Objectives and Benefits of Molecular Breeding in Forage Species

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Key points

1. The amount of resources and information provided by forage crop genomic programs has dramatically increased during the past few years.
2. Trait-based forward genetic procedures such as mapping and expression profiling have successfully provided new candidate genes or genome regions affecting forage quality. Respective information can easily be transferred across related forage species.
3. Since several genes in major biochemical pathways related to forage traits have been isolated, gene-based reverse genetic approaches (transformation, association studies) are promising.
4. Most genetic experiments are conducted under simplified "artificial" conditions such as on single-spaced plants. Therefore, transferability of respective genetic information to breeding practice needs to be demonstrated.

Keywords: forage genomics, forward genetics, reverse genetics, digestibility

Introduction

The major goals in forage production are (i) to maximize dry matter yield, and (ii) to achieve a high level of forage quality. Since both goals might be negatively correlated, the ultimate goal is to produce a maximum yield of metabolizable energy. Several factors need to be considered when determining forage quality, such as the fed animal species, the forage plant species and cropping system, and the method(s) used for forage quality evaluation that determine the feed - animal interaction. In addition, the optimum diet of animals depends on the product, such as beef or milk for cattle. Ruminants have a much better capability to digest fibrous carbohydrates compared to monogastrics and to convert poor quality protein and nonprotein nitrogen sources (Van Soest, 1974). Furthermore, intake and digestion by animals depends on forage properties such as its dry matter concentration, particle size, and the ensiling process.

In most forage grasses and legumes, the above-ground parts are harvested before or during flowering. An exception is forage maize, which is harvested after seed-set. Dry matter yield can be easily determined, whereas quality evaluation is rather difficult. The direct approach to evaluate the quality of a given forage crop is the conduct of animal feeding trials to maximize the yield of the intended product such as milk or beef. Different parameters were developed for these traits such as Mega Joule “Net-Energy-Lactation“ ((MJ) NEL) (Groß, 1979, Weißbach, 1993) for milk production and “Kilo Starch-Units“ (kStE) (Zimmer *et al*., 1980) for beef production and “Metabolizable Energy“ (ME) (Menke and Huss, 1987), all reflecting the energy density (J/kg) of forage dry matter (Boberfeld, 1986). Nevertheless, this direct approach of quality evaluation has a number of limitations. Animal trials are rather time-consuming, laborious, and consequently expensive. Hence, it is not possible to handle thousands of plant genotypes, as required in breeding programs. Furthermore, feeding trials depend on a number of additional factors, such as the animal species and genotypes employed, and the mode of feeding forage genotypes, impairing the generalization of the results. In consequence, a number of indirect biological, chemical, and physical methods for

quality evaluation have been developed. Furthermore, prediction of the breeding value of forage plant genotypes based on molecular markers would be highly desirable.

Biological methods for quality evaluation can be subdivided into field, *in vitro*, and enzymatic methods. Field methods score the expected nutritive value of plant communities (Boberfeld, 1986) based on the species composition. In case of forage maize, the proportion of ears in total dry matter has been used for quality evaluation (Zscheischler, 1990). A widely used *in vitro* rumen digestion analysis was developed by Tilley and Terry (1963) using a two-step procedure - first rumen liquor and subsequently peptic hydrochloric acid to estimate the *in vitro* digestibility of organic matter. Another *in vitro* test employing rumen liquor determines gas production, protein and fat content to estimate NEL or StE (Menke and Steingass, 1987). Enzymatic methods use cellulase together with peptic hydrochloric acid to estimate NEL and StE (Kirchgessner and Kellner, 1981).

Since digestibility is mainly limited by poorly digestible cell wall components, chemical methods for forage quality evaluation focus on the breakdown and characterization of cell wall fractions within the organic matter. Using detergents Van Soest (1974) separated cell complexes into soluble cell content and insoluble "neutral detergent fibre" (NDF) representing mainly the cell wall fraction. By acidic detergents further fractionation into a lignin ("acid detergent lignin": ADL) and cellulose fraction (ADF-ADL; ADF: "acid detergent fibre") is possible. ADF values can be converted into NEL and StE estimates by convenient equations (Kirchgessner and Kellner, 1981).

All above mentioned approaches are too laborious for routine quality evaluation of large numbers as required in plant breeding. Near-infrared reflectance spectroscopy (NIRS) (Norris *et al*., 1976) helps to overcome this limitation. By this method, large sample numbers can be investigated with low effort. Infrared spectra of ground materials (1400 to 2600 nm) can be employed to estimate a number of quality parameters, if suitable calibrations exist based on animal trials, biological or chemical methods. However, with the availability of an increasing number of both tools and knowledge at the genome and gene level, the prospects for efficient breeding strategies based on genotypic rather than on phenotypic information have rapidly changed during the past few years.

The most important forage species belong to two families, the monocot grasses (Gramineae) and the dicot legumes (Leguminosae). Both families include more than 500 genera with annual, biennial, and perennial species. Within these families, the most important agronomic species include *Lolium perenne*, *Lolium multiflorum*, *Festuca arundinacea, Festuca pratensis*, *Trifolium repens, Trifolium pratense,* and *Medicago sativa*. These forage crop species also receive most attention with regard to the development and application of molecular tools, in addition to the "forage model species" *Lotus corniculatus* and *Medicago truncatula*. The rapid development of molecular genetic tools in these species is well documented in the past volumes of "Developments in Plant Breeding" devoted to forage crops (Spangenberg, 2001; Hopkins *et al*., 2003).

The objectives of this paper are to describe the current status of (i) plant genomics activities in forage crops, (ii) known biochemical pathways and respective genes affecting forage quality with regard to reverse genetic approaches, (iii) activities on forward genetic approaches with regard to forage quality, and (iv) prospects and limitations on the implementation of molecular tools into forage crop breeding.

Forage crop genomics: tools

The ambition of plant genomics (Lander, 1996) is to provide structural information on whole genomes and in multi-parallel experimental approaches to (i) achieve a holistic view on biological processes, (ii) to accumulate information across experiments and species in order to investigate the function and interaction of genes, and, (iii), to transfer information to crops by transgenic approaches or by creating "designer" plants (Pelemann and van der Voort, 2003) based on functional DNA markers (Andersen and Lübberstedt, 2003).

For major crops more than 300.000 gene-derived EST (expressed sequence tag) sequences per species have been generated (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). Complete plant genomes have been sequenced so far for the model species *Arabidopsis thaliana* and rice (The *Arabidopsis* Genome Initiative 2000; Goff *et al*., 2002; Yu *et al*., 2002). More than 100,000 ESTs each have been generated for the model legumes *L. corniculatus* and *M. truncatula* (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). For forage crops, only a limited number of ESTs has been released to public databases (5,800 ESTs for *L. multiflorum*, 6,500 ESTs for *M. sativa*). However, substantial numbers of so far non-released ESTs (>10,000) have been or are currently generated for *L. perenne* (Sawbridge *et al*., 2003; Asp *et al*., 2003), *F. arundinacea* (Zhang and Mian, 2003), and *T. repens* (Spangenberg *et al*., 2003). Furthermore, a comprehenseive collection of "gene thresher" genomic sequences has been produced for *L. perenne* (http://www.vialactia.co.nz/news/newsitem.asp?id=61), and provided to Cold Spring Harbour Laboratory for annotation. Finally, BAC libraries have been reported for *L. perenne*, *F. arundinacea*, and *T. repens* (Farrar *et al*., 2005; Spangenberg *et al*., 2003; Donnison *et al*., 2002).

To improve complex traits such as forage quality, this structural genomic information has two major implications. Firstly, for those species with comprehensive genome sequence information available, it has become feasible to apply the 'forward genetic' approach of map-based gene isolation, as compared to previous attempts where it was basically impossible to go beyond mapped QTL. Secondly there is the possibility of information transfer across related species due to the evolutionary conserved gene order in chromosome blocks or even chromosomes (Devos and Gale, 2000). This approach of using syntenic relationships to identify relevant genes in forage crops is extremely promising due to the close relationships existing within the grass and legume families. Close syntenic relationships among different grass (Alm *et al*., 2003) and legume (VandenBosch and Stacey, 2003) species have been demonstrated. The concept for identifying orthologous sequences has meanwhile successfully been used in forage grasses (e.g., Armstead *et al*., 2004; Jensen *et al*., 2004).

In the frame of "functional genomics", efficient tools for multi-parallel and rapid testing of gene function including microarray-based expression profiling (Aharoni and Vorst, 2002), comprehensive mutant collections and virus induced gene silencing (VIGS) (Constantin *et al*., 2004) have been developed for plants. For the model species *A. thaliana*, the ambition is to characterize the function of all genes of this species until 2010. Comprehensive functional genomics projects are also underway for *L. corniculatus* and *M. truncatula* (VandenBosch and Stacey, 2003). The term "function" relates to some basic characteristics of genes (e.g. mutant phenotype, biochemical properties, expression pattern of selected genotypes). Functional genomics will provide new candidate genes at high speed for several traits due to better understanding of their biochemical role. Genes of interest can, in principle, be identified for any forage crop by exploiting information based on sequence homology or conserved map position

provided from model species. Recently, an increasing number of genomic tools have been developed for the major forage crops. These include gene-derived markers (e.g., Faville *et al*., 2004; Lübberstedt *et al*., 2003; Sledge *et al*., 2003; Saha *et al*., 2004) and microarrays (Spangenberg *et al*., 2001, 2003). Moreover, transformation for *in vivo* validation of gene function is established for the major forage crops (Spangenberg *et al*., 2001) and, more recently, VIGS has been established for legume species (Constantin *et al*., 2004).

Traits: forward genetics

Despite rapid progress in the last decade in generating sequences and tools in plant genomics, the function of more than 90% of all genes is still unknown even for the model species *A. thaliana*. Thus, reverse genetics approaches can currently only include a minor fraction of all possible genes. Forward genetic approaches are based on traits of interest (e.g. forage quality characters), and genome regions or genes are associated with trait variation. Quantitative trait loci (QTL) mapping combines conventional "black box" quantitative genetics with a marker gene-based approach. Several QTL mapping studies for forage traits have been conducted, e.g., in maize (Lübberstedt *et al*., 1997; 1998; Barriere *et al*., 2003), and ryegrass (Cogan *et al*., 2004). Combined with the availability of BAC libraries, map-based gene isolation has generally become possible. Another option is the comparison of QTL with candidate gene locations in order to identify the most promising known candidate genes (Barriere *et al*., 2003, Cogan *et al*., 2004). These map-based approaches can be extended to introgression lines as shown for ryegrass and *Festuca* in the EU project SAGES (http://www.iger.bbsrc.ac.uk/SAGES/). This approach is promising in forage crops since it makes use of synteny both to exploit genomic tools across related species, and gene materials to broaden genetic variation.

Additional genomic tools have already been employed in forward genetic approaches to monitor genes associated with forage traits. In maize, publicly available microarrays have been used to compare expression profiles of bm isogenic lines or extremes from QTL mapping populations (Lübberstedt *et al*., 2004). Since several hundred genes were found to be significantly differentially expressed between these genetic contrasts in relation to cell wall digestibility, the next crucial step is to identify the most relevant candidates by comparison of expression profiling experiments across different genetic contrasts, or by comparison of map positions of differentially expressed genes with QTL locations. For ryegrass and white clover, programs for forage plant gene discovery based on expression profiling have been announced (Spangenberg *et al*., 2001; http://www.dafgri.dk). It can be foreseen that VIGS or TILLING will be useful for the identification of additional genes affecting forage quality.

Pathways and genes: reverse genetics

Prerequisite for reverse genetics approaches is the availability of proven or "qualified" candidate genes affecting traits. Forage quality is determined by cell wall properties or cell content composition. The digestibility of cell walls mainly in grasses but also in legumes is often limited by the content and composition of the lignin fraction. With respect to cell content, water soluble carbohydrates such as fructans are most relevant in grasses, whereas proteins and tannins are major determinants of legume quality.

The lignin biosynthetic pathway is well characterized (Boudet *et al*., 1995), especially the common phenylpropanoid pathway starting with the deamination of phenylalanine and providing hydroxycinnamoyl CoAs. The enzymes involved in the common phenylpropanoid

pathway are phenylalanine ammonia-lyase (PAL), cinnamate hydroxylase (C4H), coumarate hydroxylase (C3H), caffeic O-methyltransferases (COMT), ferulate hydroxylase (FA5H), and hydroxycinnamate CoA ligases (4CL). In total 34 genes have been identified in the *A. thaliana* genome coding for enzymes in the monlignol biosynthesis (Raes *et al*., 2003). The end products of this common pathway, the hydroxycinnamoyl CoAs, are the precursors of the major classes of phenolic compounds which accumulate in plant tissues, e.g. flavonoids, stilbenes, phenolamides as well as lignins. Subsequently, cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are specifically involved in biosynthesis of the lignin monomers p-Coumaryl, Coniferyl, and Sinapyl alcohol. In maize, genes for COMT (Collazo *et al*., 1992) and CAD (Halpin *et al*., 1998) have been isolated. Defect alleles of both genes have been shown to correspond to brown midrib mutations (COMT: bm3; CAD: bm1) known for long time (Barriere and Argillier, 1993). Several independent studies on bm1 and bm3 have proved already the concept of increasing silage quality by altering the lignin biosynthetic pathway (Barriere and Argillier, 1993) and reducing lignin content. However, the application of bm mutants in plant breeding has been hampered so far by their strong negative pleiotropic effects on yield characters and lodging. Therefore, one current target for improving feed quality is the identification of optimal alleles both for quality but also agronomic performance at well characterized genes such as COMT (Fontaine *et al*., 2003, Lübberstedt *et al*., 2004). Another target is the identification and evaluation of additional promising candidate genes affecting lignification and cell wall formation.

Lignins exhibit a high degree of structural variability depending on the relative proportion of three monolignols, different types of interunit linkages, and the occurrence of non conventional lignin units within the polymer (Boudet and Grima-Pettenati, 1996). Polymerization of monolignols involves peroxidases and laccases in an oxidation step but is generally not well understood (Boudet *et al*., 1995). Laccase was the first enzyme shown to be able to polymerize lignin monomers in vitro (Freudenberg *et al*., 1958). Several studies indicated that laccase and laccase-like activities are closely correlated with lignin deposition in developing xylem (Davin *et al*., 1992). Nersissian and Shipp (2002) identified 19 laccases in the genome of *A. thaliana*. In case of peroxidases, association between allelic variation at one Prx locus with forage digestibility in maize has been demonstrated (Guillet-Claude *et al*., 2004). Thus, a minimum of 70-80 candidate genes code for enzymes that are directly involved in lignin formation, based on the small *A. thaliana* genome. Furthermore, gene families involved in cellulose or hemicellulose formation might also affect cell wall digestibility (Barriere *et al*., 2003, Ralph *et al*., 2004). In conclusion, the challenge is not in finding candidate genes but in the identification of the most promising genes among those candidates for transgenic or marker-based approaches.

The digestibility of grasses as a general trait becomes markedly reduced during the course of the growing season. This reduction is largely caused by an increase in the content of poorly digestible cell wall structural components. In parallel, there is a decrease in the content of soluble carbohydrates – "sugars". Varieties of ryegrass with a high stable level of carbohydrates in the form of fructans have been shown to retain a high degree of digestibility throughout the growing season. Poorly digestible structural components create an imbalance between carbohydrate and protein levels during ruminant fermentation, leading to a loss of nitrogen (ammonia) to environment. Grass varieties with an increased level of soluble carbohydrates will lead to a more efficient uptake of proteins in ruminants, and thus, more efficient milk and meat production. Fructans are polymers of fructose, and have a general structure of a glucose linked to multiple fructose units (polyfructosylsucroses). In contrast to the uniform structure of bacterial fructans, plant fructans represent five major classes of

structural distinct fructans according to the linkages between the fructose units; inulins, levans, inulin neoseries, levan neoseries, and mixed type levans. It is now known that four out of five different fructosyltransferases (FT), each with their own specificity, are needed to synthesize the wide variety of fructans found in plants; 1-SST (1-sucrose:sucrose fructosyltransferase), 1-FFT (1-fructan:fructan fructosyltransferase), 6-FFT (6-fructan:fructan fructosyltransferase), 6-SFT (Sucrose fructosyl 6-transferase) and 6G-FFT (fructan:fructan 6G-fructosyltransferase). Other enzymes that are involved in fructan degradation are fructanhydrolases (FH), and invertases. Fructanhydrolase is a beta-fructofuranosidase and can uncouple fructose units from fructans with sucrose as an end product. Invertase, which is active in the vacuole, cleaves one sucrose molecule into glucose and fructose. It's also capable of cleaving fructose molecules from smaller fructans. This fructan hydrolysing activity decreases with a higher degree of polymerization of the fructan. The type of fructans and FTs varies among different monocot species. Levan type fructans are abundant in *Triticum*, *Hordeum* and *Bromus*, whereas inulin types are characteristic to e.g. *Lolium* species. Interestingly, bifurcose (a product of 6-SFT and precursor to the levan type and one of the routes to the levan neoseries) has not been found in *Lolium* species. According to this, four enzymes would be necessary to account for the synthesis of the fructans identified in *L. perenne,* namely 1-SST, 1-FFT, 6G-FFT and 6FFT (Parvis *et al*., 2001). Genes coding respective enzymes in forage crops have been isolated recently (Gallagher *et al*., 2004; Chalmers *et al*., 2003).

Forage legumes are highly digestible as compared to forage grasses. However, proteolysis and microbial deamination might lead to protein loss in the rumen, not fully compensated by post-ruminal absorption (Robbins *et al*., 2002). High digestion rates may result in protein foaming and rumen pasture bloat as a digestive disorder (Gruber *et al*., 2001). Moreover, amino acid composition of the protein fraction determines it's nutritive value (Spangenberg *et al*., 2001). A major role for reducing the high digestion rates has been assigned to condensed tannins (Gruber *et al*., 2001). Condensed tannins are polymeric flavonoids with protein-precipitating properties. Whereas high amounts of condensed tannins are detrimental to ruminant digestion, moderate levels (2-3% of dry matter) improve forage legume quality by reducing ruminal digestion rates and avoidance of protein foaming, and thus lead to higher rates of protein conversion into animal products (Robbins *et al*., 2002). Highly nutritious species such as white clover and alfalfa have a low level of endogeneous tannins as compared to tanniferous forages like Lotus. The initial steps in condensed tannin biosynthesis belong to the general flavonoid pathway and include enzymes like chalcone synthase. Whereas genes coding for enzymes of the general flavonoid have been isolated for long time, the first genes coding for enzymes of the condensed tannin specific pathway such as Leucoanthocyanidin reductase have been isolated more recently (Tanner *et al*., 2003). As for the lignin biosynthesis pathway, regulatory genes coding for transfactors have been envisaged as targets to manipulate tannin content, such as myb- or myc-like genes (Gruber *et al*., 2001).

Once qualified candidate genes affecting forage quality have been identified and isolated, these genes can be used to (i) create new genetic variation not available in elite germplasm of forage crops, or (ii) monitor and exploit existing genetic variation in a more targeted way. Transgenic approaches have been successfully employed both for improving cell wall digestibility and cell content composition in different forage crops, either by overexpression of novel genes, or by suppression of genes using antisense or RNAi technology (Spangenberg *et al*., 2001). One of the most obvious examples is the production of condensed tannins in alfalfa or white clover by overexpression of genes from the tannin biosynthesis (Gruber *et al*., 2001). Another major research area is the down-regulation of genes from the lignin

biosynthesis pathway (Ralph *et al*., 2004). Down-regulation of the maize bm3 orthologue coding for COMT was shown to successfully alter both lignin content and composition in grasses (Chen *et al*., 2003). However, implementation of transgenic approaches for variety production suffers from a lack of acceptance in several countries, and requires extensive risk evaluation (Wang *et al*., 2003), which is especially crucial for the mostly outbreeding forage crops endogeneous in the relevant production areas. An alternative, at least for the knock-out approach, is the generation and screening of new genetic variation by TILLING (McCallum *et al*., 2000). However, public TILLING populations are currently not available for forage crops, but in preparation for maize (http://genome.purdue.edu/maizetilling/), ryegrass (http://www.intl-pag.org/pag/13/abstracts/PAG13_W100.html), and *M. truncatula* (May *et al*., 2003).

The second important reverse genetics area is the monitoring of allelic variation with a view to the development of functional markers (Andersen and Lübberstedt, 2003). In this context, association studies have recently been adapted to plants from human genetics and proven valuable to identify sequence motifs within genes affecting a trait of interest (Flint-Garcia *et al*., 2003). Thornsberry *et al*. (2001) identified in a pioneering study nine SNP or INDEL polymorphisms in the maize dwarf 8 gene associated with flowering time. More recently, polymorphisms within CCoAOMT-2 (Guillet-Claude *et al*., 2004a), a peroxidase (Guillet-Claude *et al*., 2004b), and COMT (Lübberstedt *et al*., 2004) were associated with cell wall digestibility in maize. Association studies based on candidate genes are especially promising in species with a generally low linkage disequilibrium (Flint-Garcia *et al*., 2003), as can be expected for outcrossing forage crops. Studies on systematic allele-sequencing and association studies in ryegrass are currently ongoing in the EU project GRASP (http://www.grasp-euv.dk). However, sequence motifs showing association with the trait of interest need further validation (Andersen and Lübberstedt, 2003) before converting them into robust functional markers.

Molecular breeding: benefits

Independent of the breeding programme, the process of breeding new cultivars includes three phases: I) generation of genetic variation, II) development of genetic components for producing new varieties (such as inbred lines in hybrid breeding), and III) testing of experimental varieties (Becker, 1993). Molecular breeding benefits all three phases, but is also useful in the context of variety registration and protection as well as for the characterization and management of genetic resources. The major approaches provided by genomics are based on transgenes or markers. The predictive value of markers depends on whether they are random DNA markers, gene-derived or functional markers (Andersen and Lübberstedt, 2003).

The major benefit of transgenic approaches is a broadening of genetic variation, especially if respective genes are lacking in the target species (Spangenberg *et al*., 2001). Markers are useful to establish heterotic groups (Riday and Brummer, 2003), and to assign genotypes or populations to heterotic groups. This topic might become increasingly relevant, if forage crop breeding moves from population or synthetic breeding to hybrid breeding (Riday and Brummer, 2003). Furthermore, markers might assist identification of genetically divergent parent genotypes or populations with a maximum usefulness (Lamkey *et al*., 1995; Schnell, 1983) to generate better varieties. Finally, recurrent selection programs might benefit from the application of markers ensuring, e.g., a sufficient level of genetic variation over several selection cycles.

Marker-assisted selection (MAS) and backcrossing (MAB) are major applications of molecular markers. MAS is especially promising for traits with low heritability (Lande and Thompson, 1990), whereas MAB allows tracing of favourable alleles, which is especially useful in case of recessive gene action. In case of MAB, including transfer of trangenes across genotypes and populations, markers are useful for background selection (Frisch *et al*., 2001) to recover the elite parent background efficiently in a short time. For MAS, an increasing number of candidate gene-derived or even functional markers (Andersen and Lübberstedt, 2003) will become available in the near future, as demonstrated for ryegrass recently (Faville *et al*., 2004). Functional markers will reduce the risk of a Type 3 error in declaring QTL-marker associations (Dudley, 2003), i.e., declaring in case of a significant QTL the wrong marker allele as being linked to a favourable QTL allele. In addition, markers can be employed to predict the performance of components of hybrid or synthetic varieties using BLUP (Bernardo, 2002).

Transgenic approaches might be employed for controlled crosses in view of hybrid seed production but also in the context of controlled flowering of grasses to increase forage quality by reducing the stem fraction. This would, in addition, minimize pollen flow of transgenic plants to natural populations but also pollution with pollen allergens (Spangenberg *et al*., 2001). Furthermore, transgenic traits or bar-codes can be employed for variety description and protection (Spangenberg *et al*., 2001). During the final steps of variety production markers can be used to (i) reduce the amount of experimental testing, (ii) confirm hybridity, and (iii) fulfil DUS criteria in variety registration (Tommasini *et al*., 2001). Finally, gene bank collections might benefit from molecular markers to describe and maintain genetic resources, as well as to establish core collections.

Levels of complexity: limitations

Forage crop breeding is characterized by several layers of complexity. At the trait level, direct evaluation of feed quality is too costly to be performed for several genotypes or populations as required in breeding programs. Therefore, indirect biological and chemical methods have been established, meanwhile substituted by more rapid physical methods like NIRS, often calibrated to the indirect chemical or biological methods. Genetic markers based on results obtained with, e.g., NIRS, are currently developed and implemented in breeding programs. Thus, the increasing distance between original animal trials and current indirect methods require re-calibration to avoid artefacts based on the indirect methods used.

At the feed or plant level, a given variety is often only part of the animal diet fed together with minerals, additives etc. If used for grazing, a single variety typically is only part of a mixture between different grasses and legumes. Forages are grown in swards and not at single-spaced plant level as a number of genetic experiments, with quite variable cultivation regimes (e.g., regarding the number of cuts). Further aspects adding to the complexity are different ploidy levels within crops such as ryegrass, and symbiosis with endophytes or root nodule bacteria. In contrast, many genetic experiments are performed under simplified conditions to establish sound phenotype – genotype associations, preferably (i) at the diploid level, (ii) in monoculture, (iii) at per se level, and (iv) for single spaced plants. Therefore, a crucial question is, to what extent are results obtained in "artificial" experimental situations transferable and, thus, valuable to operational breeding programmes?

For some forage crops such as alfalfa, commercial varieties are mainly tetraploid although diploids also exist. In this case, QTL mapping in diploids is much more straightforward. However, QTL detected at diploid level might not be functional at the autopolyploid level. A well known example for differences in gene action at diploid and tetraploid level is the presence of gametophytic self-incompatibility in diploid potatoes, whereas autotetraploid potatoes generally are self fertile (Becker, 1993). Furthermore, autopolyploids generally have enlarged cells and vegetative organs as compared to diploid forms (Becker, 1993). This implies that tetraploid performance can only partly be predicted based on "diploid information". Similarly, prediction of genotype or family performance to be grown in swards based on information obtained at the single-spaced plant level might be poor. Posselt (1984) reported a generally lower heterosis for agronomic traits for ryegrass in swards as compared to spaced plants. Furthermore, low correlations were found in ryegrass for seed yield components evaluated in plots versus single plants (Elgersma, 1990). Thus, depending on the trait of interest, the mode of testing genotype or family performance is essential with regard to the transferability of information for breeding of superior varieties under practical conditions.

Genome regions increasing GCA within a given synthetic are of highest priority for synthetic breeding. Hence, evaluation of testcross rather than per se performance (after cloning of mapped genotypes) will be preferable. In hybrid breeding, per se performance of inbreds is of minor interest compared to that of hybrid performance. In an experiment on mapping of QTL for forage traits in maize, four segregating populations were established within the flint heterotic pool and evaluated for forage traits after testcrossing to elite dent tester inbreds at the hybrid level (Lübberstedt *et al*., 1997a, b, 1998). The predictive value of QTL was evaluated by comparing QTL results across testers within one population or across populations using the same tester. The three small validation populations had zero, one, or both parent lines in common with the large calibration population. Generally, the number of common QTL across populations increased with the genetic similarity of mapping populations. Almost all QTL detected in the small independent sample were also detected in the calibration population, both derived from the same cross. For unrelated mapping populations, about 70% of the detected QTL were specific to each population. However, consistency of QTL across populations as well as testers was highly trait-dependent. In conclusion, QTL or genes identified in different populations or test systems (like plots versus single spaced plants) need re-evaluation in breeding populations under practical relevant conditions.

Conclusions and perspective

During the past decade, the availability of genomic tools and information in major forage crops has dramatically increased. Numerous genes have been isolated and characterized in biochemical pathways relevant to forage quality. First studies demonstrated the usefulness of these new tools and information in both reverse and forward genetic approaches for more efficient breeding of forage crop varieties. Besides continued development of genomic tools and their application in basic research, the next challenge will be the implementation of these resources in experimental procedures delivering relevant information for practical breeding. Besides implementation of experimental approaches such as haplotype of association mapping (Flint-Garcia *et al*., 2003) in forage crops, this will require phenotypic testing close to agronomic practice.

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