

## Review

# Advances in Molecular Breeding of Forage Crops: Technologies, Applications and Prospects

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**Abstract:** Molecular breeding has revolutionized the improvement of forage crops by offering precise tools to enhance the yield, quality, and environmental resilience. This review provides a comprehensive overview of the current technologies, applications, and future directions in the field of forage crop molecular breeding. Technological advancements in the field, including Quantitative Trait Loci (QTL) mapping, Genome-Wide Association Studies (GWASs), genomic selection (GS), and genome-editing tools such as CRISPR-Cas9, have significantly advanced the identification and incorporation of beneficial traits into forage species. These approaches have dramatically shortened the breeding cycles and increased the efficiency of developing cultivars with improved yield, disease resistance, stress tolerance, and nutritional profiles. The implementation of these technologies has led to notable successes, as demonstrated by case studies on various forage crops, showcasing enhanced forage quality and adaptability to challenging environmental conditions. Furthermore, the integration of high-throughput phenotyping with advanced bioinformatics tools has streamlined the management of large-scale genomic data, facilitating more precise selection and breeding decisions. Looking ahead, this review explores the potential of emerging technologies, such as the application of artificial intelligence in predictive breeding, along with the associated ethical and regulatory considerations. While we stand to gain benefit from these innovations, the future of molecular breeding in forage crops must also confront the challenges posed by climate change and the imperative of sustainable agricultural practices. This review concludes by emphasizing the transformative impact of molecular breeding on the improvement of forage crop and the critical need for ongoing research and collaboration to fully realize its potential.



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**Keywords:** forage crops; molecular breeding; quantitative trait loci (QTL) mapping; genomic selection (GS); CRISPR-Cas9; high-throughput phenotyping (HTP); artificial intelligence (AI)

## 1. Introduction

Forage crops play an integral role in sustainable agriculture, serving as a cornerstone in ruminant diets and contributing significantly to soil conservation, carbon sequestration, and biodiversity. As global change imposes stresses on ecosystems, there is an increasing demand for improved varieties of forage crops that are resilient to environmental challenges, including drought, salinity, and extreme temperatures. The traditional breeding methods have enhanced the forage species, but are constrained by long breeding cycles, the complex nature of traits in forage crops, and the polyploidy of many forage species, which complicates genetic improvement. The urgency for the molecular breeding of forage crops is underscored by the need to expedite the development of improved cultivars with enhanced nutritional quality, increased yield, and greater stress tolerance. Molecular breeding tools, including marker-assisted selection, genomic selection, and advanced biotechnologies like CRISPR/Cas9 genome editing, offer the potential to surpass the limitations of traditional breeding by enabling precise and targeted genetic improvements. These tools can assist in dissecting complex traits and expedite the breeding process, ensuring the swift introduction of forage varieties adapted to changing climates and capable of supporting

livestock production systems. The subsequent sections will elaborate on the innovative molecular breeding technologies and their applications to forage crops.

## 2. Quantitative Trait Loci (QTL) Mapping

Quantitative Trait Loci (QTL) mapping is a statistical technique utilized in genetics to pinpoint the genomic regions associated with specific quantitative traits. These complex traits, influenced by multiple genes, exhibit continuous variation across populations. The main objective of QTL mapping is to connect the genetic markers with phenotypic traits, thus unraveling the genetic blueprint of complex traits [1]. Following the identification of QTLs, the subsequent steps include validation and fine mapping to accurately identify the specific genes implicated [2]. QTL mapping has become a pivotal tool in the genetic analysis of forage crops, shedding light on the complex genetic makeup underlying pivotal agronomic characteristics, including the yield potential, forage quality, and resilience to biotic as well as abiotic stressors [3]. The application of this methodology has been instrumental in the development of novel cultivars endowed with enhanced attributes. This progress has been accelerated by utilizing detailed, high-density genetic maps in conjunction with the latest advances in next-generation sequencing technologies, which together have propelled the precision and efficiency of forage crop breeding programs [3]. Table 1 lists the QTL loci identified in forage crops from recent studies.

Yield maximization remains the principal objective for forage crop improvement programs. Significant advancements have been made in identifying the QTLs influencing yield determinants. For example, a comprehensive genetic map for rye (*Secale cereale* L.) was constructed, identifying a significant QTL linked to spike length, a critical yield determinant [4]. In intermediate wheatgrass, a synergistic approach that combines genetic and physical mapping has uncovered QTLs associated with the traits related to domestication, clarifying the genetic intricacies underlying its domestication [5]. For alfalfa (*Medicago sativa* L.), a key forage crop, several QTLs regulating yield and its sub-traits have been identified, laying the groundwork for future yield improvements [6]. An innovative high-density genetic map for *Elymus sibiricus* was developed, addressing the widespread issue of seed shattering and highlighting the key QTLs related to seed yield [7]. In perennial ryegrass (*Lolium perenne* L.), the extensive mapping of a self-compatibility locus was conducted, producing diagnostic markers essential for implementing marker-assisted selection for this trait [8]. Furthermore, the integration of QTL mapping with RNA-Seq has facilitated the identification of genes involved in alfalfa leaf development, a trait that significantly influences both the forage yield and quality [9]. An extensive linkage map for *Stylosanthes guianensis* was also constructed, identifying the QTLs affecting yield and quality traits, showcasing the efficacy of marker-assisted selection in the genetic enhancement of these characteristics in the tropical forage legumes [10]. Collectively, these studies are poised to transform the yield and quality improvement strategies through the application of QTL mapping and marker-assisted selection to forage crop breeding.

Our understanding of complex agronomic traits has advanced, with the efficacy of DNA marker-assisted selection in enhancing alfalfa's biomass productivity being validated, particularly under a condition of water deficit. The findings, however, indicated the variability in performance across distinct genetic backgrounds [11]. Utilizing a nested association mapping population proved pivotal in identifying the QTLs influencing the regrowth vigor of switchgrass (*Panicum virgatum* L.), an essential trait for ensuring consistent biomass yields across multiple harvests [12]. Furthermore, the timing of flowering, a critical factor in biomass accumulation, has been thoroughly investigated. Choi et al. (2023) pinpointed a QTL affecting flowering time in switchgrass [13], whereas Jiang et al. (2022) identified several QTLs that control this trait in alfalfa [14]. These groundbreaking discoveries provide essential insights that are crucial in developing breeding strategies aimed at optimizing biomass production.

**Table 1.** The QTL loci identified in forage crops from recent studies.

Species	QTLs	Map Position	LOD	Associated Traits	Candidate Genes	Reference
<i>Secale cereale</i>	1	LG4	6.11	spike length	ScWN4R01G329300 and ScWN4R01G329600	[4]
<i>Thinopyrum intermedium</i>	111		15.00	grain production traits	42 possible domestication orthogenes	[5]
<i>Medicago sativa</i>	21		>3.00	yield and yield components		[6]
<i>Elymus sibiricus</i>	29			seed-related traits	30 candidate genes for seed shattering	[7]
<i>Lolium perenne</i>	1	LG5	7.17	self-compatibility	seven candidate genes	[8]
<i>Medicago sativa</i>	24			leaf size	seven candidate genes	[9]
<i>Stylosanthes guianensis</i>	52		>2.50	important agronomic traits		[10]
<i>Panicum virgatum</i>	10			regrowth vigor		[12]
<i>Panicum virgatum</i>	1			flowering time	PvHd1	[13]
<i>Medicago sativa</i>	38		>3.00	flowering time	seven candidate genes	[14]
<i>Boehmeria nivea</i>	9	CF7 and CF13	>4.05	crude fiber and crude protein	whole_GLEAN_10016511	[15]
<i>Medicago sativa</i>	83			quality traits	cellulose synthase-like protein A1, E3 ubiquitin-protein ligase	[16]
<i>Trifolium pratense</i>	1			resistance to southern anthracnose		[17]
<i>Trifolium pratense</i>	8			rot resistance		[17]
<i>Medicago sativa</i>	5	4D, 6B, 6D, and 8C	3.00	Verticillium Wilt Resistance	two putative candidates of nucleotide-binding site leucine-rich repeat disease resistance genes	[18]
<i>Lolium multiflorum</i>	1	LG4		bacterial wilt resistance		[19]
<i>Brachiaria grasses</i>	3		>3.00	aluminum tolerance	30 genes	[20]
<i>Panicum virgatum</i>	3			surface wax	50 candidate genes	[21]
<i>Eragrostis curvula</i>	2			diplospory		[22]
<i>Megathyrsus maximus</i>	47	LG4	6.11	complex Traits and Apospory	55 candidate gene	[23]

Forage quality, which includes nutrient density and digestibility, is of critical importance. Recent research has illuminated the genetic framework that determine the levels of crude protein and fiber content in ramie (*Boehmeria nivea* (L.) Gaud.), identifying the QTLs and candidate genes that could be utilized to improve the forage quality via marker-assisted selection strategies [15]. Additionally, the genetic loci associated with crude protein content and fiber composition were identified in alfalfa, highlighting the complex genetic mechanisms that influence these traits that are integral to forage quality [16].

Enhancing stress tolerance is crucial for the resilience and sustainability of forage crop production systems. The QTLs that confer resistance to southern anthracnose and clover rot have been discovered in red clover (*Trifolium pratense* L.), setting the stage for genomic-assisted breeding approaches [17]. The construction of high-density linkage maps and the identification of QTLs related to resistance against *Verticillium* wilt in alfalfa offer genetic markers to aid in the creation of new alfalfa varieties with improved disease resistance [18]. Furthermore, pooled DNA sequencing has revealed SNPs linked to a major QTL for bacterial wilt resistance in Italian ryegrass (*Lolium multiflorum* Lamk.), providing molecular

tools for marker-assisted selection to enhance resistance in this forage species [19]. The studies on *Brachiaria* grasses have identified QTLs that contribute to tolerance against high aluminum concentrations by mapping the transcriptional responses to aluminum stress [20]. A recent study uncovered QTLs that the control leaf surface wax load in switchgrass, a key trait for plant resilience to various environmental stresses [21]. Collectively, these research efforts highlight the intricate genetic basis of stress tolerance in forage crops across a range of biotic and abiotic stressors. They also illustrate the importance of QTL mapping in identifying the genetic loci for breeders to target, paving the way for the development of forage varieties that are more resilient and productive.

Additionally, the utilization of QTL mapping has illuminated the genetic foundations of apomixis, an asexual mode of seed reproduction. The locus responsible for diplospory has been accurately identified in the species *Eragrostis curvula*, representing a critical advancement in the incorporation of apomixis into plant breeding programs [22]. Concurrently, the genomic regions associated with the regulation of complex traits and apospory have been precisely mapped in guinea grass, advancing our understanding of the genetic mechanisms that govern apomictic reproduction [23].

### 3. Genome-Wide Association Studies (GWASs)

Integrating Genome-Wide Association Studies (GWASs) into forage crop breeding programs has significantly improved our understanding of the genetic factors influencing yield, quality, and stress resistance. GWASs enable the association of genetic variants across the genome with complex phenotypes by analyzing diverse germplasm collections, providing a powerful method to identify the genetic markers for breeding programs. Table 2 presents a summary of the GWAS findings for forage crops from recent studies.

**Table 2.** A summary of GWAS findings for forage crops from recent studies.

Species	GWAS Model or Software	Chromosomal Location	SNPs	Associated Traits	Candidate Genes	Reference
<i>Lolium perenne</i>	FarmCPU, MLM, and BLINK	Hv_chr4H and Hv_chr6H	3	leaf growth reduction under water deficit	phytochrome B and a MYB41 transcription factor	[24]
<i>Cenchrus purpureus</i>	BLINK.R and MLM		35	agronomic, morphological, and water-use efficiency		[25]
<i>Lotus corniculatus</i>	MLMM, FarmCPU, and BLINK	Chr6_22318549, chr5_19909005, and chr3_691967	3	CNglcs content and growth	LjMTR, LjZCD, LjZCB, and LjZCA	[26]
<i>Medicago sativa</i>	Six different models EMMAX		28	quality	20	[27]
<i>Sorghum sudanense</i>	(emmax-intel64-20120205.gz)	Sobic.001G012300	1	HCN-p	CYP79A1	[28]
<i>Lolium perenne</i>	MLM		hundreds	phenology		[29]
<i>Trifolium subterraneum</i>	PLINK v1.90b3.42	Chr1, 2, 3, 5, 6, and 7	8	Boron toxicity tolerance	respiratory burst oxidase-like protein etc.	[30]
<i>Trifolium pratense</i>	MLMM	LG1, LG2, LG3, and LG6	8	freezing tolerance	10	[31]

Table 2. Cont.

Species	GWAS Model or Software	Chromosomal Location	SNPs	Associated Traits	Candidate Genes	Reference
<i>Lolium perenne</i>	MLM		8	heading date, plant width, and plant biomass and water-soluble carbohydrate accumulation	a number of candidate genes	[32]
<i>Avena sativa</i>	MLM	Mrg21, Mrg05, Mrg17, Mrg18, and Mrg20	9	hullessness and lemma color		[33]
<i>Boehmeria nivea</i>	EMMAX	Chr5, 4, and 13	5	FWP, FWPC, LDWP, and DWP	9	[34]

In a study of forage yield, GWASs were proven crucial for identifying the markers closely associated with biomass accumulation in perennial ryegrass, a critical grass species for temperate agroecosystems. A GWAS was conducted to identify the DNA polymorphisms strongly correlated with reduced leaf growth under water scarcity conditions and closely linked to the genes coding for phytochrome B and the MYB41 transcription factor. These findings are crucial for breeding programs aimed at enhancing biomass production during mild summer drought periods, which are expected to become more frequent due to global climate change [24]. Similarly, the genetic architecture underlying complex traits in Napier grass (*Cenchrus purpureus*), a tropical perennial grass, was explored through the evaluation of 84 genotypes for agro-morphological and nutritional traits across varying soil moisture levels. This study identified more than 35 QTL regions associated with these traits, providing markers that are valuable for genetic improvement and future QTL exploration [25]. Moreover, the resequencing of global *Lotus corniculatus* accessions has clarified the population structure and identified the genetic loci associated with the accumulation of cyanogenic glycosides and growth traits. By employing GWASs, this research identified the candidate genes and an SNP site crucial for influencing these traits, thus aiding the molecular breeding efforts of *L. corniculatus* [26].

Forage quality traits, such as fiber digestibility and lignin content, are critically important and influenced by environmental factors. GWASs have enabled the identification of markers linked to these quality attributes, as demonstrated in a study on alfalfa that found two markers consistently associated with nine fiber digestibility traits in various environments. A single marker associated with lignin content was identified in various contexts, revealing candidate genes that are potentially involved in lignin biosynthesis and cell wall formation [27]. These markers and genes offer the potential for genetically enhancing forage quality in alfalfa, pending marker validation. A study by Li et al. (2023) on sudangrass (*Sorghum sudanense*), a hybrid forage known for its significant biomass and low dhurrin content, uncovered a QTL closely linked to hydrocyanic acid potential (HCN-p), with associated SNPs pointing to the CYP79A1 gene, a key player in dhurrin synthesis. This finding is pivotal in understanding the genetic underpinnings of the dhurrin content in sudangrass, as opposed to sorghum (*Sorghum bicolor* (L.) Moench), which plays a critical role in ensuring the safety and quality of forage [28].

Resistance to stress, particularly to water deficit, is a critical breeding objective in the context of changing climatic conditions. A GWAS was utilized to identify the genetic variants that confer enhanced tolerance to water scarcity. High-throughput genome-wide genotyping was employed to identify significant correlations with agronomic and adaptive characteristics in perennial ryegrass, including multiple markers linked to phenological traits, potentially contributing to environmental adaptation. These correlations are crucial for leveraging natural genetic diversity to develop improved grassland species adapted to regional climates [29]. Apart from drought, boron toxicity tolerance is a significant trait for



forages grown in the alkaline soils of arid and semi-arid regions. A GWAS utilizing a diverse panel of subterranean clover (*Trifolium subterraneum* L.) identified SNPs on chromosomes 1, 2, and 3 associated with boron toxicity tolerance. These SNPs align with haplotype blocks suggesting potential genes for boron toxicity tolerance assays, offering markers for pasture improvement programs [30]. In red clover, the loci associated with freezing tolerance (FT) were identified through a GWAS within a collection of European germplasm. The study noted considerable variability in FT and identified multiple loci linked to this trait, which may aid in developing molecular tools for breeding more resilient red clover varieties for cold climates [31].

The integration of a GWAS with additional genomic tools is demonstrated by Harper et al. (2019), who combined a BAC-based physical map with a GWAS on a collection of European ecotypes of perennial ryegrass. This approach identified the loci significantly associated with agriculturally important traits, including the heading date, plant width, biomass, and water-soluble carbohydrate content, advancing the search for candidate genes within large genomic regions [32]. A GWAS was performed on a collection of oat (*Avena sativa* L.) landraces and cultivars, examining traits including hulllessness and lemma color. This study revealed significant correlations with the markers located on different linkage groups, uncovering genetic diversity and population structure patterns, which have important implications for oat breeding at high altitudes [33]. In ramie, a GWAS examining six forage traits demonstrated consistent associations across these traits and identified the candidate genes potentially involved in nitrogen metabolism, growth, and development. This provides a foundation for breeding ramie varieties with improved forage qualities [34].

#### 4. Genomic Selection

Genomic selection (GS) has emerged as a transformative approach in plant breeding, offering the potential to accelerate genetic improvements for complex traits such as forage yield, quality, and stress tolerance. GS is distinguished from QTL mapping and GWASs by not focusing on identifying the specific genetic loci linked to traits. Instead, it leverages all the available marker data across the genome, including Single-Nucleotide Polymorphisms (SNPs), Microsatellites (also known as Simple Sequence Repeats or SSRs), and Insertion–Deletions (indels), to predict the genetic merit of individuals. The primary aim is to estimate the breeding values of individuals for selection purposes, rather than unraveling the genetic basis of traits. This methodology enables breeders to more efficiently identify the individuals with desirable traits, even when these traits are influenced by complex genetic architectures involving multiple genes with minor effects.

In the field of forage crop research, GS has been applied to various species, each presenting unique challenges due to their complex genomes. For instance, triticale, a hybrid crop from the crossbreeding of wheat and rye, has shown significant improvements through GS application, despite its large and complex genome. A study by Ayalew et al. (2022) analyzed dense marker data to explore the genetic diversity and population structure of cultivated triticale [35]. The study revealed a significant R genome substitution with the D genome and demonstrated the potential of GS to enhance the forage yield, achieving a mean accuracy of 0.52. While this is not perfect (a value of 1 would indicate perfect prediction), an accuracy of 0.52 indicates that GS can make the selections more accurate than random. Similarly, substantial progress has been made in applying GS to perennial grasses, which are typically characterized by obligate outcrossing. A recent study explored the challenges and successes of implementing GS in species such as perennial ryegrass, switchgrass, and timothy (*Phleum pratense* L.), observing that GS has the potential to significantly enhance the genetic gain for traits with varying heritabilities. The authors also highlighted the importance of utilizing enough markers and considering the genotype-by-environment interactions in GS models [36]. The potential of GS for various nutritive traits in perennial ryegrass, which showed moderate-to-high predictive abilities, suggests the practicality of implementing GS for these traits. This highlights the potential applications of multi-trait

GS in forage crop research [37]. The efficacy of genotyping-by-sequencing and GS in crested wheatgrass breeding revealed moderate prediction accuracies for the agronomic, morphological, and nutritive traits, indicating GS's potential applicability to non-model species [38]. GS has also been implemented in alfalfa to predict the canopy height and dry matter yield using family bulks. This strategy considered the interaction between the genotype and environment, leading to improved predictive capabilities [39]. The global alfalfa diversity panel was analyzed, identifying the genomic selection signatures specifically in Chinese varieties. Additionally, genomic associations with root development were discovered, offering valuable insights into improving the alfalfa traits [40].

The recent findings highlight the potential of advanced computational approaches to enhance GS in complex genomes. Six GS models were evaluated for *Panicum maximum*, an autotetraploid forage crop, demonstrating diverse applications. Specifically, GS-TD (Genomic Selection considering Tetraploid Dosage) and GS-DD (Genomic Selection considering Diploid Dosage) models were compared using 1223 informative markers. The results indicated that GS-TD outperformed GS-DD in predictive abilities, though all the models showed a relatively comparable performance. This research provides bioinformatic and modeling guidelines to account for tetraploid dosage effects, suggesting that applying these insights could improve the recurrent selection program for *P. maximum* [41]. Additionally, a machine learning method utilizing a joint learning approach for polyploid grasses resulted in over 50% accuracy improvements compared to those of the traditional methods [42]. Decision support software was used to simulate the effects of GS on genetic gain in terms of the dry matter yield for perennial ryegrass. The findings revealed significant improvements in the genetic gain rates and, in certain scenarios, enhanced cost-effectiveness [43]. Furthermore, multi-trait genomic prediction models were introduced, potentially improving the predictive accuracy for traits like dry matter yield and water-soluble carbohydrates in perennial ryegrass [44].

Integrating GS with high-throughput phenotyping and advanced statistical models offers a promising strategy for accelerating the breeding cycles and enhancing the genetic potential of forage crops. The review covered the status and potential future applications of GS in tropical forage crops. This review emphasized the critical role of utilizing genotyping, phenotyping, and envirotyping strategies to enhance the genetic gains. The authors discussed promising preliminary results from implementing GS in tropical forage crops. Additionally, they highlighted the importance of establishing adequately sized training populations [45]. The study also examined the application of imaging phenotyping and GS in advancing bromegrass breeding. The authors assessed the potential of these technologies to promote genetic gains and facilitate adaptation in future bromegrass breeding programs [46].

## 5. Genome Editing

Genome editing, particularly CRISPR/Cas9 technology, has undergone significant advancements in forage crop improvement. These advancements have demonstrated the potential to enhance traits, such as the biomass yield, forage quality, disease resistance, and stress tolerance, which are vital for the sustainability of livestock industries and bio-fuel production. A recent review provided a comprehensive summary of the advances in CRISPR/Cas9 technology for plant genome editing, particularly emphasizing its applications in forage crops and the technology's precision and efficiency. The progress in targeted mutagenesis across various forage crops was also evaluated, discussing the potential benefits and challenges associated with the use of CRISPR/Cas9 in forage breeding [47].

Robust transformation protocols have been developed for perennial ryegrass, facilitating advancements in functional genomics and breeding for this important grass species [48]. A chromosome-level, allele-aware genome assembly for cultivated alfalfa has been developed, alongside the establishment of an efficient CRISPR/Cas9-based genome editing protocol. This enables the precise induction of tetra-allelic mutations and the inheritance of traits without transgenes, potentially accelerating the molecular breeding efforts [49].

The high-efficiency gene editing of perennial ryegrass has been demonstrated using an *Agrobacterium*-mediated system with a single Cas9-sgRNA transcript. By controlling Cas9 and sgRNA expressions via the maize Ubiquitin 1 (*ZmUbi1*) promoter, a 29% editing efficiency for the *PHYTOENE DESATURASE* (*PDS*) was achieved, with most plants exhibiting bi-allelic mutations [50].

In alfalfa, the multiplexed CRISPR/Cas9-mediated mutagenesis of the *FLOWERING LOCUS Ta1* (*MsFTa1*) has been utilized to delay flowering, resulting in an enhanced forage biomass yield and quality. A polycistronic tRNA-gRNA system successfully introduced mutations, delaying flowering and increased the forage biomass yield by up to 78% in fresh weight and 76% in dry weight compared to those of the control plants. This method also led to a reduction in lignin content and an increase in crude protein and mineral contents, particularly in the plant stems, potentially enhancing forage digestibility and the nutritional value [51]. In sheepgrass (*Leymus chinensis* (Trin.) Tzvel.), an efficient *Agrobacterium*-mediated CRISPR/Cas9 genome editing platform has been developed. By targeting the *Teosinte Branched1* (*TB1*) gene, an increase in tiller number and biomass was observed, demonstrating the method's effectiveness for gene function studies and breeding in sheepgrass [52]. The study also investigated genome evolution and initial breeding in sheepgrass, suggesting that genome editing could play a pivotal role in enhancing the yield-related traits. The knockout of monocot-specific miR528 using CRISPR/Cas9 resulted in increases in the tiller number and growth rate, offering a framework for the genetic improvement of this species and potentially other Triticeae grasses [53].

In a follow-up study, the challenges of polyploidy in bahiagrass (*Paspalum notatum* Flügge) were addressed using CRISPR/Cas9 technology to target the magnesium chelatase (*MgCh*) gene, a key enzyme in chlorophyll biosynthesis. The study demonstrated a high rate of successful mutagenesis and the identification of progeny plants with consistent genetic edits, suggesting the potential of this technology to expedite the development of novel bahiagrass cultivars [54]. The effects of CRISPR/Cas9-mediated modifications on the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* (*MsSPL8*) in alfalfa revealed significant phenotypic changes, including reduced leaf sizes and earlier flowering [55]. These findings suggest that the spectrum of phenotypic expression is influenced by the degree of allelic mutations, indicating the variability in *MsSPL8*'s function depending on the genotype, environmental factors, or particular alleles. Furthermore, an in vitro regeneration protocol for sudangrass was developed in conjunction with the use of CRISPR/Cas9 to precisely edit the *Caffeic acid O-methyltransferase* (*COMT*) gene, which is crucial in lignin synthesis. This affects the digestibility of forage and the efficiency of bioethanol biomass conversion [56]. The successful modification of the *COMT* gene represents a significant advancement in improving the quality of forage and biomass in sudangrass, offering considerable benefits for agricultural biotechnology and the sustainability of bioenergy production.

The investigation into CRISPR/Cas9-mediated genome editing's potential to enhance disease resistance in alfalfa underscored its value as a precise genetic alteration tool in breeding. The procedures outlined for generating a single nonhomologous end-joining-derived indel at a targeted genomic locus demonstrate the method's precision and efficiency [57]. In a related study, CRISPR/Cas9 was used to knock out the isoflavone synthase gene in red clover, leading to significant reductions in isoflavone concentrations, without impacting nodulation, indicating its potential role in rhizosphere defense over rhizobial signaling [58].

## 6. The Integration of High-Throughput Phenotyping (HTP) and Deep Learning

High-throughput phenotyping (HTP) has emerged as a transformative approach in forage breeding, enabling the rapid and precise measurement of complex traits, such as the biomass yield. The integration of unmanned aerial vehicles (UAVs) and sophisticated machine learning techniques has proven pivotal in the recent advancements of HTP applications for forage crops.

Sharma et al. (2022) demonstrated the application of UAV-based remote sensing coupled with machine learning to estimate the above-ground biomass in oats. Using a UAV



equipped with a multispectral sensor (DJI Matrice 600 hexacopter, SZ DJI Technology Co., Ltd., Shenzhen, China; MicaSense RedEdge-MX camera, MicaSense, Inc., Seattle, WA, USA) over experimental fields, researchers calculated vegetation indices (VIs) and employed machine learning algorithms to develop biomass estimation models. Although the accuracy varied across various locations, the study highlighted the potential of integrating UAV remote sensing with machine learning for biomass estimation in oat breeding nurseries [59]. The predictability of alfalfa biomass was confirmed using UAV-based photogrammetry (DJI Phantom 4 Pro UAV, SZ DJI Technology Co., Shenzhen, China; Double 4 K Multi-Spectral Ag camera, Sentera Inc., St Paul, MN, USA) and automated plot segmentation. This study developed a predictive model based on multispectral camera images from a UAV, which accounted for a significant proportion of the biomass variation. This study also indicates that UAV-based HTP could improve the efficiency of biomass selection in alfalfa breeding [60]. Similarly, UAV-based imagery and spatial analysis were used in selecting alfalfa for herbage accumulation (HA). UAV imagery captured prior to each harvest enabled the calculation of various VIs, which exhibited high correlations with HA. Spatial analysis significantly improved genetic parameter estimation, demonstrating the effectiveness of HTP in alfalfa phenotyping [61]. The application of multispectral sensors and unmanned aerial systems (UAS) was also assessed for evaluating the biomass in perennial ryegrass field trials, identifying significant correlations between the biomass yield and Normalized Difference Vegetation Index (NDVI) [62]. These technologies demonstrated the potential in replacing visual scoring and serving as valuable proxies for yield estimation.

Deep learning applications have been extended to forage phenotyping, as demonstrated by de Oliveira et al. (2021), employing convolutional neural networks (CNNs) for estimating dry matter yield in a guineagrass breeding program. Several CNN architectures were evaluated, some yielding high-level heritability and genetic correlations between the observed and HTP-derived traits. Specifically, the pre-trained ResNeXt50 CNN architecture yielded optimal results for indirect selection [63]. The application of deep learning techniques and UAV-based RGB imagery for phenotyping biomass yield in forages was investigated. Comparative analysis of several CNN models revealed the AlexNet model to exhibit a high correlation and minimal mean absolute error in biomass estimation [64]. In mixed sward environments, Bateman et al. (2020) introduced an innovative CNN architecture, the local context network (LC-Net), achieving a high segmentation accuracy; however, its biomass estimation accuracy was limited, suggesting that incorporating additional data sources could enhance the accuracy [65].

Taken together, these studies suggest that HTP, enabled by UAV-based remote sensing and deep learning techniques, offers significant potential for improving both the efficiency and accuracy of biomass assessments in forage breeding programs. Although challenges, such as variable accuracy across different environments, persist, ongoing advancement in these technologies and methods are expected to further improve the capabilities of HTP in forage breeding programs.

## 7. The Future of Molecular Breeding in Forage Crops

The future of forage crop breeding lies in technological innovation and addressing pressing global challenges. Artificial intelligence (AI) is substantially advancing predictive plant breeding by facilitating the integration of complex multi-omics data through machine learning and deep learning techniques. This advancement enables the development of accurate predictive models of plants' performance. The concept of integrated genomic-enviromic prediction (iGEP) introduced by Xu et al. (2022) exemplifies this advancement by extending genomic prediction to encompass environmental data alongside genotypic and phenotypic information. This integration yields more refined phenotype predictions by considering the intricate interactions between the genotype, enviromic, and genotype–environment (G×E) interactions, which are crucial for successful plant breeding [66]. Furthermore, AI's capacity to manage and interpret the inherent complexity of multi-omics data has been demonstrated, leading to significant advancements in plant

phenotyping [67]. This integration promises to revolutionize the field by enabling more comprehensive analyses, improved prediction, and enhance the management of plant diseases and stress responses. As AI models evolve, they are poised to play a pivotal role in the developing new forage varieties tailored to the specific needs of diverse ecosystems and agricultural practices.

The introduction of CRISPR/Cas9 genome editing technology has prompted critical inquiries regarding biosafety, biodiversity, and the ownership of genetic resources, particularly with respect to precise genome editing in forage crops. The biosafety risks associated with CRISPR variants were explored, which include off-target effects and the unintended gene transfer to non-target organisms. This also proposes strategies for mitigating these risks and methods for the detection of edited genes [68]. Within the European Union context, the European Court of Justice's ruling underscored the necessity for a case-specific environmental risk assessment (ERA) for genome-edited plants. The ruling advocates integrating considerations of traits introduced by genome editing and method-related unintended effects into the ERA. This aims to provide specific guidance for a focused assessment and monitoring approach for genetically edited plants [69]. Such considerations are essential to ensure the biosafety and sustainability of agricultural practices involving genome-edited crops, including forage crops.

Climate change poses a significant threat to global food security, and forage crops are no exception. Rising temperatures, shifting precipitation patterns, and increased incidences of pest and disease necessitate developing more resilient forage crops varieties. Molecular breeding techniques, such as marker-assisted selection, genomic selection, and CRISPR-Cas9, serve as powerful tools to enhance forage crops' stress tolerance. Identifying and selecting the genes that confer resistance to biotic and abiotic stresses enables breeders to develop varieties more suited to thrive in changing climates [70].

In summary, the advancement of forage crop improvement is being shaped by significant advances in molecular breeding technologies and the integration of AI into predictive breeding. These innovations could significantly accelerate the development of forage varieties with an enhanced yield, nutritional value, and resilience to environmental stressors. However, these advancements also bring challenges. AI and machine learning stand to revolutionize breeding by analyzing complex data to enhance the performance. Ethical and regulatory frameworks need to be established for the governance of genome editing technologies such as CRISPR/Cas9 to ensure responsible application and the equitable distribution of benefits. Given the threat of climate change to food security, molecular breeding techniques, such as marker-assisted selection based on QTL mapping and GWASs, along with GS, are instrumental in developing stress-tolerant forage varieties. These methods enable breeders to identify and utilize the genes that confer resistance to stressors, thereby developing forage crops that are better equipped to fulfill the future agricultural demands.

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