0.0	22.1	9.9	2.5
16/01/2004	0.0	27.2	8.1	12.5
17/01/2004	0.0	27.6	15.3	13.7
18/01/2004	0.0	24.1	15.3	1.4
19/01/2004	4.0	20.8	14.3	0.0
20/01/2004	19.4	14.3	9.8	0.0
21/01/2004	35.8	20.9	11.6	1.1
22/01/2004	1.2	22.3	12.1	2.4
23/01/2004	0.8	19.9	12.2	2.7
24/01/2004	2.6	24.8	15.3	7.3
25/01/2004	0.0	22.1	13.5	4.9
26/01/2004	0.0	23.1	12.3	2.5
27/01/2004	0.0	25.6	13.5	11.3
28/01/2004	3.8	26.9	12.8	8.7
29/01/2004	0.0	27.9	15.7	9.0
30/01/2004	0.4	25.7	16.6	1.4
31/01/2004	2.2	26.1	18.5	2.4
1/02/2004	18.4	26.3	18.5	0.2
2/02/2004	27.2	23.8	18.5	1.1
3/02/2004	4.0	24.1	18.7	1.5
4/02/2004	0.0	22.6	17.3	0.6
5/02/2004	2.2	19.9	14.4	2.1
6/02/2004	0.0	22.2	13.8	7.5
7/02/2004	0.0	21.8	15.2	3.4
8/02/2004	2.8	20.9	13.7	3.7
9/02/2004	0.0	21.7	12.8	8.3

Date	Rain (mm)	Max (°C)	Min (°C)	Sun (h)
11/02/2004	6.6	24.6	16.8	0.7
12/02/2004	4.6	21.4	13.2	8.2
13/02/2004	0.0	23.4	11.7	8.9
14/02/2004	26.8	20.1	13.0	2.6
15/02/2004	58.2	15.0	12.4	0.0
16/02/2004	24.0	16.3	11.8	1.1
17/02/2004	7.6	17.7	12.0	3.2
18/02/2004	0.2	20.1	11.9	4.5
19/02/2004	37.8	21.2	12.5	0.8
20/02/2004	2.0	17.0	13.2	7.2
21/02/2004	5.6	21.1	11.7	1.3
22/02/2004	0.0	19.6	15.0	4.7
23/02/2004	9.6	20.7	12.6	1.1
24/02/2004	0.6	18.8	12.2	10.1
25/02/2004	0.0	18.7	9.1	12.1
26/02/2004	0.0	19.2	6.4	7.9
27/02/2004	4.6	19.8	10.6	1.4
28/02/2004	45.8	18.8	13.4	0.0
1/03/2004	1.2	19.5	11.4	3.0
2/03/2004	20.0	21.7	10.4	4.6
3/03/2004	1.2	16.9	10.4	5.2
4/03/2004	0.0	17.7	9.1	1.8
5/03/2004	0.0	20.6	5.5	12.1
6/03/2004	0.0	19.6	11.9	4.3
7/03/2004	0.0	24.3	11.6	10.3
8/03/2004	0.0	22.6	9.9	8.6
9/03/2004	0.0	21.6	12.6	8.6
10/03/2004	0.0	21.7	10.3	9.6

Appendix C Sample collection dates and number of samples collected 'Samson' Treatment 1.

NISDs → vernalisation → warm LDs

Date	Duration of treatment[a]		Sampling of Samson[b]		
			Dissections	Cryo-storage (md)	Cryo-storage (pm)
31/07/07	0 d				
14/08/07[c]	14 d	NISD	1		1
4/09/07	35 d	vernalisation	1		1
17/10/07	64 d	vernalisation	1	1	1
19/10/07	2 d	LD	1		1
23/10/07	6 d	LD	1	1	2
25/10/07	8 d	LD	1	1	2
27/10/07	10 d	LD	1		2
29/10/07	12 d	LD	2	1	2
31/10/07	14 d	LD	1	1	2
2/11/07	16 d	LD	1	1	2
4/11/07	18 d	LD	1		2
6/11/07	20 d	LD	1	1	2
8/11/07	22 d	LD	1	1	2
11/11/07	25 d	LD			2
13/11/07	27 d	LD	1	1	2
15/11/07	29 d	LD	1	1	2
17/11/07	31 d	LD	1		2
19/11/07	33 d	LD	1	1	2
21/11/07	35 d	LD	1		2
23/11/07	37 d	LD	1	1	2
25/11/07	39 d	LD	1		2
29/11/07	43 d	LD	3	1	2
3/12/07	47 d	LD	1	1	2
11/12/07	55 d	LD	1	1	1
18/12/07[d]	62 d	outside	50		
20/12/07[d]	64 d	outside	61		
21/12/07[d]	65 d	outside	10		

[a] NISD – non-inductive short days, LD – long days. [b] midday (md) samples were collected between 1400 and 1600, and evening (pm) samples were collected between 1830 and 1930. Daylight hours ran from 0600 to 2000 (14 hour photoperiod). [c] one sample was collected across all treatments as all treatments were the same up until this date. [d] samples analysed on 18th to 21st December were examined intact with reproductive tillers recorded.

Appendix D Sample collection dates and number of samples collected for 'Impact' Treatment 1.

NISDs → vernalisation → warm LDs

Date	Duration of treatment[a]		Sampling of Impact[b]		
			Dissections	Cryo-storage (md)	Cryo-storage (pm)
31/07/07	0 d				
14/08/07[c]	14 d	NISD	1		1
4/09/07	21 d	vernalisation	1		1
17/10/07	64 d	vernalisation	1	1	1
19/10/07	2 d	LD	1		1
23/10/07	6 d	LD	1	1	2
25/10/07	8 d	LD	1	1	2
27/10/07	10 d	LD	1		2
29/10/07	12 d	LD	2	1	2
31/10/07	14 d	LD	1	1	2
2/11/07	16 d	LD	1	1	2
4/11/07	18 d	LD	1		2
6/11/07	20 d	LD	1	1	2
8/11/07	22 d	LD	1	1	2
11/11/07	25 d	LD			2
13/11/07	27 d	LD	1	1	2
15/11/07	29 d	LD	1	1	2
17/11/07	31 d	LD	1		2
19/11/07	33 d	LD	1	1	1
21/11/07	35 d	LD	1		1
23/11/07	37 d	LD	1	1	2
25/11/07	39 d	LD	1		2
29/11/07	43 d	LD	3	1	2
3/12/07	47 d	LD	1	1	2
11/12/07	55 d	LD	1	1	2
20/12/07[d]	64 d	outside	65		
21/12/07[d]	65 d	outside	102		

[a] NISD – non-inductive short days, LD – long days. [b] midday (md) samples were collected between 1400 and 1600, evening (pm) samples were collected between 1830 and 1930. Daylight hours ran from 0600 to 2000 (14 hour photoperiod). [c] one sample was collected across all treatments as all treatments were the same up until this date. [d] samples analysed on 20th and 21st December were examined intact with reproductive tillers recorded.

Appendix E Sample collection dates and number of samples collected for 'Samson' and 'Impact' Treatment 2.

NISDs → vernalisation → NISDs

Date	Duration of treatment[a]		Sampling[b]	
			Dissections	Cryo-storage
31/07/07	0 d			
14/08/07[c]	14 d	NISD	1 per cultivar	1 per cultivar
4/09/07	21 d	vernalisation	1 per cultivar	1 per cultivar
17/10/07	64 d	vernalisation	1 per cultivar	1 per cultivar
19/10/07	2 d	NISD	1 per cultivar	1 per cultivar
23/10/07	6 d	NISD	1 per cultivar	1 per cultivar
25/10/07	8 d	NISD	1 per cultivar	1 per cultivar
27/10/07	10 d	NISD	1 per cultivar	1 per cultivar
29/10/07	12 d	NISD	1 per cultivar	1 per cultivar
31/10/07	14 d	NISD	1 per cultivar	1 per cultivar
2/11/07	16 d	NISD	1 per cultivar	1 per cultivar
4/11/07	18 d	NISD	1 per cultivar	1 per cultivar
6/11/07	20 d	NISD	1 per cultivar	1 per cultivar
8/11/07	22 d	NISD	1 per cultivar	1 per cultivar
13/11/07	27 d	NISD	1 per cultivar	1 per cultivar
17/11/07	31 d	NISD	1 per cultivar	1 per cultivar
19/11/07	33 d	NISD	1 per cultivar	1 per cultivar
23/11/07[d]	37 d	NISD	1 per cultivar	1 per cultivar

[a] NISD – non-inductive short days. [b] most samples were collected between 1600 and 1700. Daylight hours ran from 0800 to 1700 (9 hour photoperiod). [c] one sample was collected across all treatments as all treatments were the same up until this date. [d] remaining plants were discarded after this date due to low light in NISDs.

Appendix F Sample collection dates and number of samples collected for 'Samson' and 'Impact' Treatment 3

NISDs → NISDs → (warm LDs)

Date	Duration of treatment[a]		Sampling[b]	
			Dissections	Cryo-storage
31/07/07	0 d			
14/08/07[a]	14 d	NISD	1 per cultivar	1 per cultivar
4/09/07	21 d	vernalisation	1 per cultivar	1 per cultivar
17/10/07[b]	64 d	vernalisation	1 per cultivar	1 per cultivar

[a] one sample per cultivar was collected across all treatments as all treatments were the same up until this date. [b] Remaining plants were discarded on this date due to low light conditions in the short day room retarding growth