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Genetics and Molecular Breeding in *Lolium/Festuca* grass species complex

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Summary

Perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*L. multiflorum*) are regarded as ideal grass species for use as animal forage in temperate grassland agriculture. Ryegrasses establish and grow quickly, and provide dense swards of highly nutritious and easily digestible forage that can be turned into healthy meat and animal products for human consumption. However, their use is restricted since they lack persistency especially in marginal areas and locations that are subject to summer and winter stresses and drought stress. Close relative species from within genus *Festuca* are much better adapted to such abiotic stresses, but by contrast do not compare well in animal forage provision to *Lolium* species, as they show poor establishment and comparatively lower quality characteristics. *Lolium* and *Festuca* species hybridize naturally, and exhibit high frequencies of gene exchange in the hybrid condition. Intergeneric hybrids (*Festulolium*) between *Lolium* and *Festuca* species are being used to broaden the gene pool and to provide the plant breeder with options to combine high quality traits with broad adaptations to a range of environmental constraints. *Festulolium* varieties have promise as novel grasses with high forage quality and resistance to environmental stress and thereby can improve grassland productivity, persistency and benefit incomes. Recent progress on *Festulolium* breeding programs is described here. Conventional forage grass breeding programs rely on basis observable phenotype using the natural genetic variation found between and within varieties or ecotypes. Genetic improvement of forage grasses by conventional breeding programs is very slow due to the obligate outbreeding and perennial nature of grasses. Advances in genomics and gene

manipulation can complement and enhance conventional plant breeding programs. Many studies concerning implementation of DNA markers, high-throughout gene discovery, genome-wide gene expression analysis, and gene manipulation are being currently conducted for forage grasses. Recent progress on molecular and genomic research activity in the genus *Lolium* and *Festuca* is reviewed.

Key words: comparative genomics, gene cloning, DNA marker, *Festuca*, *Festulolium*, functional genomics, *Lolium*, QTL analysis, transgenesis

Introduction

Dairy farming and beef production in Japan depend largely on imported feed from abroad. In such circumstances, recently, large number of problems have arisen for animal production, including environmental pollution with animal wastes, serious animal diseases such as foot and mouse disease and bovine spongiform encephalopathy (BSE). The self-sufficiency rate of animal feed has been dramatically decreasing for past decades. In contrast, rice over-production has led to increase of uncultivated paddy fields. Forage grasses are considered the best alternative crops in a paddy field. Consequently, self-sufficient forage production is one of the essential pre-requisites for animal production with security and safety. Therefore it is priority to develop new forage varieties with higher productivity, forage quality and tolerance to summer and winter stresses and this is important for responsibilities associated with sustainable environmental and economic development.

The *Lolium-Festuca* group is composed of some highly herbage productive, nutritious,

persistent, seed-productive, and well-adapted grasses that are widely used for agricultural and amenity purposes, and for stabilization of soils (Jauhar 1993). Italian ryegrass (*Lolium multiflorum* Lam.), perennial ryegrass (*Lolium perenne* L.), meadow fescue (*Festuca pratensis* Huds.) and tall fescue (*Festuca arundinacea* Schreb.) are the most important species for temperate grassland agriculture in the world including Japan, providing the basis for grassland production systems (Barnes et al. 1995). The traits considered a priority for future grass varieties are improvement in dry matter yield and seed yield, herbage feeding value, for example, *in vitro* dry mater digestibility, the ratio of crude protein, water-soluble carbohydrate, fiber, concentration of alkaloid toxins, and traits associated with good persistency, including tolerance to a range of abiotic and biotic stresses (Wilkins and Humphreys 2003).

These forage grasses exhibit gametophytic self-incompatibility and hence require cross-pollinated breeding systems (Lundqvist 1962; Cornish et al. 1979). Varieties of forage grasses are synthetics developed by polycrossing selected parental clones (plants). The improvement of open-pollinating species depends essentially upon a change of gene frequency leading towards the fixation of favorable alleles whilst still maintaining some degree of heterozygosity. In this regard, the practice of recurrent selection has been seen to offer a useful breeding approach for these open-pollinating species (Burton 1992; Vogel and Pederson 1993). Recurrent selection programs have been demonstrated in perennial ryegrass to be effective for improvement to tolerance of summer stress in the warmer region of Japan (Yamada et al. 1999, 2001).

Intergeneric crosses involving the *Lolium-Festuca* species complex could potentially lead to a combination of the enhanced palatability and forage quality of *Lolium*

species with the higher resistance to disease and tolerance to stress environments such as cold and drought that are typical of *Festuca* species (Thomas and Humphreys 1991). In Europe and USA, a range of *Lolium* x *Festuca* hybrids (*Festulolium*) have been developed as novel temperate forage grasses (Humphreys et al. 2003). In Japan, *Festulolium* breeding has been recently initiated in three breeding stations of the National Agriculture and Bio-oriented Research Organization (NARO) (Yamada and Takamizo 2004).

Conventional forage grass breeding programs are performed on the basis of selecting observable phenotypes using natural genetic variation occurring within and between varieties and ecotypes. Genetic improvement of forage grasses by conventional breeding programs is very slow due to the obligate outbreeding and perennial nature of temperate grasses. However, the development of high-resolution genetic maps based on DNA-based molecular genetic markers shortens the time required previously for trait selection and makes it possible to target specific chromosomal locations responsible for agriculturally important traits such as flowering time, winter hardiness and forage quality. DNA marker assisted selection (MAS) technologies bring precision to plant breeding and target gene combinations that govern specific agronomic traits. Genomic and molecular genetic studies also provide available functional information on genes that regulate agriculturally important traits, such as tolerance to biotic and abiotic stresses and forage quality. The transformation approaches offer a future opportunity to generate unique genetic variation in the even that, the required variation is either absent or has very low heritability. The obligate outbreeding and perennial nature of forage grasses clearly present major difficulties to research in genomics and molecular genetics. However, many studies are currently being conducted using high-throughput genomics and molecular genetic technologies (Spangenberg et al. 1998; Spangenberg 2001;

Hopkins et al. 2003).

In this paper, recent progress on intergeneric hybridization programs between *Lolium* and *Festuca* are reviewed. The authors also describe recent progress on genomics and molecular research activities such as implementation of DNA markers, high-throughout gene discovery, genome-wide gene expression analysis, and gene manipulation in the genus *Lolium* and *Festuca* including recent advances by the authors's Institutes in Japan, Australia and the UK.

Intergeneric hybridization between *Lolium* and *Festuca*

The genus *Festuca* L. and its the closely allied genus *Lolium* L. have long fascinated agronomists, evolutionists, and plant breeders, and these genera are among the most widely studied of the non-cereal grasses. Intergeneric hybrids between closely related *Lolium* and *Festuca* species are being used to broaden the gene pool and provide plant breeders with options to combine high quality traits with broad adaptation to a range of environmental constraints (Humphreys et al. 2003, 2004). The species complex has an enormous wealth of genetic variability and in hybrids high potential for genetic exchange, thereby offering unique opportunities for the production of versatile hybrid varieties with new combinations of useful characters suited to modern grassland farming (Thomas et al. 2003). *Lolium* and *Festuca* species share valuable and complementary agronomic characters: for example *L. perenne*, offers good regrowth and nutritive value and is a good species for grazing whilst, *F. pratensis*, is more persistent and winter-hardy (Humphreys et al. 1998a). *Lolium* and *Festuca* species hybridize naturally, and as hybrids regularly exchange genes at high frequency. Some *Festulolium* cultivars have been developed as novel temperate forage grasses in both Europe

and USA (Table 1).

1) Amphidiploid breeding program

Intergeneric hybrids between diploid *L. multiflorum*, *L. perenne* and *F. pratensis* species have very low fertility. Doubling of the chromosome number of the F_1 hybrids leads to a restoration of fertility. Partially male and female fertile F_1 hybrids can be also obtained by crossing autotetraploid forms of *Lolium* sp. and *F. pratensis*. The first amphidiploid *Festulolium* cultivars, 'Elmet' (*L. multiflorum* x *F. pratensis*) and 'Prior' (*L. perenne* x *F. pratensis*) were bred at the Welsh Plant Breeding Station, UK (now Institute of Grassland and Environmental Research) in the early seventies (Thomas and Humphreys 1991). Some commercial cultivars were developed in Central and Eastern Europe (Zwierzkowski 2004, Table 1). In the USA, cultivar, 'Spring Green' has been developed from *F. pratensis* x *L. perenne* hybrids (Casler et al. 2001). An F_1 *Festulolium* hybrid between male sterile *L. multiflorum* and *F. arundinacea* revealed good performance for persistency and forage quality in the warmer region of Japan (Arakawa et al. 2004). An efficient seed production system is a prerequisite for commercialization of the F_1 *Festulolium* cultivar in use of male sterile system. Three *Festulolium* varieties have been evaluated in the northern Tohuku region of Japan (Yonemaru et al. 2004). Polish *Festulolium* cultivars showed high forage yield and quality comparable to *L. multiflorum* as well as better persistency, drought resistance, and winter hardiness compared to *L. multiflorum* (Zwierzkowski 2004).

2) Introgression breeding programs

A major problem for amphidiploid breeding is the high level of homoeologous pairing

between the different genomes, that leads to genetic instability and loss of hybridity in later generations. To overcome these problems and reduce transfer of deleterious *Festuca* traits, selective introgression of genes for desirable traits from *Festuca* into *Lolium* has become a favoured methodology. This process involves the transfer of small segments of alien *Festuca* chromatin into the recipient *Lolium* genome (Humphreys 1989; Humphreys and Pašakinskien_ 1996), and has been successfully employed to produce novel *Festulolium* lines (Humphreys and Thomas 1993; Humphreys et al. 2005). Intergeneric hybrids between *L. multiflorum* (2x) and *F. arundinacea* (6x) were used in the first putatively successful introgression breeding program conducted at the University of Kentucky, USA, in which two cultivars, 'Kenhy' (Buckner et al. 1977) and 'Johnstone' (Buckner et al. 1983) were developed, although in both cases, the inclusion of *Lolium* genes was never confirmed. 'Kenhy' showed improved palatability and a lower fiber content than tall fescue 'Kentucky 31'(Buckner et al. 1979). Introgression procedures for the transfer of genes for drought resistance from *F arundinacea* var. *glucescens* (2n=4x=28) into *L. multiflorum* (2n=2x=14) using DNA markers derived from *F. glucescens* were described by Humphreys et al. (2005), and has also been achieved recently from *F. arundinacea* (http://www.iger.bbsrc.ac.uk/SAGES2/sages2.html). An introgression-derived *Festulolium* cultivar, 'Felina' exhibited better tolerance to summer stress in the warmer region of Japan (Uchiyama et al. 2004).

3) Androgenesis

Androgenesis was found to be an effective procedure for selection of *Lolium-Festuca* genotypes comprising gene combinations rarely or never recovered by conventional backcross

breeding programs (Leśniewska et al. 2001; Humphreys et al. 2003) (Fig. 1). Androgenesis from *Festuca-Lolium* complex had been studied using different parental hybrids such as *F. pratensis* x *L. multiflorum* (Leśniewska et al. 2001; Rapacz et al. 2004) and *L. multiflorum* x *F. arundinacea* (Humphreys et al. 1997, 1998b; Pašakinskienė et al. 1997; Zwierzykowski et al. 1999; Zare et al. 1999). Following androgenesis from *F. pratensis* x *L. multiflorum* amphidiploid cultivars (2n=4x=28), over 80% of the resulting androgenic plants had 14 chromosomes and were likely to be dihaploids (n + n = 14) with a single genome of *Lolium* and *Festuca* (Leśniewska et al. 2001). Androgenic plants derived either from F_8 hybrids of *F. pratensis* x *L multiflorum* (2n=4x=28) or from pentaploid F_1 hybrids of *L.multiflorum* x *F. arundinacea* (2n=5x=35), contained rare gene combinations that contributed to drought and/or freezing tolerance in excess of the parental genotypes (Humphreys et al. 2000). Recent research has demonstrated that genotypes with high winter hardiness or snow mould resistance were recovered at equal frequency in androgenic populations derived from both resistant and susceptible parent genotypes and cultivars (Rapacz et al. 2005). Androgenesis can therefore enhance gene expression and allow the realization of the potential of a genotype. In androgenic populations derived from a *F. arundinacea* x *L. multiflorum* hybrid, Zwierzykowski et al. (1998) reported chromosome variation to be much wider than that transmitted by conventional backcrossing. An androgenic population derived from a pentaploid *L. multiflorum* x *F. arundinacea* (2x=5x=35) showed extreme diversity in freezing-tolerance (Zare et al. 1999). Another population derived from amphidiploid *F. pratensis* x *L. multiflorum* (2n=4x=28) cultivars showed variation for winter survival, freezing tolerance and resistance to cold-induced photoinactivation of photosystem II (PSII) (Rapacz et al. 2004). For turf grass breeding androgenesis has been applied to reduce the ploidy level

to diploid in intergeneric tetraploid hybrids of *F. pratensis* with *L. perenne* and *L. multiflurum* (Kopecky et al. 2005).

An introgression breeding program on intergenic hybridization between *L. perenne* and *F. pratensis* using androgenesis has been recently initiated at the National Agricultural Research Center for Hokkaido Region (NARCH). Successful androgenesis in *Festulolium* hybdids, *L. perenne* x *F. pratensis* was accomplished using PG-96 medium for embryo/callus induction, and a large numbers of dihaploids were produced (Guo et al. 2005). The PG-96 medium was composed of relatively complex organic acids and vitamin compounds (Guo et al. 1999). Modified PG-96 induction medium promoted regeneration from isolated microspores of timothy and rye (Guo and Pulli 2000a, b). Androgenic progeny showed a large variation in freezing tolerance, 7 % of 292 progeny exceeding that of freezing hardy *F. pratensis* despite containing chromosomes of *L. perenne*, a more freezing-sensitive species and more than 60% of flowering progeny produced dehiscent anthers with pollen stainability ranging from 5% to 85% (Guo et al. 2005). Dihaploids with both freezing tolerance and fertility potential have been backcrossed onto diploid *L. perenne*. Superior plants with high freezing tolerance have been found in the back-crossed progeny (Yamada et al. unpublished). These valuable breeding materials will be used in *Festulolium* introgression breeding programs to accelerate the breeding process and provide novel robust new forage grasses for cultivation in marginal areas such as the eastern part of Hokkaido region of Japan.

4) Somatic hybridization

Somatic hybridization is a technique based upon protoplast fusion, which enables hybridization between sexually incompatible species or genera. This technique may also

provide an alternative approach in order to create novel genetic combination between *Festuca* and *Lolium* species. A specific objective is the creation of novel cytoplasmic combinations, e.g., a mixture of both parental cytoplasms. Symmetric somatic hybrids (Takamizo et al. 1991) and asymmetric somatic hybrids (Spangenberg et al. 1994) have been produced between *F.arundinacea* and *L. multiflorum,* but no further research regarding their progeny with respect to agronomic traits were reported. Recently, Takamizo et al. (2004) reported the yield and forage quality of progeny derived from somatic hybrid plants between *F. arundinacea* and *L. multiflorum.* Intermediate-type progeny derived from somatic hybrids plants showed good forage quality, but lower yield in comparison to *Festuca*-type progeny.

5) GISH and DNA-based genetic marker technology for introgression programs

The chromosomes of *Lolium* and *Festuca* species can be discriminated using genomic *in situ* hybridization (GISH) (Humphreys et al. 1997, Mizukami et al. 1998). The GISH technique provides the means to identify segments of alien chromosomes introduced into the recipient species, and has proved to be a powerful tool for determining their chromosome location (Thomas et al. 1994, Humphreys et al. 1995; Humphreys and Pašakinskienė 1996; Pašakinskienė 2004). However, there are potential difficulties in the identification of very small introgressed chromosome segments using the GISH technique (Humphreys et al. 1998a). By a combined approach of GISH analysis to identify selected stress resistant plants that carry single *Festuca* chromatin segments and subsequently, the association of amplified fragment length polymorphisms (AFLP) or similarly suitable molecular markers with these alien segments, it is possible to 'tag' genes responsible for desirable agronomic traits in breeding programs (King et al. 1998, 2002; Armstead et al. 2001; Humphreys et al. 2005).

Molecular DNA markers based on Southern hybridization such as restriction fragment length polymorphisms (RFLP) as well as PCR-based markers such as randomly amplified polymorphic DNA (RAPD), AFLP and simple sequence repeats (SSR) have been developed for grass species. Yamada and Kishida (2003) applied rice cDNA RFLP probes from the activity of the Rice Genome Program (RGP) of Japan to forage grasses in order to investigate genetic variation within and between varieties of grasses, and to identify variety-specific RFLP markers for use in breeding programs exploiting intergeneric hybridization of *Lolium* and *Festuca*. RFLP analysis is a highly labour-intensive methodology compared to the PCR-based methods. Recently, SSR markers have been developed in perennial ryegrass (Jones et al. 2001; Kubik et al. 2001), Italian ryegrass (Hirata et al. 2000) and tall fescue (Saha et al. 2004). SSR markers provide the current marker system of choice due to their abundance, ubiquitous distribution in plant genomes, high level of reproducibility, ease of PCR-based analysis, and detection of co-dominant multiallelic loci. Momotaz et al. (2004) have analyzed the genetic polymorphism of multiple genotypes derived from taxa of the *Lolium*/*Festuca* complex using these distinct sets of SSR markers and applied these data to investigate introgression and genetic relatedness in *Festulolium* accessions. In addition, sequence tagged site (STS) markers were generated in *L. perenne* from sequence-characterized barley and oat sequences (Taylor et al. 2001). STS markers were also developed from genomic sequences of *Lolium* and related species of Poaceae (Lem and Lallemand 2003). A selection of genomic RFLP markers of Italian ryegrass have been converted into STS markers (Inoue and Cai 2004). STS markers may be useful to monitor gene introgression program for intergeneric hybridization between *Lolium* and *Festuca* (Humphreys et al. 2005). STS markers may also offer a reliable PCR-based system for

mapping orthologous loci across distantly related species and hence, to assist the alignment of genetic linkage maps from divergent species.

Genetic maps and QTL analysis

The majority of traits of interest to forage grass breeders, such as dry matter yield, forage quality and environmental stress tolerance, show continuous phenotypic variation and are controlled by a variable number of quantitative trait loci (QTL). Substantial advances have been made in the genetic improvement of plant populations through artificial selection of quantitative traits. Most of this selection has been on the basis of observable phenotype, without knowledge of the genetic architecture of the selected characteristics. In major crop species, the development of high-resolution genetic maps has made it possible to identify the chromosomal regions, or in some instances, the individual sequence variants that are responsible for trait variation. There have been relatively few reports to date of QTL analysis for agronomic traits in forage grasses, due to the absence of a sufficiently well developed genetic map.

1) Genetic maps

An enhanced molecular marker-based genetic linkage map of perennial ryegrass has recently been constructed through the activities of the International *Lolium* Genome Initiative (ILGI) (Forster et al. 2001), using the p150/112 one-way pseudo-testcross mapping population. The current map contains 109 RFLP loci detected by heterologous probes from wheat, barley, oat and rice. Comparative genetic mapping has allowed the alignment of the perennial ryegrass genetic map with those of wheat, rice and oat, revealing substantial conserved synteny with

the genomes of Poaceae species (Jones et al., 2002a). The syntenic chromosomal regions are represented as a concentric circle alignment (Fig. 2). At the macrosyntenic level, each of the 7 linkage groups of perennial ryegrass chiefly corresponds to one of the seven basic homeologous chromosome groups of the Triticeae cereals, and they have been numbered accordingly. Seven linkage groups of perennial ryegrass also correspond to 12 linkage groups of rice.

The p150/112 genetic map has been further enhanced by the assignment of nearly 100 polymorphic perennial ryegrass simple sequence repeat (LPSSR) loci (Jones et al. 2001) to locations on each of the seven linkage groups (Jones et al. 2002b). As a consequence, a total of more than 200 co-dominant genetic markers have been mapped in the p150/112 family, along with about 200 AFLP loci, permitting detailed genetic analysis of traits that vary within this population.

Two genetic mapping populations of perennial ryegrass have been independently developed as successors to the p150/112 population, and have been aligned to the reference map using common markers (Armstead et al. 2002, 2004; Faville et al. 2004). These genetic maps contain functionally-associated molecular marker information through the inclusion of gene-associated cleaved amplified polymorphic sequences (CAPS) markers, and both RFLP and SSR markers from expressed sequence tags (ESTs), respectively. In addition, high-density molecular marker-based genetic maps have also recently been constructed for other species of *Lolium* and *Festuca* (Alm et al. 2003; Inoue et al. 2004a; Warnke et al. 2004; Sim et al. 2005: Saha et al. 2005). Genetic maps are consequently available for detailed dissection of complex phenotypes to resolve the locations of pleiotropic and interacting genetic factors.

2) Flowering time

Heading and flowering time is an important character that impacts on yield, forage quality, and persistency. The genetic control of flowering time in *Arabidopsis* has been well characterized through the interaction of photoperiod, vernalization, autonomous and gibberellic acid-dependent pathways (e.g. Mouradov et al. 2002; Henderson et al. 2004). In rice, a short-day flowering plant, QTL analysis for heading date was undertaken using progeny derived from a single cross combination between *Oryza sativa* ssp. *japonica* and *O. sativa* ssp. *indica* cultivars and this enabled 14 QTLs controlling flowering time to be identified (Yano et al., 2001). Three heading QTLs (*Hd1, Hd3* and *Hd6*) were mapped at high resolution using near isogenic lines (NILs) and were isolated by a map-based cloning approach (Yano et al. 2000; Kojima et al. 2002; Takahashi et al. 1998). In cereals, genes that regulate the timing of flowering can be divided into three categories based on their interaction with environmental signals: vernalization response genes (*Vrn*) that regulate flowering using low temperature; photoperiod response genes (*Ppd*) that regulate flowering using day length; and 'earliness per se' factors that appear to be largely independent of these controls (Laurie 1997). Conserved genomic locations for genes involved in processes such as vernalization and photoperiodic induction have been identified between species by comparative genetic studies (Dubcovsky et al. 1998; Laurie et al. 2004). A detailed physical and genetic map of the *Vrn-A^{m}1* (*Vrn1*) region was constructed for the diploid wheat, *Triticum monococcum* L. and found to be colinear with the corresponding region of rice chromosome 3 (Yan et al. 2003). A candidate gene for *Vrn1* was isolated by positional means, and was identified as a relative of the *Arabidopsis apetala-1* gene class (*AP1*). Allelic variation

between spring and winter-type growth habit wheat at the *AP1* gene was observed only in the promoter region, suggesting that variation of gene expression was the causal factor for differences between these two varietal groups. A second vernalization gene, designated *Vrn-A^{m}2*, was assigned to the distal region of chromosome 5A^{m}L within a segment translocated from homoeologous group 4 (Dubcovsky et al. 1998). Yan et al. (2004) reported the positional cloning of the *Vrn2* gene, which encodes a dominant repressor of flowering that is down-regulated by vernalization. Loss of function at *Vrn2*, whether by natural point mutation or deletion, resulted in lines with spring-type growth habit, which do not require vernalization to flower.

A single QTL for heading date was observed on linkage group (LG) 4 in the p115/120 reference family of perennial ryegrass (Yamada et al. 2004). However, a number of QTL positions for heading date have been reported from the analysis of single mapping populations in other Poaceae species (Hayes et al. 1993; Laurie et al. 1995; Bezant et al. 1996; Börner et al. 2002). The number of QTLs and their relative importance may vary according to the origin of the genotypes used to construct mapping families. Studies on geographical populations of *Lolium* species covering the climatic range from the Mediterranean region to northern and central Europe revealed a regular cline in flowering responses to temperature and photoperiod (Cooper 1960). The heterozygous parent of the p150/112 mapping population was derived from a cross between eastern European (Romanian), southern European (north Italian ecotypes) and northern European ('Melle' or 'S23') genotypes, and might be expected to represent a variety of response genes. In rice, the major heading date QTL *Hd6*, which is associated with inhibition of flowering under long day conditions, is located on chromosome 3 (Yamamoto et al. 2000) and encodes an α-subunit of CK2

(Takahashi et al. 2001). Comparative genetic mapping studies between rice and wheat based on the colinearity of four common RFLP markers (Kato et al. 1999) have revealed that the rice *Hd6* locus region on chromosome 3 is syntenic with the *VrnA1* region on chromosome 5AL. RFLP-based mapping of the wheat CK2□ gene (*tck2a*) probe detected a genetic locus closely linked (by 1.1 cM) to *VrnA1* (Kato et al. 2002). Two putative CK2α genes (*Lpck2a-1* and *Lpck2a-2*) genes have been isolated from a cDNA library constructed with mRNA isolated from cold-acclimated crown tissues of *Lolium perenne* using sequence information derived from the *tck2a* gene. The *Lpck2a-1* CAPS marker was assigned to LG 4 of the p115/120 reference family near to the location of the QTL for heading date, while the *Lpck2a-2* CAPS marker was assigned to LG 2 (Shinozuka et al. submitted). The location of the *Lpck2a-1* locus supports the inference of conserved synteny between perennial ryegrass LG 4, the Triticeae homoeologous group 5L chromosomes and the corresponding segment of rice chromosome 3 (Yamada et al. 2004).

A mapping population consisting of 184 F_2 genotypes from a cross between a genotype from the perennial ryegrass synthetic variety 'Veyo' and a genotype from the perennial ryegrass ecotype 'Falster' was measured for vernalization response as days to heading under artificially controlled condition (Jensen et al. 2005). In total, five QTLs were identified on LGs 2, 4, 6 and 7. A CAPS marker derived from the putative orthologue of the *Triticum monococcum VRN1* gene co-located with a major QTL on LG 4 for vernalization response (Jensen et al. 2005). This data further confirms the presence of flowering time gene orthologues and corresponding QTLs on LG 4.

Heading date QTLs were also identified in one of the second generation reference populations, derived from self-pollination of an F_1 hybrid obtained by crossing individuals

from partially inbred lines developed from the two agronomically contrasting cultivars 'Aurora' and 'Perma' (Turner et al. 2001). Genetic mapping of the F_2 (Aurora x Perma) population identified seven linkage groups with a total map length of 628 cM (Armstead et al., 2004), extending the studies of Armstead et al. (2002) and consistent with the ILGI reference map (Jones et al. 2002a). A major QTL accounting for up to 70% of the variance was identified on LG 7, along with additional small QTLs on LG 2 and 4 (Armstead et al. 2004). The genomic region associated with the major QTL on LG 7 shows a high degree of conserved synteny with the *Hd3* region of rice chromosome 6.

Two annual ryegrass plants from the cultivar 'Floregon' were crossed with two perennial ryegrass plants from the cultivar 'Manhattan'. From the resultant F_1 populations, two random plants were chosen and crossed to develop a pseudo-F_2 mapping family (ψF_2[MFA-4 x MFB-2]). A total of 235 AFLP markers, 81 RAPD markers, 16 grass comparative anchor probe RFLPs, 106 SSR markers, 2 isoenzyme loci and 2 morphological characteristics, 8-h flowering and seedling root fluorescence were used to construct a male map 537cM in length and a female map 712 cM in length, each with 7 LGs (Warnke et al. 2004). The genetic linkage map of the ψF_2 (MFA-4 x MFB-2) population was enhanced and extended existing comparative relationships of ryegrass with other Poaceae species via heterologous RFLP markers (Sim et al. 2005). Two major QTLs influencing photoperiodic control of flowering were identified in locations syntenic with flowering control regions of the wheat and barley genomes (Warnke et al. 2003a,b). In Italian ryegrass, a two way pseudo-testcross F_1 population derived from a pair cross between single individuals selected from cultivars 'Nioudachi' and 'Nigatawase' was used for linkage map construction (Inoue et al. 2004a) and some QTLs for heading date were identified (Inoue et al. 2004b). QTLs

identified on LGs 4 and 7 may be in conserved regions with those identified in perennial ryegrass.

3) Morphological characters

The morphogenesis of individual grass plants within a grazed sward plays a key role in determining herbage yield, persistence and recovery from grazing. In vegetative plants, plant morphogenesis is described by three key variables: leaf appearance rate, leaf elongation rate and leaf lifespan. The expression of each of these traits is under both genetic and environmental control (Lemaire and Chapman 1996), and leaf development in *Lolium* has been demonstrated to be under genetic control in a number of studies (Hazard et al. 1996). Structural characteristics of plants such as tiller number, leaf number and leaf size are the result of these morphogenetic traits, and their measurement in breeding programs allows a dissection of the complex herbage yield trait as well as predictions of the response to grazing.

QTLs were detected for morphological traits such as plant height, tiller size, leaf length, leaf width, fresh weight at harvest, plant type, spikelet number per spike and spike length using the p150/112 genetic map (Yamada et al. 2004). A number of traits were significantly correlated, and coincident QTL locations identified. For example, coincident QTL for plant height, tiller size and leaf length were identified on LG 3. The rice *SD1* semi-dwarfing gene, that launched the 'green revolution', encodes a gibberellin biosynthetic enzyme (*GA20ox*), and was assigned to the long arm of rice chromosome 1 (Sasaki et al. 2002). A CAPS marker developed for the perennial ryegrass ortholocus of the *GA20ox* gene was mapped to LG 3 close to the plant height QTL, in a region of conserved synteny with rice chromosome 1 (Kobayashi et al. unpublished). This finding provides further evidence for

the utility of the candidate gene-based marker approach.

In Italian ryegrass, a total of 17 QTLs for six traits related to lodging resistance and heading date were detected by single interval mapping (SIM), while 33 independent QTLs from the male and female parents were detected by composite interval mapping (CIM) (Inoue et al. 2004b). The QTL for plant height on LG 1 is potentially located in conserved regions with that identified in perennial ryegrass.

4) Winter hardiness

Winter hardiness is the outcome of a number of interacting factors that may include vernalization requirement, photoperiod response, low-temperature tolerance and resistance to snow moulds. An understanding of the genetic basis of these component traits permits more efficient selection based on closely linked molecular marker loci. In the Triticeae cereals, QTL analysis has identified a limited number of conserved genome regions as responsible for the winter hardiness character. The most consistently identified region, on homoeologous group 5 chromosomes, contain QTLs for vernalization response, low temperature tolerance and photoperiod sensitivity (Sutka and Snape 1989; Pan et al. 1994; Galiba et al. 1995: Sutka et al. 1999; Cattivelli et al. 2002). Close genetic linkage between the major genes influencing winter hardiness and genes regulating cold-induced sugar production was observed in wheat (Galiba et al. 1997). Recently, comprehensive measurements of low temperature tolerance and vernalization requirement were used for analysis of a new 'winter' x 'spring' barley population, and a QTL for accumulation of proteins encoded by COR (COld Regulated) genes on chromosome 5H (*Cor14b, tmc-ap3*) was coincident with a QTL for low temperature tolerance (Francia et al. 2004). C-repeat

binding factor (CBF) family genes were also mapped in this region (Francia et al. 2004). In wheat, *CBF3* was also linked to the frost-tolerance locus *Fr-A2* on chromosome 5A (Vágújfalvi et al. 2003). In *Arabidopsis thaliana*, the transcription factors encoded by CBF family genes have been shown to be key determinants of low temperature tolerance (Thomashow et al. 2001).

No significant QTLs for winter survival in the field were identified in the reference map of p150/112 perennial ryegrass (Yamada et al. 2004). However, a QTL for electrical conductivity corresponding to frost tolerance (Dexter et al. 1932) was located close to a heading date QTL in a region is likely to show conserved synteny with chromosomal regions associated with both winter hardiness and flowering time variation in cereals, as described above.

The F_2 (Aurora x Perma) genetic map population was also used to identify QTLs for traits relating to winter hardiness, as well as sugar content. Snow mould-resistant varieties accumulate higher levels of fructan and metabolise them at slower rates compared to susceptible varieties (Yoshida et al. 1998). Many of the snow mould fungi, such as *Typhula* spp., *Microdochium nivale* and *Myriosclerotinia borealis*, can co-infect on single plants, and their interactions may obscure the respective effects on plant survival (Matsumoto and Araki 1982; Matsumoto et al. 1982). The use of fungicides with a limited spectrum of activity may clarify these specific effects. *Typhula* snow moulds such as *T. ishikariensis* and *T. incarnata* generally occur in the deep snow environment of the western region of Hokkaido, Japan, including Sapporo. In this environment, control of *S. borealis* and *M. nivale* infections with the fungicide iminoctadine-triacetate is an effective method for evaluation of resistance to *Typhula* snow moulds (Takai et al. 2004). Scores of winter survival were measured in the F_2

(Aurora x Perma) population using this control regime, and QTLs for this trait were identified on LGs 2, 4, 6 and 7. Fructan content was also measured by high performance liquid chromatography (HPLC) using crown tissues from plants grown outdoor in December. QTLs for content of high molecular fructan with more than eight degrees of polymerization (DP) were observed on LGs 1, 2 and 4. QTLs for winter survival in LGs 2 and 4 are close to QTLs for high molecular weight fructan content.

Two major QTLs for freezing tolerance (*Frf*) and 4 QTLs for winter survival have been identified in the closely related pasture grass species meadow fescue (*Festuca pratensis* Huds.) (Rognli et al. 2002). Comparative mapping with heterologous wheat anchor probes indicated that *Frf4_1* on LG 4 of *F. pratensis* was orthologous to the frost-tolerance loci *Fr1* and *Fr2* in wheat. The QTLs for winter survival, by contrast, were located on LGs 1, 2, 5, and 6 (Rognli et al. 2002).

The gene for a putative glycine-rich RNA binding protein, *LpGRP1*, was isolated from a cDNA library constructed from crown tissues of cold-treated perennial ryegrass plants (Hisano et al. submitted). An RFLP locus detected by the *LpGRP1* cDNA probe was mapped to a distal location on LG 2 in the p150/112 population. A full length sucrose synthase cDNA (*LpSS*) from perennial ryegrass was isolated and CAPS marker for this was mapped on LG 7 in the F_2 (Aurora x Perma) population (Bhowmik et al. in preparation).

Fructosyltransferase genes involved in fructan biosynthesis such as 1-SST, 1-FFT and 6G-FFT were isolated from perennial ryegrass and characterized by heterologous expression in the *Pichia pastoris* system (Hisano et al. in preparation). *Lp1-SST* and *Lp1-FFT* mapped to the upper region of LG 7 in the F_2 (Aurora x Perma) genetic map, but failed to show coincidence with any fructan content QTLs. The *Lp1-SST* gene (Chalmers et al. 2003) was

also assigned to the equivalent region of LG 7 as a single nucleotide polymorphism (SNP) locus in the F_1 (NA_6 x AU_6) second-generation reference family (Faville et al. 2004). However, *Lp6G-FFT* mapped to LG 3 close to a QTL for low-molecular weight fructan content (Hisano et al. in preparation). As previously described, evidence from comparative genome studies suggests that the terminal part of LG4 in perennial ryegrass may contain a region of conserved synteny with the long arms of the Triticeae homoeologous group 5 chromosome. It is possible that allelic variation in regulatory genes such as those for the CBF transcription factor family may contribute to the QTLs for winter survival and fructan content observed on LG 4. We are currently isolating CBF genes from perennial ryegrass with the intention pf performing mapping studies.

5) Forage quality

Quality is the most important of all agronomic traits for forage due to the nutritive requirements of grass-fed livestock. The genetic control of nutritive value parameters in grass species has been reviewed (e.g. Casler 2001), and genetic variation for specific traits has been established. Digestibility is generally considered to be the most important temperate grass nutritive value trait for either live-weight gain (Wheeler and Corbett 1989) or dairy production (Smith et al. 1997). Deliberate attempts to improve dry matter digestibility (DMD) in forage crop species have led to rates of genetic gain in the range of 1 - 4.7 % per annum as a proportion of the initial population means (Casler 2001). Progress in simultaneous improvement of yield and DMD in forage grasses has, however, been variable (Wilkins and Humphreys 2003).

Forage quality may be directly evaluated by feeding trials using animals, but this

approach is costly, laborious and limited for small quantities of herbage from breeding experiments. Indirect methods of assessment include *in vitro* digestibility with rumen liquor (Menke et al. 1979; Tilly and Terry 1963), enzymatic digestion (De Boever et al. 1986) and chemical analysis of cellular components (van Soest 1963). The development of near infra-red reflectance spectroscopy (NIRS) analysis for prediction of forage quality has facilitated rapid and non-destructive evaluation of samples from plant breeding programs. NIRS has been used to develop calibrations to predict a wide range of forage quality traits (Marten et al. 1984; Smith and Flinn 1991) including crude protein (CP) content, estimated *in vivo* dry matter digestibility (IVVDMD), neutral detergent fibre (NDF) content (Smith and Flinn 1991), and water-soluble carbohydrate (WSC) content (Smith and Kearney 2000) in perennial ryegrass. NIRS estimates of DMD and related nutritive value traits have been reported in a range of forage systems (e.g. Carpenter and Casler 1990; Hopkins et al. 1995; Smith et al. 2004).

Lübberstedt et al. (1997, 1998) published the first QTL analysis devoted to forage quality in maize. QTL for cell-wall digestibility and lignification traits in maize were also investigated in two recombinant inbred lines (RILs) progeny by Méchin et al. (2001). Cardinal et al. (2003) detected 65 QTLs related to fiber and lignin content in maize. The best options for breeding of grasses for improved digestibility was assessed based on a search for genome locations involved in forage quality traits through QTL analysis (Ralph et al. 2004).

Ground herbage samples from genotypes of the p150/112 population were measured for quality traits such as CP, IVVDMD, NDF, estimated metabolisable energy (EstME) and WSC by NIRS analysis (Cogan et al. 2005). A total of 42 QTLs were observed in six different

sampling experiments varying by developmental stage (anthesis or vegetative growth), location or year. Coincident QTLs were detected on LGs 3, 5 and 7. The region on LG 3 was associated with variation for all measured traits across various experimental datasets. The region on LG 7 was associated with variation for all traits except CP, and is located in the vicinity of the lignin biosynthesis gene loci *xlpomt1* (caffeic acid-*O*-methyltransferase), *xlpccr1* (cinnamoyl CoA-reductase) and *xlpssrcad2.1* (cinnamyl alcohol dehydrogenase).

WSC provides the most readily available source of energy for grazing ruminants. In the F_2 (Aurora x Perma) population, high molecular fructan which constitutes the major part of the WSC pool, was analyzed in samples collected during spring and autumn in tiller bases and leaves with replication of data over years (*ie* collection one replicate each year for several years) (Turner et al. unpublished). Correlations between traits did not always lead to corresponding cluster of QTL and some traits revealed no reproducible QTLs. Tiller base QTLs were identified on LGs 1 and 5, and leaf QTLs on LGs 2 and 6 (Humphreys et al. 2003).

Improvements of forage quality may also be obtained by alteration of the content and ratios of minerals in grasses, to prevent metabolic disorders. Grass tetany (hypomagnesaemia) is caused by low levels of magnesium in the blood of cattle or sheep. Varieties of Italian ryegrass and tall fescue with markedly different levels of magnesium have proved to be very effective in maintaining levels of blood magnesium in grazing sheep (Moseley and Baker 1991) and cattle (Crawford et al. 1998). Milk fever, caused by low blood calcium, produces animal welfare and production problems that could be addressed by reducing potassium content of forage without reducing calcium and magnesium concentrations (Sanchez et al. 1994). Variation in mineral content in grasses may be strongly influenced by genetic factors

and is amenable to QTL analysis. Herbage samples of the p150/112 population from four sampling experiment were analyzed for mineral content (aluminum, calcium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, sodium, sulfur and zinc) by inductively-coupled plasma mass spectroscopy (ICP-MS) and a total of 45 QTLs were identified (Cogan et al. submitted). QTL clusters were observed on LGs 1, 2, 4 and 5. QTLs for the important traits for control of grass tetany, and magnesium content were detected on LGs 2 and 5. Field herbage samples from the F_1 (NA_6 x AU_6) population were also analyzed for mineral content by ICP-MS. A total of 14 QTLs were identified on the NA_6 map, and 9 QTLs were identified on the AU_6 genetic map. A number of clustered QTL locations showed coincidence between the two different populations.

6) Disease resistance

Crown rust (*Puccinia coronata* f. sp. *lolii*) is the most serious foliar disease of ryegrasses and causes severe losses in forage yield and quality. Dumsday et al. (2003) mapped a major QTL for crown rust resistance on LG 2 in perennial ryegrass in region of conserved synteny with the Pca crown rust resistance region from the diploid oat species *Avena strigosa*. In addition, two closely linked QTLs for crown resistance were identified on LG 1 from analysis of the F_1 (NA_6 x AU_6) population (Forster et al. 2004). In Italian ryegrass, three crown rust resistance genes loci, *Pc1, Pc2* and *Pc3* were identified at the molecular level (Fujimori et al. 2004; Fujimori 2004). Three AFLP markers were tightly linked to *Pc1* and these DNA markers will be useful for the selection and development of resistant varieties (Fujimori et al. 2004). Roderick et al. (2003) reported the transfer of genes from crown rust resistance from *F. pratensis* to chromosome 5 of *L. multiflorum*.

7) Future prospects

Although the existing QTL information in forage grasses is relatively underdeveloped compared to other major crops such as rice and barley, the recent establishment of detailed molecular genetic maps is rapidly stimulating QTL analysis and trait dissection. The current genetic maps are largely populated by anonymous genetic markers, with limited diagnostic value. The next generation of molecular genetic markers for forage grasses will be derived from expressed sequences, with an emphasis on functionally-defined genes associated with biochemical and physiological processes that are likely to be correlated with target phenotypic traits (Andersen and Lübberstedt 2003; Forster et al. 2004; Faville et al. 2004). The large-scale gene sequence collections generated by ESTs provides the resource for functionally-associated marker development. Single nucleotide polymorphism (SNP) markers can in principle be developed for any gene, and show the benefits of locus-specificity, high data fidelity and high-throughput analysis. Comparative genomics with other Poaceae species such as rice, wheat, barley and maize will support the development of such high value molecular markers through orthologous QTL detection and co-location of candidate genes. Alternatively linkage disequlibrium (LD) based method may allow the detection of accuracy QTLs for traits of interest. Genome-wide association studies will soon become possible and could open new frontiers in our understanding of complex traits (Hirschhorn and Daly 2005; Wang et al. 2005). Based on the population biology of perennial ryegrass (outbreeding with relatively large effective population sizes, at least for ecotypic populations), LD is expected to extend over relatively short molecular distances (Forster et al. 2004). In this instance, it should be possible to identify diagnostic variants for the selection of individual parental

genotypes on the basis of superior allele content. This will allow more efficient use of germplasm collections for parental selection. In addition, such 'perfect' markers will allow highly effective progeny selection. The Primary Industries Research Victoria (PIRV), Australia and the Institute of Grassland and Environmental Research (IGER), UK have commenced the preliminary association analysis in *L. perenne*. The progress at IGER in association mapping flowering time genes in *Lolium perenne* has been reported recently (Skøt et al. 2005). Accuracy in phenotypic assessment will be essential for precise detection of significant associations. The stability of QTLs and other genetic effects across different environmental conditions is also an important target of study. International collaborations of NARCH with IGER and PIRV are being conducted to achieve this objective.

Functional Genomics

Recently, two complete plant genome sequences, one from a dicot, *Arabidopsis thaliana* and one from a monocot, rice (*Oryza sativa*) have been completed. This progress has opened a floodgate of relevant information for molecular genetics and breeding in flowering plants.

An EST is generated by single-pass sequencing of cDNA and provides a starting point for elucidation on gene function. ESTs have been developed for many organisms (http://www.ncbi.nih.gov/dbEST/index.html). Sawbridge et al. (2003) reported the generation of more than 44,000 ESTs from 29 cDNA libraries of *L. perenne* representing a range of plant organs and developmental stages. The ESTs were analyzed by BLAST searches, categorized functionally, and were subjected to a cluster analysis leading to the identification of a unigene set corresponding to 14,767 genes. A total of 1,500 ESTs have been generated from a cDNA library constructed from crown tissues of cold-treated *L.*

perenne plants (Yamada et al. unpublished). In *L. multiflorum* 5,922 ESTs have been generated from seven cDNA libraries from various tissues and leaves under biotic and abiotic stresses (Ikeda et al. 2004). In *F. arundinacea*, a heat stressed shoot cDNA library was constructed and an EST collection is underway (Zhang et al. 2004). The first forage grass gene chip with 15K ryegrass unigenes was constructed recently by Primary Industries Research Victoria, Australia, and AgResearch, New Zealand (Spangenberg et al. 2004). This cDNA-based microarray will be highly for novel gene expression profiling and promoter discovery in ryegrasses and fescues. An international consortium program on transcriptome analysis in *Lolium* and *Festuca* species (ITIFT) has been proposed (Spangenberg pers. comm.).

Resistant (R) gene-mediated disease resistance in plants has been studied intensively (Hulbert et al. 2001). Nearly all of the known R gene belong to one of six major classes (Jones 2001). Although these genes confer resistance to a range of pathogens that are taxonomically unrelated, most of them encode members of the nucleotide-binding (NB)-leucine-rich repeat (LRR) class (Jones and Jones 1997). Initial step towards the cloning R gene analogues (RGAs) from Italian ryegrass have been undertaken (Ikeda 2005). In this study, clones amplified from NBS-LRR-type RGAs by using primers designed from sequence motifs conserved among R genes were sequenced and primer sets of unique NBS-LRR sequences developed. This study may be useful for development of the R gene-mediated disease resistant markers in forage grass species.

Genetic transformation

Conventional forage grass breeding has been based on the use of natural genetic variation as

found between and within ecotypes or created through sexual recombination. Gene technology and the production of transgenic plants offer the opportunity to generate unique genetic variation. Application of transgenesis to forage plant improvement has been focused on the development of transformation events with unique genetic variation and in studies on the molecular dissection of plant biosynthetic pathways and developmental processes of high relevance for forage production (Spangenberg et al. 1998; Spangenberg et al. 2001). The biolistic methodology, based on particle bombardment, has been the most successful means of transforming grasses. A protocol for rapid production of large numbers of independently transformed fertile perennial ryegrass plants from commercially important forage and turf-type cultivars has been developed using the biolistic procedures (Altpeter et al. 2000). Takahashi et al. (2002) have also developed a transformation technique in Italian ryegrass cv. 'Waseaoba' using microprojectile bombardment of embryogenic calli. A successful *Agrobacterium*-mediated transformation method has been reported for tall fescue and Italian ryegrass (Bettany et al. 2003) and more efficient *Agrobacterium*-mediated transformation has been reported for tall fescue (Lee et al. 2004; Wang and Ge 2005).

Fructans, a polymer of fructose and a major component of nonstructural carbohydrates is accumulated in temperate grasses. The increased level of soluble carbohydrates appears to improve the nutritional value of grasses, particularly during summer when grasses suffer a major decline in digestibility. The soluble carbohydrate composition of Italian ryegrass has been altered by transformation with the *Bacillus subtilis sac B* gene (Ye et al. 2001). Lignification of plant well walls has been identified as the major factor responsible for lowering digestibility of forage tissues. Molecular breeding for improved digestibility by down-regulating monolignol biosynthetic enzymes through transgenesis has been explored.

Forage digestibility of tall fescue has been improved by transgenic down-regulation of cinnamyl alcohol dehydrogenase (Chen et al. 2003) and caffeic acid *O*-methyltransferase (Chen et al. 2004). Sulphur-containing (S-) amino acids, methionine and cysteine, are among the most limiting essential amino acids in ruminant animal nutrition. A sulphur-rich albumin gene from sunflower has been expressed in leaves of transgenic tall fescue (Wang et al. 2001).

Transgenic approaches have been explored to enhance resistance to diseases. A rice chitinase (*Cht-2; RCC2*) gene has been introduced into Italian ryegrass and bioassay of leaves in transgenic plants indicated increased resistance to crown rust (Takahashi et al. in press). The coat protein-mediated transgenic resistance approach has also been applied. Resistance of perennial ryegrass to ryegrass mosaic virus (RMV) has been increased by transformation with an RMV coat protein gene (Xu et al. 2001).

Hayfever and seasonal allergic asthma due to grass pollen are environmental diseases that afflict many peoples around the world. Manipulation of pollen allergens in grasses is one of the important solutions for this disease. Transgenic plants with reduced levels of the main ryegrass pollen allergens (Lolp1 and Lolp2) have been generated for *L. perenne* and *L. multiflorum* (Petrovska et al. 2004).

Fructan accumulation is also associated with winter hardiness. Transgenic perennial ryegrass plants that over-expressed the wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose-fructan 6-fructosyltransferase (6-SFT) and sucrose-sucrose 1-fructosyltransferase (1-SST), respectively, under the control of CaMV 35S promoter have been produced using a biolistic transformation (Hisano et al. 2004). Transgenic plants that accumulated a greater amount of fructan than non-transgenic plants showed increased

tolerance to cellular freezing. The results suggest that the over-expression of the genes involved in fructan synthesis serves as a novel strategy to produce freezing-tolerant grasses (Hisano et al. 2004). Transgenesis is powerful tool for the elucidation of genetic mechanism in metabolic pathways. Further analysis of the perennial ryegrass fructosyltransferase genes will involve detailed molecular genetic studies based on gene silencing (Vance and Vaucheret 2001) to determine the effects of these genes on fructan biosynthesis.

Forage grasses are wind-pollinated and outcrossing species. Significant spread of the transgenes to other species or wild populations of the same species would be inevitable without strict control of pollination. Wang et al. (2004) reported pollen-mediated transgene flow using T_1 and T_2 progenies derived of transgenic tall fescue plants (Wang et al. 2003 a,b). No transgene was detected at 200 m distance in any direction. This experiment indicated that the isolation distance 300 m is enough to prevent transgene flow to neighboring plants, at least small-scale field trials.

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* In Japanese with English summary

Legends of figures

Fig. 1. Introgression program using androgenesis in the combination of *Lolium perenne* and *Festuca pratensis*

Fig. 2. Concentric circle alignment of Poaceae genomes (from Jones et al. 2002a)

Table 1. *Festulolium* cultivars developed in Europa and USA.

Hybrid combination	cultivar name	Type	Country	year
L. multiflorum x *F. pratensis*	Elmet	amphidiploid	United Kingdom	1973
	Perun	amphidiploid	Czech Republic	1991
	Rakopan	amphidiploid	Poland	2001
F. pratensis x *L. multiflorum*	Paulita	amphidiploid	Germany	1986
	Paulena	amphidiploid	Germany	1995
	Punia	amphidiploid	Lithuania	1997
	Felopa	amphidiploid	Poland	1998
	Sulino	amphidiploid	Poland	1998
	Agula	amphidiploid	Poland	2002
L. perenne x *F. pratensis*	Prior	amphidiploid	United Kingdom	1973
	Spring Green	amphidiploid	USA	2001
L. multiflorum x *F. arundinacea*	Kenhy	introgression	USA	1977
	Johnstone	introgression	USA	1983
	Felina	introgression	Czech Republic	1988
	Hykor	introgression	Czech Republic	1991
	Korina	introgression	Czech Republic	1997
	Be_va	introgression	Czech Republic	1989

From Z. Zwierzykowski (2004).

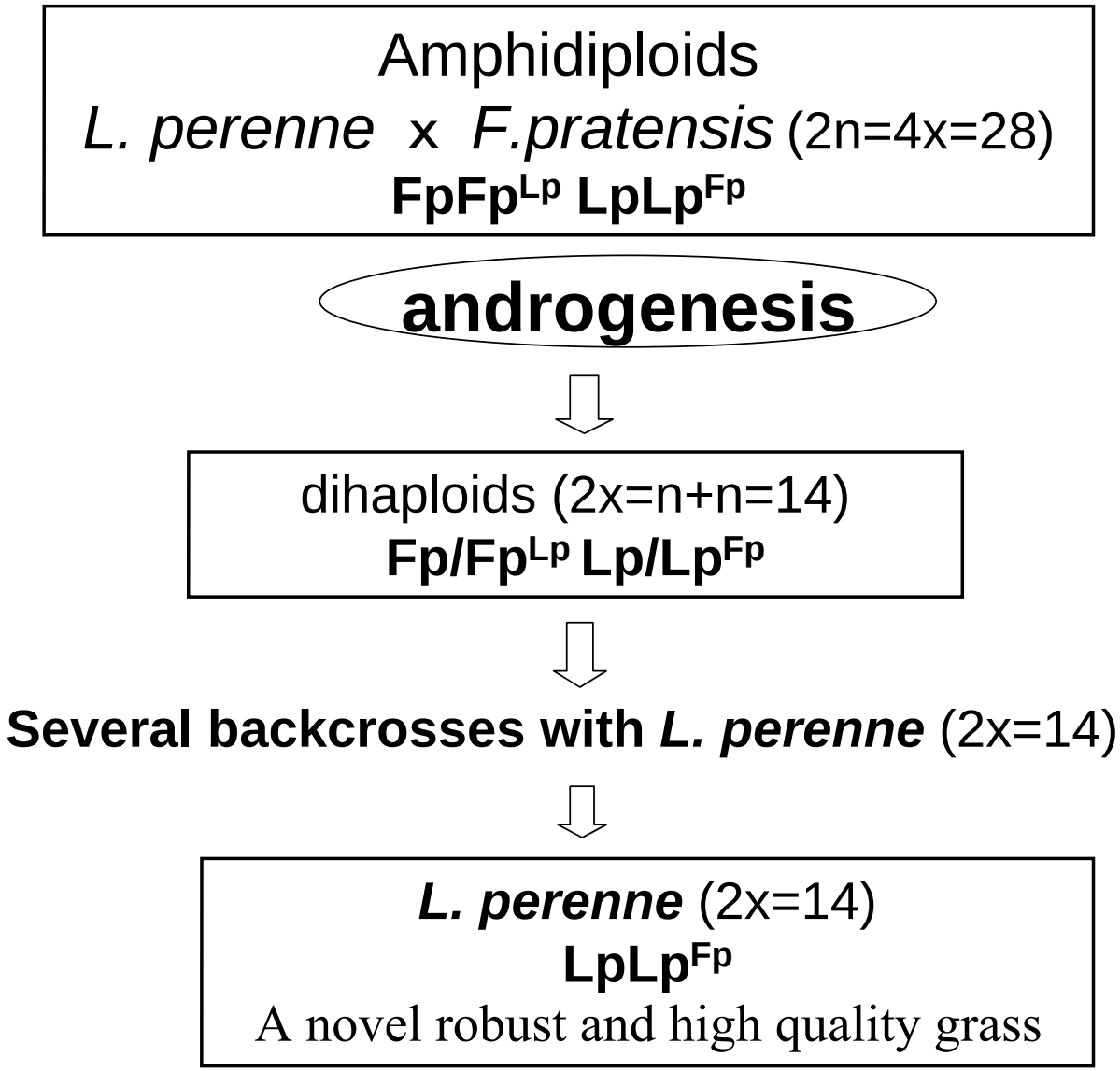
Amphidiploids
L. perenne x F.pratensis (2n=4x=28)
FpFpLp LpLpFp
androgenesis
dihaploids (2x=n+n=14)
Fp/FpLp Lp/LpFp
Several backcrosses with L. perenne (2x=14)
L. perenne (2x=14)
LpLpFp
A novel robust and high quality grass

Fig 1.

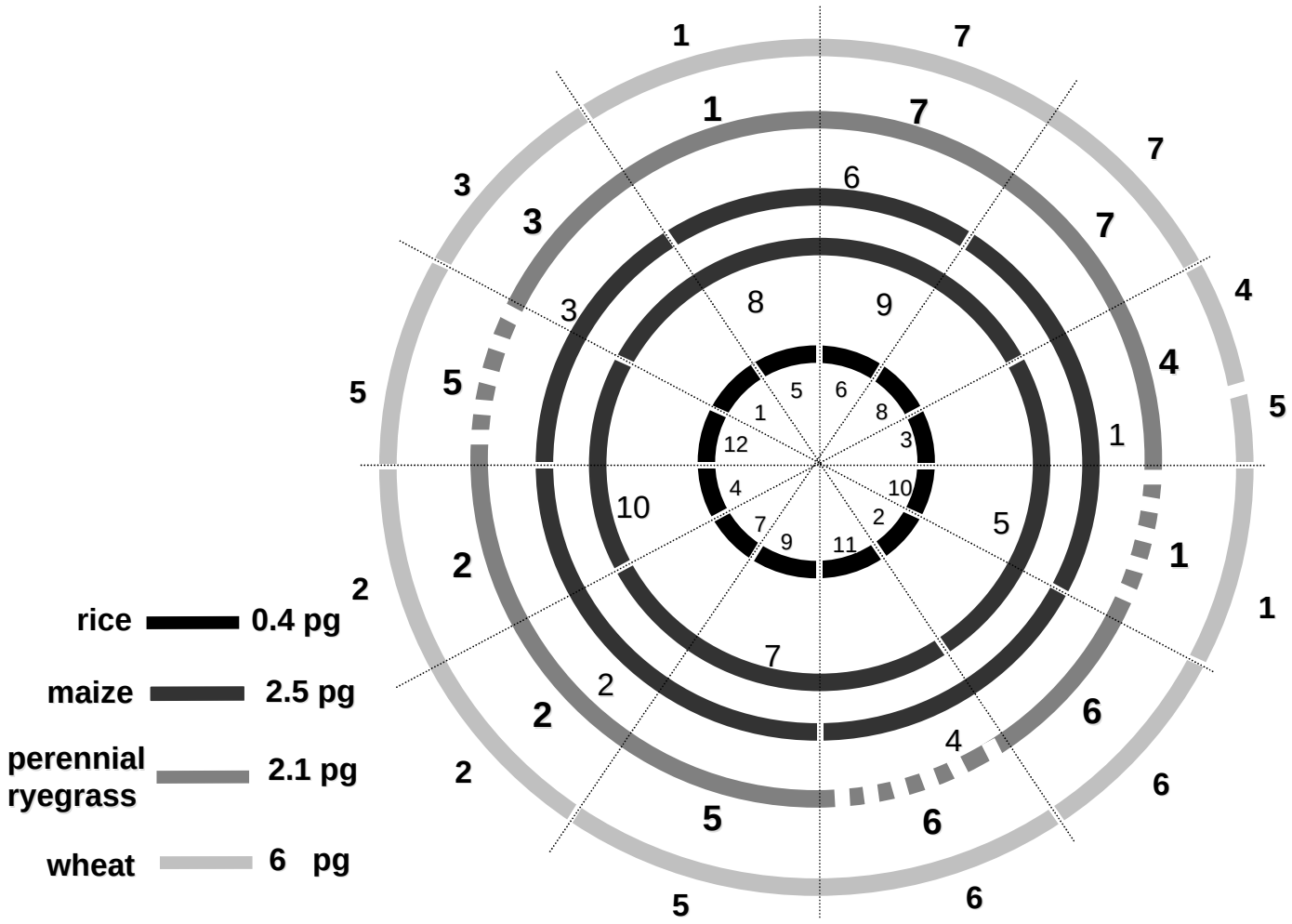

Fig.2