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REVIEW ARTICLE

Revolutionizing livestock sustainability: Pioneering breeding strategies for superior forage biomass and quality

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Abstract

Livestock primarily rely on forage crops as a source of feed and nutrition. The milk productivity of a cow or meat production in goat/sheep could directly be associated with the availability of a sufficient quantity of quality green fodders with essential nutrients in a balanced ratio. Feeding the cereal/grass: legume fodders in the required proportion will not only improve productivity but also the reproductive capacity of animals. However, many countries of the world experience a huge gap between demand and availability of green fodder. In this context, emphasis should be placed on developing efficient forage genotypes with increased biomass and quality as per the requirements of animals, duly considering their digestibility. Breeding approaches encompassing required classical approaches, including wide hybridization to exploit natural genetic variability, biotechnological tools such as transgenic technology, marker-assisted selection, genomic selection, and various omics techniques alongside high-throughput phenotyping using multispectral cameras, would help to sustain livestock productivity by meeting out the present and future fodder requirements coupled with enhanced nutrients.

Keywords

forage biomass; quality improvement; QTL mapping; genomics; MAS

Introduction

Over the past thirty years, global milk production has surged by over 77%, rising from 524 million tonnes in 1992 to 930 million tonnes in 2022. India leads the world in milk production, contributing 22% of the total output, followed by the United States, Pakistan, China, and Brazil. In recent years, developing countries have significantly increased their share of global dairy production, primarily due to a rise in the number of milk-producing animals rather than an increase in productivity per animal (1). For example, in India, the growth in milk production is mainly attributed to the expansion of the cattle population, as livestock productivity remains considerably lower than in other major milk-producing nations. India's average milk yield per cow is 1,538 kg per lactation, compared to the global average of 2,238 kg and the European average of 4,250 kg per lactation (2). The primary cause of the low productivity of Indian livestock is malnutrition or undernutrition, which is attributed to a major gap in the demand and supply of nutritious animal feed. Most cows and buffaloes in India are dependent upon crop residues

such as wheat and paddy straws as their staple feed (3,4) due to inadequate production of lush green forages in the country (5). The low crude protein, high fiber, high lignin, and silica levels in wheat and paddy straws are major nutritional constraints for using them as animal feed (6). To meet nutritional requirements, total mixed rations (chaffed crop residues, mainly wheat straw supplemented with oilcake, beans, and cereal grains) are fed in specific proportions to provide a balanced diet (3). To maintain milk production, the livestock are generally fed with concentrates. Although it increases milk production, it causes rumen acidosis in dairy animals. The practice of concentrated feeding unbalances the gut microbiota, which releases more toxins, resulting in damage to the liver (7).

Green fodder provides vitamins and minerals to dairy animals, as well as enhances digestion. Integrating the green fodder feeding system with livestock management significantly reduces the milk production cost, whereas the availability of high-quality forage throughout the year is the critical success factor in the livestock industry. However, breeding for nutritional quality in forage is considered the second most important objective, next to biomass yield. Unlike forage biomass, nutritional quality traits are neglected as the determinant factor for market price (8).

High-quality forage encompasses an adequate amount of minerals, carbohydrates, crude protein, sulphur amino acids, high palatability, and minimal anti-nutritional factor, which is responsible for livestock production and reproductive success (8). Although forage nutritional quality improvement is possible through conventional and advanced plant breeding approaches, substantial progress has not been achieved due to the greater heterogeneity, polyploidy, apomixes, and self-incompatibility of forage crops (9). In this situation, innovative strategies such as transgenic breeding, marker-assisted selection, genomic selection, and integrated omics approach hold immense potential in improving nutrient compositions.

Major nutritional components in forage crops

Nutritionally, the foremost vital components of forage crops are carbohydrates, proteins, and lipids. In addition to these, the other components, such as vitamins and minerals, are crucial for plant function and can have a definite nutritional impact (Fig. 1) (10).

Carbohydrates

Carbohydrates are the primary source of digestible energy present in forages (10). A major fraction of 50-80% of the dry matter present in grass forages is carbohydrates (9,10). The usual forms of carbohydrates present in forage are plant fiber (structural carbohydrate), monosaccharides (non-structural carbohydrates), starch (storage molecules), and disaccharides. According to their status as cell wall constituents, solubility, and storage or structural polysaccharides (i.e., non-starch polysaccharides (NSPs) versus starch), the carbohydrates found in plant-based diets are classified as fibrous or non-fibrous (11). These complex carbohydrates further break down into simpler sugars by

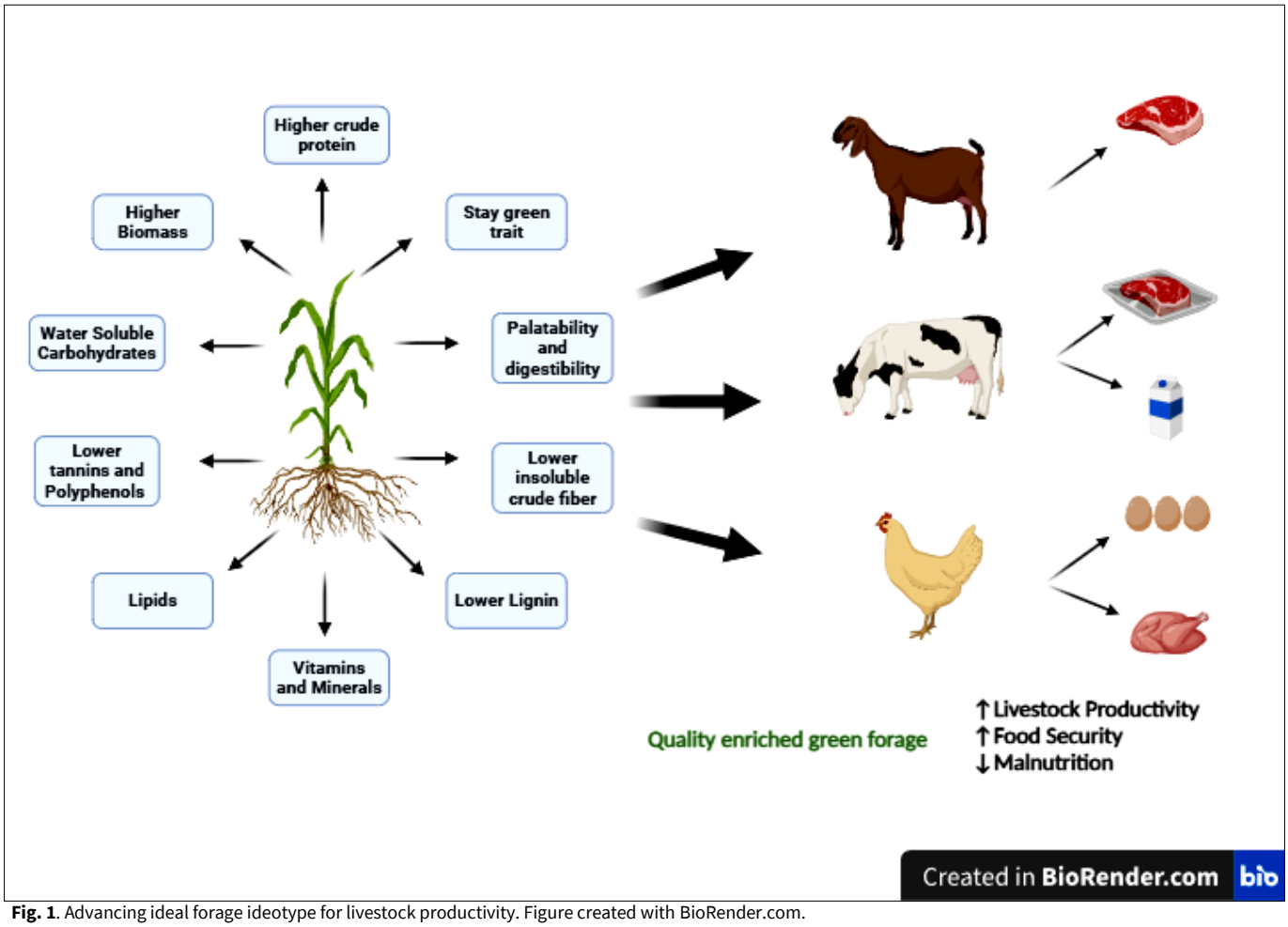

Fig. 1. Advancing ideal forage ideotype for livestock productivity. Figure created with BioRender.com.

the cleavage of glycosidic bonds by the animals (both ruminants and non-ruminants) or by microbial digestion (ruminants only) (9). The microbial fermentation of carbohydrates in the gastrointestinal tract of herbivorous animals produces short-chain fatty acids (acetic, propionic, and butyric), also known as volatile fatty acid, which contributes approximately 70% to the ruminants' caloric requirements (12). Lignin content in forage cell walls adversely affects fiber digestion in ruminants, and it depends on the physiological maturity of the crop. Grass cell walls appear to be more severely inhibited by lignification than do legumes (13). Hence, carbohydrates play a vital role in animal health, growth, and reproduction, as well as in improving the quality of animal products (10).

Protein

In forages, enzyme proteins are the most crucial nutritional constituents for the well-being and growth of plants. The biosynthesis of all other nutritional components, including carbohydrates, lipids, and proteins, is dependent upon the activity of these enzymes. Forage proteins do not differ from other herbaceous proteins in terms of structure or composition. Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) makes up about 40–60% of the protein found in plant leaves, with the remaining 40–60% being a complicated mixture of about 20,000 distinct proteins. As a consequence, it is difficult to alter the concentration and composition of protein to enhance nutritional benefits to animals (10). The source of nitrogen to the animal body is mainly from forage crude protein (9).

Some other compounds that alter protein digestibility are condensed tannins and polyphenol oxidase. Proanthocyanidins or condensed tannins (CT) decrease the degradation of forage protein in the rumen. It can be beneficial as excess protein degradation causes bloating in animals. However, in the animal's midgut or digestive tract, high CTs prevent the absorption of protein, which results in a total loss of nutritional value (14). For desired digestibility, a CT concentration of 2%–4% is ideal (15). Similarly, Polyphenol oxidase (PPO) reacts with phenol to form o-quinones, which further bind with dietary protein to form o-quinone-protein complexes and reduce the protein degradation rate in the rumen as well as in silage (16). The PPO has a positive impact on forage crops as it safeguards the plant protein and glycerol-based PUFA by inhibiting the activity of proteases and lipases (8).

Lipids

Lipids in forage crops are mostly present in the form of PUFA (Polyunsaturated fatty acid), which includes linoleic acid (LA) and alpha-linolenic acid (ALA). These particular fatty acids act as a precursor of beneficial fatty acids (17) and a substantial proportion of them is identified as alpha-linolenic acid (18). The major source of leaf lipid deposition is the cell membrane, which contains about 3.5% dry matter (19). The fatty acids are responsible for the overall quality of meat and dairy products, such as the flavour development during cooking (20), the colour and shelf life of meat, and the hardness or spreadability of butter (21). Studies have shown that cows fed with fresh herbage yield milk with a higher content of alpha-linolenic acid and conjugated linoleic acid than preserved forage. The impact of forage on alpha-linolenic acid concentrations in ruminant products depends upon two factors: enhancing the precursor supply and mitigating the degree of biohydrogenation (17).

Minerals and vitamins

Minerals and vitamins play a crucial role in enhancing animal health and performance. The vital minerals Na, K, Ca, P, Mg, S, Cu, Co, Zn, Fe, Mn, Mo, I, and Se, as well as Vitamins A and E, are abundant in forages. Generally, the sufficiency, deficiency, or toxicity of minerals and vitamins in animal feed can often be identified by particular signs and symptoms. However, the subclinical amounts can significantly impact feed intake, digestibility, and overall animal performance, all without apparent signs (22). Major minerals such as Calcium, Phosphorus, Chloride, Potassium, Magnesium, Sodium, and Sulfur are required in larger amounts, usually in grams per day. On the other hand, trace elements like copper, iron, iodine, manganese, selenium, zinc, and cobalt are needed in smaller amounts, usually in milligrams per day. Some other elements are important either due to the potential risk of toxicity (Cd, F, Pb) posed by them or due to their interactions with the accessibility of essential elements (Mo interactions with Cu) (23).

Generally, vitamins are classified based on their solubility in either water (B and C group) or in lipid solvents (A, D, E, and K). In addition to this, vitamins for adult ruminants are categorized based on either by self-supply (via the rumen or endogenous supply) (K, C, D, and B group); or by supply from the feed (A and E). Thus, ruminants have a particular dietary dependency, primarily concerning vitamins A and E (24).

Considering the importance of the nutritional compositions discussed, carbohydrates are the main sources of energy that play a vital role in animal health and productivity. Protein from the fodder is the primary source of protein in milk production, as well as the growth and development of animals. Whereas minerals and vitamins are critical in the body functioning of the animals, which ultimately reflects on milk/meat production. Hence, forage crop genotypes developed for quality improvement have to be necessarily evaluated for the presence of appropriate levels of these nutrients for the sustenance of livestock productivity.

Genetic diversity for nutritional traits

The collection and conservation of forage germplasm were primarily focused at the Australian Tropical Forage Genetic Resource Center at Brisbane (ATCFC), Commonwealth Scientific and Industrial Research Organization (CSIRO), and the International Center for Tropical Agriculture (CIAT, Columbia) along with the International Board for Plant Genetic Resource (IBPGR) and national research institutes located in Africa, Asia, and America. To increase genetic diversity, the introduction or exchange of germplasm with unique characteristics led to the development of many landmark varieties. For example, the development of

tropical forage legume cultivar Stylo (*Stylosanthes guianensis* (Aubl.) Sw. var. *guianensis*) is more productive and has almost equivalent fodder quality compared to other legumes such as Canavalia CIAT 17009 (*Canavalia brasiliensis*) and BRA 9690 (*Aeschynomene histrix*) (25)

Recently, most of the forage crops were domesticated, and the improvement of landraces started in the early 20th century (26). The domestication of forages was highly systematic when compared to grain crops. Because of this, forage species exhibit far higher genetic diversity than grain crops (25, 27). Wild species can be a valuable source of desirable quality traits and be used to improve cultivated forage crops through wide hybridization. In the case of maize, wild teosinte types (*Zea mays* ssp. *mexicana*) are used to enhance the methionine contents in forage. Wild species of maize possess higher protein compared to cultivated types. Also, the protein content of inter-subspecific hybrids of maize and teosinte is substantially higher than either of the parents (28). Similarly, *Pennisetum typhoides* x *P. purpureum*, a successful interspecific triploid between pearl millet and napier, resulted in higher biomass and quality for both species (29). In line with this, *Sorghum bicolor* × *S. sudanense* hybrids recorded high protein, high dry matter yield, and reduced HCN content (30). *Lotus tenuis* × *L. corniculatus* hybrids registered reduced amounts of proanthocyanidins (PAs) in edible tissues to prevent ruminants from bloating. The Lolium–Festuca complex's genetic diversity is derived from its mandatory outbreeding mating system, high potential for hybridization between related species, and the lack of major genetic bottlenecks caused by domestication (26). *F. glaucescens* protein degrades slowly (protein's half-life under *in vitro* rumen like conditions is 19.2 hr.). This trait of *Festuca* combined with the complementary trait for high leaf protein in *L. multiforum* (protein's half-life is 2.3 hr) significantly increased the protein half-life of the *Lolium* parents (31). A wild species, *Arachis cardenasii* is known for its high fodder value. ICG11563 ($2n = 2x = 20$), a diploid line of *Arachis cardenasii* was crossed as a male parent with cultivated variety VRI4 ($2n = 4x = 40$), a tetraploid of *A. hypogaea.* Subsequent backcrosses of F_1 with VRI 4 resulted in five BC_1F_1 hybrids with vigorous growth, prostrate habit, and broad and dark green leaves with high palatability. These hybrids were suitable for multiple cutting and had 98% success in vegetative propagation (32).

Landraces are potential donors for valuable traits, and characterization of landraces through gene-specific markers can be done to assess genetic diversity. The North Eastern Himalayan region of India is a well-known center of diversity for maize, with landraces from this area exhibiting good agronomic performance and acclimatization to stress conditions. A total of 26 maize landraces screened for β-carotene polymorphism using the crtRB1 3'TE marker and identified two landraces, CAU-M66 and CAU-M16, with enhanced β-carotene content (33).

Exploration and conservation of nutritive grass fodder species in their native areas are crucial for habitat restoration, preventing animals from moving out of their habitat and affecting agricultural crops. An exploration study was conducted by (34) to evaluate the nutritional potential of wild grass fodder in the Elephant reserve of the Western Ghats. The nutrient-rich species are *Cynodon dactylon, Oplismenus burmanniifor, Dichanthium aristatum, Heteropogon contortus, and Themeda triandra* were identified for elephants. The higher crude protein content was found in *Cynodon dactylon* (11.94%). These species are highly recommended for fodder bank development in the elephant corridors.

Selecting crop species or genotypes with complementary functional traits is considered as an effective approach to improve both productivity and yield stability in mixtures (35,36). By incorporating species with diverse root and shoot architectures, mixtures can better exploit available resources. For example, (37) integrating deep-rooting forbs such as "*Cichorium intybus*" with grass-legume mixtures increased biomass production. Forage chicory (*Cichorium intybus* L.), a mineral-rich perennial herb with high palatability and a healthy fatty acid profile (38), supports cattle grazing on temperate swards, aiding in maintaining or boosting milk production during summer (39). Chicory grazing does not cause bloat and helps to reduce internal parasites, lowering the need for anthelmintics (40). Integrating chicory into the traditional ryegrass and white clover (RGWC) mixed pasture systems improved forage yield, yield stability (41), dairy production, and environmental sustainability (38). Another study reported that binary mixtures of white clover (*Trifolium repens*) and chicory produced significantly higher dry matter yields than white clover monocultures, ryegrass-chicory mixtures, and multi-species mixtures of ryegrass, white clover and chicory across both marginal and fertile lands (41). Similarly, including chicory in white clover-based grasslands improved yield stability compared to clover pure stands and clover-ryegrass mixtures during low precipitation years, and this effect was reversed under high precipitation (42). Hence, in mixed pastures, forage yield depends on plant species' genotype, botanical composition, cropping sites, and environmental conditions (41).

In another experiment, the milk yield was found to be increased by feeding the RGWC with afternoon chicory (CHPM) in comparison with the other treatments, such as RGWC alone and RGWC with morning chicory (CHAM) (38). Milk from chicory-fed cows had higher polyunsaturated fatty acid (PUFA) levels. Chicory also increased urination frequency and reduced urinary nitrogen, highlighting its environmental benefits without compromising productivity.

To assess the molecular diversity of forage crops, various markers such as RAPD (Random Amplified Polymorphic DNA) (43), RFLPs (Restriction Fragment Length Polymorphism) (44), SCoT (start codon targeted polymorphism) (45), SRAP (sequence-related amplified polymorphism) (46), DArT (Diversity Arrays Technology) (47), SSR (simple sequence), ISSR (inter short simple repeat) (48) and SNP's have been employed. Some of the examples are given in Table 1.

Table 1. Genetic diversity studies using various molecular markers.

Forage species	Molecular marker	Variability reported for quality traits	References
Timothy (*Phleum pratense* L.)	SSR	Dry matter yield	(103)
Pearl Millet	SSR and GBS identified SNP	Green forage yield (GFY), dry forage yield (DFY), crude protein (CP) and invitro dry matter digestibility (IVDMD)	(104)
Fodder maize	SSR	Biomass yield	(105)
Maize	SSR and SNP	β – carotene content	(106)
Bermudagrass	Simple sequence repeat (SSR)	Plant height, biomass, moisture content, crude ash, crude fiber (CF),	(107)
Grass Pea	SSR	Biomass yield, plant height, CP, IVDMD and lignin	(108)
Pearl Millet	SNP and silicoDArT	Dry weight yield and metabolizable energy	(47)

Molecular markers employed by the scientists indicated the presence of greater genetic diversity for nutritive traits such as crude protein, crude fiber, crude fat, acid detergent fiber (ADF), and neutral detergent fiber (NDF) coupled with higher biomass and invitro-dry matter digestibility (IVDMD) which offers greater scope for the crop breeders to exploit it from the primary and secondary gene pools of different forages offering a huge potential for selection and improvement of forage species besides revealing a scope for parental selection to exploit maximum heterosis in forage biomass and quality.

Integrated breeding strategy for forage quality improvement

Breeding programs aimed at improving forage crops face numerous obstacles and challenges. These include substantial genotypic and phenotypic heterogeneity among individuals, varying degrees of polyploidy (from low to high), self-incompatibility, apomixes, in-breeding across grasses, and few agronomic traits linked with distinct genes (9). Also, the conventional breeding of forage crops often takes 10 years to develop a cultivar, and it is mostly based on traits that are poorly understood, which makes the breeding process cumbersome and time-consuming (49). Therefore, the integration of conventional plant breeding with molecular sciences and biotechnology offers a pathway to enhance the efficiency of forage breeding, particularly for improving nutritional traits (Fig. 2).

Molecular tools for augmenting nutritional status of forage crops

Conventional breeding methods have contributed to the improvement of forage plants for the past decades. In the biotechnological era, molecular techniques show promise,

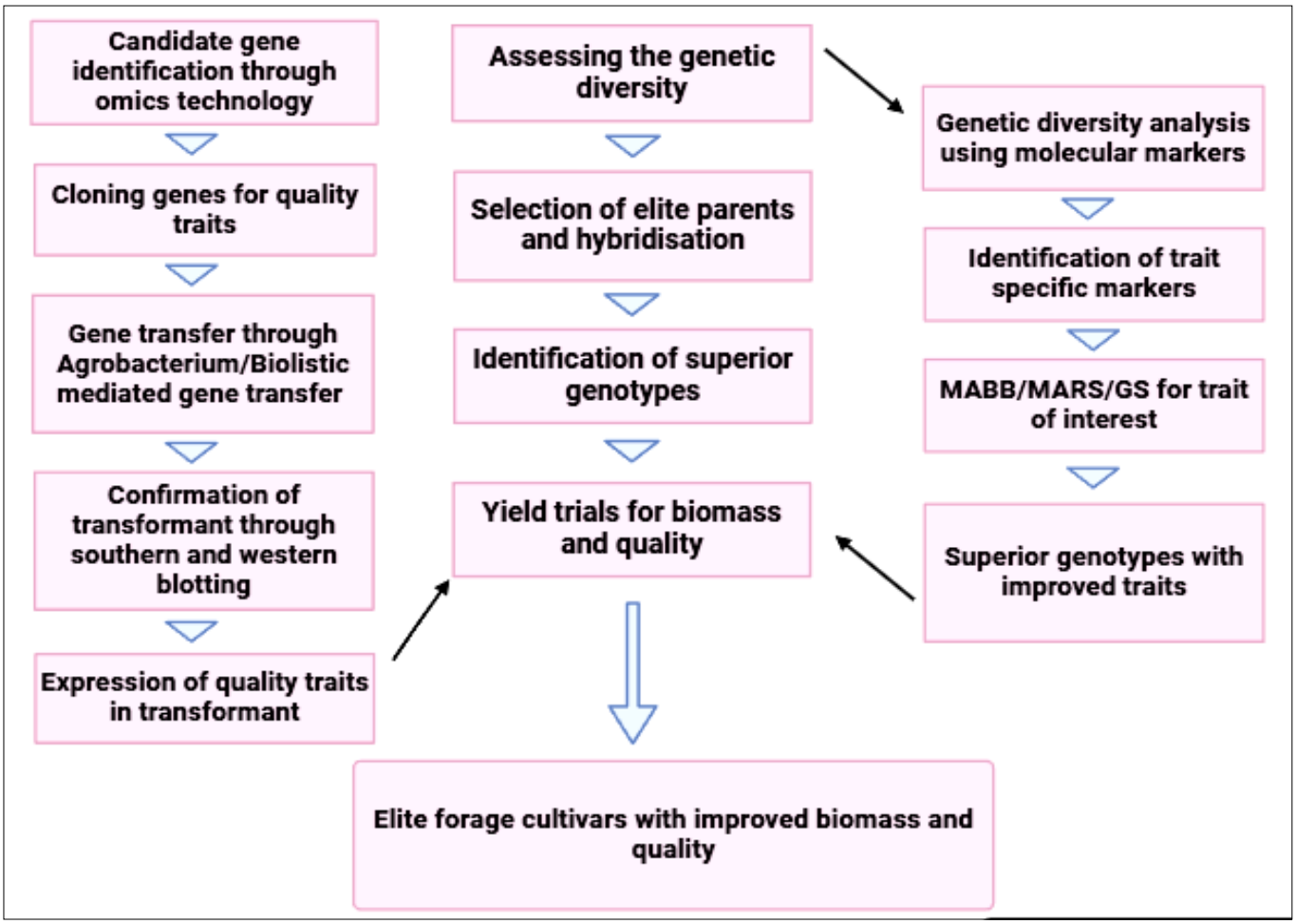

Fig. 2. Integrated approaches for enhancing quality and biomass in forage crops. Figure created with BioRender.com.

particularly in accelerating the breeding of fodder crops, which are typically perennial plants (50,51).

Transgenic approaches

Some of the notable examples of transgenes responsible for improving forage quality are furnished. The major water-soluble carbohydrates are the fructans. Based on sucrose as the substrate, fructan metabolism is catalysed by fructosyltransferases for biosynthesis and fructan exohydrolases for breakdown. Thus, digestibility can be enhanced by increasing fructan metabolism (52). For example, in ryegrass, the fructan biosynthesis pathway has been altered by expressing fructosyltransferases through the biolistic transformation method. It led to an incline in water-soluble carbohydrates, metabolizable energy, and *in-vivo* dry matter digestibility, as well as a decline in the NDF concentrations (53).

Using RNA antisense (AS) technology, (54) genetically modified maize plants were developed with reduced COMT (caffeic acid O- methyltransferase) activity under the control of maize *Adh1* (alcohol dehydrogenase) promoter. These COMT-AS lines showed a decrease in lignin content at the flowering stage. Similarly, (55) downregulated maize O-methyltransferase gene (OMT) by expressing antisense sorghum OMT driven by maize ubiquitin-1 (*Ubi*) promoter. T1 transgenic plant showed a decrease in lignin content by 17% and increased digestibility by 72-76% on a whole plant basis.

Overexpression of two genes of *Medicago truncatula, phytase (MtPHY1)* and *purple acid phosphatase (MtPAP1),* in alfalfa, increased the phosphorus acquisition and biomass yield (56). Likewise, co-over expression of *Zygophyllum xanthoxylum* (ZX) genes, namely ZxNHX and ZxVP1- 1 in alfalfa, increased phosphorus in root and leaves as well as crude protein, crude fiber, crude fat, and crude ash, especially under stress conditions (57). In another study, the bacterial transgenes Aspartate kinase (AK) and Adenylylsulfate reductase (APR) were transferred to alfalfa to increase cysteine content by 30 % and methionine by 60% compared to wild types (58).

Downregulation of the MtSGR (STAYGREEN) gene in alfalfa through antisense RNA technology showed chlorophyll percentage up to 50% during the senescence stage and increased the amount of crude protein compared to the wild type (59). The stay-green characteristic may be advantageous in fields with standing crops of mostly senescing leaf material utilized to supply livestock with fodder (60).

Quantitative Trait Loci (QTL) Mapping

The nutritional quality traits are mainly governed by polygenes and are referred to as quantitative traits (61). The genomic regions containing genes responsible for the quantitative variation of a trait are known as quantitative trait loci (QTLs), and the method of constructing linkage maps with the help of DNA markers and conducting QTL analysis is known as QTL mapping (62). QTL mapping identifies desirable genes, determines the amount of variation due to additive, dominant, and epistatic effects, helps to understand variation mechanisms, and identifies the genetic correlation between different traits within the genomic region (63).

Forage breeders mostly depend on quantitative trait loci (QTL) for the genetic improvement of forage crops because only a few major genes are responsible for regulating forage nutritional traits. Compared to other crop species, little attention has been paid to forage crops due to their complex polyploidy nature with genomes derived from multiple progenitors and showing polysomic inheritance, so there is a huge difficulty in developing consistent linkage maps than in simple diploids (64). However, the utilization of high-density linkage maps, in combination with recent advances in next-generation sequencing technologies, accelerated the forage crop improvement programs with higher precision and efficiency (50).

Significant advancements in forage crops were made in identifying the QTL's responsible for various quality traits. For example a total of 16 QTLs (6 for crude protein, 2 for crude fiber, 2 for ADF, and 6 for NDF) have been detected through composite interval mapping in RIL (recombinant inbred line) population of soybean derived from the cross (PI 483463 and Hutcheson) (65). In another study (66), the QTL analysis in the RIL population of maize derived from the cross (Zheng58 x HD568) for six related traits using composite interval mapping revealed the presence of 6,5,10,9,8,9 QTLs for ADF, ADL/NDF, CEL/NDF, IVDMD, IVNDF and NDF respectively. In addition to this, five pairs of epistatic QTLs involving 9 loci have been identified for ADF, CEL/NDF, IVNDFD, and NDF. The study also revealed a QTL hotspot on chromosome 9, flanked by the SNP markers PZE-109016787 and PZE-109076761, with a genetic interval from 55.7 to 90.3 cM. From this result, 29.8% of QTLs showed >10% of the phenotypic variation, and 70.2% explained <10% variation. Hence, the study concluded that fewer major QTLs and many minor QTLs are associated with the biosynthesis of cells and digestibility in maize stalks.

The interspecific hybrid of Sorghum and Sudan grass is considered a high-quality forage for livestock. The hybrid inherited the quality of sorghum, being resistant to drought, and sudan grass for its high biomass production (67). From sorghum Tx623A- Sudan grass Sa hybrid (68), a high-density genetic map of length 1191.7 cM was constructed using RAD-seq and identified 1065 markers. Further, 19 QTLs for five forage quality traits (one for CP, five for NDF, three for ADF, two for ADL, and eight for HC (hemicellulose) were mapped in a RIL population between sorghum Tx623A- Sudan grass Sa. The study concluded four overlapping QTLs for NDF, ADF, ADL, and HC, which will be the candidate locus for further breeding of high-quality forage.

In maize, fiber and lignin are the major components affecting stem cell wall digestion, so to increase the feed value of maize (increasing the cell wall digestibility), it is necessary to decrease the concentration of fiber and lignin (69). Several QTL mapping studies were conducted using low-density markers such as RFLP or SSRs, and these

studies are not more focused on high-oil maize (HOM) (70). Further research conducted by (71) has proved that the stalk quality of high-oil maize is better than normal maize. Hence, 188 recombinant inbred lines derived from the cross B73 × By804 (a high oil inbred line) with high-density SNP markers were examined (70) and identified 20 QTLs for six-cell wall-related traits. QTL linked with individual traits showed 10.0%–41.1% of phenotypic variation. The identified QTLs for forage quality traits in different forage crops are reviewed here in Table 2.

The progress in the identification of QTLs for different fodder quality traits reveals ample scope for the application of marker-assisted breeding for the breeding of nutrient-rich forage cultivars. The QTLs validated the different quality traits like crude protein, crude fiber, ADF, NDF, and IVDMD, which facilitate crop breeders to opt for marker-assisted breeding for quality improvement in forages.

Marker-assisted selection

The identification or selection of plants carrying desirable

Table 2. QTLs associated with forage quality traits (data given for major QTLs showing more than 10% phenotypic variation).

Sl. no	Crop	Population	Traits	QTLs	Chr (LG)[1]	PVE (%)	References
1	Maize	RIL (B73×By8040)	ADF	adf2	2	12.20	(70)
				adf6	6	10.72	
			ADL/NDF	adl2-1	2	20.01	
				adl2-2	2	10.30	
			CEL/NDF	cel1	1	12.43	
				cel6-2	6	10.48	
			IVDMD	ivdmd2	2	13.02	
			IVNDF	ivndfd10	10	10.01	
			NDF	ndf2-1	2	13.36	
2	Maize	RIL (Zheng58×HD58)	ADF	adf2	2	11.8	(66)
				adf9-1	9	11.1	
				adf9-2	9	12.3	
			ADL/NDF	adl9-1	9	11.1	
				adl9-2	9	11.1	
			CEL/NDF	cel2-2	2	11.6	
			IVDMD	ivdmd9-1	9	18.9	
				ivdmd9-2	9	16.9	
			IVNDFD	ivndfd9-1	9	11.2	
				ivndfd10-1	10	10.4	
			NDF	ndf2-2	2	10.2	
				ndf2-3	2	11.3	
				ndf9-1	9	14.2	
				ndf9-2	9	12.7	
3	Sorghum× Sudangrass	RIL	CP	qCP4	4	16.93	(109)
			NDF	qNDF4	4	10.44	
				qNDF5	5	40.05	
			ADF ADL	qADF5.1	5	45.26	
				qADL5	5	30.67	
				qADL1	1	0.34	
			HC	qHC3.1	3	13.64	
				qHC4.2	4	15.34	
				qHC4.3	4	14.65	
				qHC3.2	3	10.00	
				qHC2	2	10.82	
4	Soyabean	RIL3613 (Dongnong L13 × Heihe 36 a)	CP	qcp-gm16-2	Gm16	15.67	(110)
		RIL6013 (Dongnong L13 × Henong 60)	CP	qcp-gm02-1	Gm02	10.90	
			NDF	qnDF-gm02-1	Gm02	10.35	
					Gm02	11.42	

5	Soyabean	RIL (PI 483463 and Hutcheson)	CP	qCP19_1	19(L)	26.46	(65)
				qCP19_2	19(L)	10.94	
			CF	qCF19_1	19(L)	21.10	
				qCF19_1	19(L)	14.07	
				qCF19_1	19(L)	29.33	
				qCF19_1	19(L)	28.92	
			ADF	qADF19_1	19(L)	12.79	
				qADF19_1	19(L)	31.43	
				qADF19_1	19(L)	21.00	
				qADF19_1	19(L)	41.72	
			NDF	qNDF19_1	19(L)	24.04	
				qNDF19_1	19(L)	28.19	
				qNDF19_1	19(L)	26.58	
6	Barley	DH lines (Step-toe and Morex)	NDF (%)	Qndfa2a	2(2H)	49.32	(111)
			ADF (%)	Qndfa2a	2(2H)	31.03	
			NO3–N (%)	Qno3a4a	4(4H)	11.35	
			N (%)	Qna2a	2(2H)	47.28	
				Qna4a	4(4H)	10.96	
			Non-NO3–N (%)	Qnona2a	2(2H)	56.72	
			ISDMD (%)	Qdmda2a	2(2H)	58.57	

LG[1]-Linkage group, **PVE (%)**-proportion of phenotypic variation explained by particular QTL, **ADF**-acid detergent fiber, **NDF**-neutral detergent fiber**, ADL/NDF**-acid detergent lignin, **CEL/NDF**-cellulose, **IVDMD**, in vitro dry matter digestibility; **IVNDFD**, *in vitro* neutral detergent fiber digestibility, **CP**-crude protein, **CF**-crude fiber, **ISDMD**-*In situ* dry matter digestibility, **NO3–N** Nitrate-nitrogen.

alleles (for a specific trait) by utilizing molecular markers linked to the gene of interest is known as marker-assisted selection (MAS). The breeding process gets accelerated due to the indirect selection of desired traits through MAS, which leads to the rapid development of improved cultivars. Selection can be done at the early seedling stage with the help of a marker system without concern about genotype × environment (G × E) interactions. MAS can be categorized into 1) Marker-assisted backcross breeding (MABC), 2) Marker-assisted recurrent selection (MARS), and 3) Genomic Selection (GS) (72).

Marker-Assisted Backcrossing (MABC)

The process of MABC involves the use of markers to choose target loci, reduce the size of the donor segment containing a target locus, and speed up the recovery of the recurrent parent (RP) genome while backcrossing (73). Compared to conventional backcross breeding, marker-assisted backcrossing maintains higher precision and efficiency and reduces the number of backcrosses required to recover the RP phenotype (74). The success of a backcross breeding program depends upon three main factors, i.e., recurrent parent selection, screening for target traits, and the number of backcrosses (75). MABC comprises three major steps: - Foreground selection, recombinant selection, and background selection. Foreground selection is performed to screen the individual plants for the target gene at the early seedling stage. Recombinant selection is carried out to select the backcross progeny carrying the target gene and markers flanking the target gene at < 5 cM on either side to avoid linkage drag. Lastly, the background selection is performed to choose the progeny with the highest recovery of the RP genome (76).

The first MAS-based product of maize developed by using MABC is “Vivek QPM hybrid 9” (a QPM version of Vivek Maize Hybrid 9’) through introgression of the opaque 2 allele (72). In Vivek QPM Hybrid 9, there was a notable improvement in nutritional content compared to Vivek Maize Hybrid 9. Specifically, tryptophan levels increased by 41%, and lysine by 30% (77). Similarly, the β-*carotene hydroxylase* (*crtRB1*) gene introgressed into 7 elite inbreds through a marker-assisted breeding program showed a 12.6-fold increase in β-carotene concentration over the recurrent parent. Improved parental lines exhibited a kernel β-carotene concentration of 21.7 mg/g, compared to the original hybrid, which had 2.6 mg/g (78). Similar studies have been conducted on maize (79, 80, 82), which resulted in improved β-*carotene* by 9.248 µg/g in UMI1230ß+-1 and 8.286 µg/g in UMI1200ß+-2.

The antinutritional factor present in maize is phytic acid, which decreases the bioavailability of nutritive minerals in animal feed. A mutant maize plant carrying *lpa2-2* allele is responsible for low phytic acid (*lpa*) in maize. For the first time, lpa2-2 specific SSR marker ‘umc2230’ has been developed, and using marker-assisted backcross breeding the *lpa2-2* allele has been transferred from low phytate mutant line ‘EC 659418 (donor parent) into well-adapted line UMI395 (recipient parent) (81). Similarly, in another study (82), the *lpa2-2* allele from EC 659418 was transferred to an elite recipient line UMI285.

Marker-assisted gene pyramiding is an approach to transfer genes from multiple parents into a single parent. One such example is the transfer of *crtRB1* and o2 (opaque 2) genes through MABC (concurrent stepwise transfer),

which results in β-*carotene, lysine,* and *tryptophan-rich maize inbreds (83).*

Marker-assisted recurrent selection (MARS)

MARS is similar to the phenotypic recurrent selection, but it also utilizes molecular markers as an indirect selection method to increase the frequency of favourable alleles within the population. The F_2 or F_3 population of the selected cross is genotyped for several markers covering the whole genome, and F_2 derived F_4 and F_5 population is phenotyped for the target trait, followed by successive recombination cycles to estimate the marker effects. It is highly advantageous compared to MABC because it screens the whole genomic regions comprising major and minor QTLs (72).

Genomic Selection (GS)

Genomic selection involves using molecular markers across the entire genome for genomic-enabled prediction (GP) to evaluate the performance of candidates for selection. In a GS programme, there are two basic populations 1) the Training population and 2) the Testing population. The genotypic and phenotypic data of the training population are combined to derive genomic estimated breeding values (GEBVs) in a testing population (only genotyped but not phenotyped). Therefore, GS aids in the prediction of genetic and breeding values by analyzing genotypic data to identify the most suitable individual for future breeding. GS offers advantages over phenotypic selection by lowering the cost per cycle and accelerating the time needed for variety development (84). Genomic selection has been carried out in forage lucerne (85) to predict yield and quality that could accelerate the development of lucerne cultivars. 227K SNPs were obtained by genotyping by sequencing (GBS), and phenotyping was carried out in different environments. With a large training population, a quality prediction of 0.6 was obtained for dry matter yield, ADF (acid detergent fiber), and protein content. Integration of QTL information to the genomic prediction model with a large number of training populations increased the predictive quality by around 0.8. A wide array of markers can be used to identify QTL, which can then be integrated into a small set of markers for genomic prediction. Therefore, the result shows that the quality of the prediction depends upon the size of the training population, the number of markers used, and the integration of QTL effects.

Integrated Omics Technologies

The efficient development of superior cultivars is made possible by omics technologies, including genomics, transcriptomics, proteomics, metabolomics, and phenomics. Enhancing the nutritional value, palatability, and digestibility of the forage crops presents abundant opportunities to improve the quality of fodder (86). By employing various omics techniques, genes, their functions, the type of RNA or protein involved, their structure, and the pathway responsible for the development of the ultimate morphological characteristic were identified (87). The genes that were identified were further manipulated or transferred to create newer varieties or hybrids with desirable characteristics (86).

Genomics is the study of genomes and genes. It is crucial for understanding genetic variation and crop performance. It is categorized into structural, functional, and epigenomics. Structural genomics investigates genome structure, chromosomal organization, and sequence variations. It facilitates the creation of detailed genetic and physical maps to identify genomic regions that influence specific traits. Functional genomics delineates gene function responsible for traits of interest. Epigenomics is the study of epigenetic modifications that influence gene expression, such as DNA methylation and histone modifications. These factors substantially contribute to the genetic improvement of crop species (88). Innovation in plant genomics has improved and quickened the breeding process in several ways (e.g., association mapping, marker-assisted selection, 'breeding by design' gene pyramiding, and genomic selection etc.) (86). Genome-wide association studies exploited in alfalfa highlighted the polygenic control of forage quality traits, and the genetic control for a given trait is different in leaves and stems. The study identified SNPs for stem protein content and also a genomic region on chromosome 8 linked with leaf ADL (acid detergent lignin) (89).

Transcriptomics is the study of the transcriptome, which comprises every RNA transcript generated within a cell or tissue by the genome of an organism. It is a powerful tool for studying gene expression in response to different stimuli over specific time frames. Initially, methods like cDNAs-AFLP, differential display-PCR (DD-PCR), and suppression subtractive hybridization PCR (SSH-PCR) were used for transcriptome analysis, but they didn't offer a complete resolution. However, advanced techniques such as microarrays, digital gene expression profiling, next-generation sequencing, RNA sequencing, and Serial Analysis of Gene Expression (SAGE) have revolutionized RNA expression profiling (90). *Elymus sibiricus*, a significant forage grass in the Qinghai-Tibet region, faces challenges in commercial seed production due to its tendency for high seed shattering. The transcriptome analysis of two contrasting *E. sibiricus* genotypes (XH09 and ZhN03) was carried out to identify the candidate genes involved in the seed shattering habit. Quantitative real-time PCR (qRT-PCR) validated the expression profiles of 10 candidate transcripts involved in cell wall-degrading enzymes, lignin biosynthesis, and phytohormone activity. Eight of these genes were up-regulated in the low seed shattering genotype ZhN03, indicating their potential role in reducing seed shattering (91).

Not all transcripts are translated into proteins, and post-translational gene regulation plays a crucial role in gene expression, and there is no correlation between transcript abundance and protein content. Thus, proteomics techniques help in analyzing total expressed proteins and their quantities, including post-translational modifications such as phosphorylation, acetylation, glycosylation, prenylation, sulfation, and ubiquitination (88). It is categorized into sequence, structural, functional, and expression proteomics. Sequence proteomics identifies the sequence of amino acids using high-performance liquid chromatography. Structural proteomics identifies protein structure

through computer-based modeling, and experimental methods, including crystallization, electron microscopy, nuclear magnetic resonance (NMR), and the X-ray diffraction of protein crystals. Functional proteomics identifies the function of the protein by different methods, such as yeast one or two hybrids and protein microarray profiling (90).

Metabolomics is the study of metabolites present in a cell or tissue under specific conditions. The metabolome reflects the cell's phenotype, is influenced by environmental effects or mutations, and affects gene expression and protein function (92). Proteomics identifies only gene products, whereas metabolomics recognizes the protein expressions metabolically and the biochemical pathway that plays a crucial role in the functioning of the gene (90). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most commonly used techniques of metabolomics. Two common MS-based analyses are gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). These high-throughput instruments play a vital role in providing necessary data for further research in the field of metabolomics (92).

Accurate phenotyping information is required for selecting a breeding program in all genomics techniques, such as linkage and association mapping, genome-wide association studies, marker-assisted selection, genome-assisted selection, haplotype-based breeding, etc. However, collecting accurate phenotyping information is challenging, particularly in large-scale breeding programs where thousands of genotypes are screened per year at multiple locations. The challenge is more complicated for fodder crops, where multi-cuts are performed every season. The development of recent phenomics tools has the potential to phenotype various agronomical and nutritional traits in fodder crops, which can aid in overcoming this challenge (86).

Crop phenomics is defined as the multidisciplinary study of high throughput accurate acquisition and analysis of multidimensional phenotypes on an organism-wide scale through crop development (93). The phenotyping platforms are categorized into three types based on their imaging level: microscopic, ground-based, and aerial. These platforms allow the characterization of phenotypic traits at different levels of plant organization, including tissue, organ, individual plant, plot, and field (94). Assessment of large breeding populations in Lucerne for herbage accumulation (HA) and determining the dry matter content by drying consumes more time and is highly expensive. The efficiency of HA yield can be increased by the use of high throughput phenotyping (HTP). Phenomics-assisted selection has been practised in Lucerne for herbage accumulation using unmanned aerial vehicles (UAVs) equipped with sensors. Estimation of four vegetation indices such as normalized difference vegetation index (NDVI), green normalized difference vegetation index (GNDVI), normalized difference red edge (NDRE), and green and red ratio Vegetation Index (GRVI) with the help of multispectral cameras showed a high correlation with HA. Additionally, data from spatial analysis controlled field variation and increased the heritability for both HA and NDVI (95). Similarly, (96) reported that UAV-based high-throughput phenotyping (HTP) enhanced the selection efficiency for alfalfa biomass in small plots with spatial models, improving the estimation of genetic parameters and the accuracy of family selection. Traditional methods for assessing forage quality traits such as CP, ADF, and NDF used hazardous chemicals (97) but have largely been replaced by near-infrared spectroscopy (NIRS), which is now the most widely used technique. However, NIRS requires proper sample preparation and standardization, as the spectral readings are highly influenced by particle size (98). In contrast, portable spectroradiometers allow in situ, non-destructive measurements with a broader spectral range (350-2500 nm), capturing visible light absorption peaks where plant pigments are identified, unlike NIRS devices (750-2500 nm) (99). Several studies have reported the use of hyperspectral devices for predicting nutritive traits such as CP, NDF, and NDFd (NDF digestibility) (100) and biomass yield (101,102).

The omics approaches discussed above can be effectively utilized in forage crops in an integrated manner to gain a precise understanding of forage genotypes and to expedite forage breeding programs, especially for nutritional traits, in a more efficient manner. Some of the additional omics studies related to forage nutritional traits are provided in Table 3.

Table 3. A summary of various omics studies in forage crops.

Crop	Omics technology	Trait of interest	References
Alfalfa	Genomics(GWAS)	SNP markers on chromosome 8 linked to acid detergent lignin in leaves.	(89)
Maize	Genomics(GWAS and QTL mapping)	QTL region identified for low-Cd accumulation.	(112)
Trifolium repens	Metabolome and transcriptome	Differential gene expression in anthocyanin and proanthocyanidin biosynthetic pathways.	(113)
Lolium perenne	GWAS	Water soluble carbohydrate accumulation.	(114)
Sorghum	GWAS	Crude protein, acid detergent fiber, neutral detergent fiber, hemicellulose and cellulose contents.	(115)
Sorghum	Transcriptome and Metabolome	Dhurrin metabolism	(116)
Oat	GWAS	β-glucan concentration	(117)
Alfalfa	GWAS	Lignin content	(118)
Oat	Metabolome	Metabolites such as Xylitol, undecylic, glutamic acid, isofucosterol, linolenic and methylmalonic were identified.	(119)
Alfalfa	GWAS	19 QTL for dry matter yield,15 for ADF content and 15 for protein content.	(85)

Future Prospects

Sustained livestock productivity is feasible only when the cattle are fed with sufficient quantity of fodder and balanced nutrients. To meet this, forage cultivars need to evolve with greater biomass and dense nutrients. Although there are challenges in conventional breeding, particularly in perennial forages, numerous varieties have evolved, which have contributed to partially narrowing down the demand-supply gap of fodder requirement. Exploitation of Bajra x Napier hybrids, a milestone achievement in wide hybridization, contributed significantly to fodder biomass and nutrition of cattle. Further crossing of cultivated fodder maize with the teosinte will open up a way for the development of multi-tillering perennial fodder maize, which would substantially improve the total productivity and nutrient composition. The enormous variability observed in the genetic resources of forages has to be harnessed through marker assisted breeding, as several QTLs have been reported for the quality traits of fodder crops. Practical exploitation of MAS would be feasible upon validation of some identified QTLs cross-verification in different populations or locations and fine mapping of the specific genomic regions. Routine regeneration tissue culture protocols are to be standardised to facilitate the faster genetic transformation of identified candidate genes for quality traits. Developments in omics technologies have to be exploited to identify the genome sequences/candidate genes responsible for enhancing the quality/nutrient traits in forages. Therefore, an integrated approach governing conventional and biotechnological tools will definitely aid the forage breeders in developing nutrient-rich forage crops without any green fodder yield penalty, which would address the bridging of the demand gap as well as the nutritional security of cattle.

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Authors' contributions

Pawan Kumar Dash collected the literatures and prepared the manuscript. K.N Ganesan edited, revised and organised the overall manuscript. N. Manivannan suggested the improvement of manuscript scientifically. S. Vellai Kumar suggested for revision of biochemical portions of the manuscript. N. Senthil suggested for improvement of biotechnological portions of the manuscript and R. Pushpam suggested the information regarding forage genetic resources.

Compliance with ethical standards

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