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Integrating Pan-Genomics, CRISPR-Cas9, and Predictive Modelling for Next-Generation Alfalfa Cultivar Development

Muhammad Abu Bakar Ghalib[1], Ayesha Khawar[2] (Corresponding Author), Kashaf Ul Nissa Bilal[3], Muhammad Ahmed[4], Muhammad Shoaib Shabbir[5]

1. Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan, *abubakarghalibpbg@gmail.com*, *https://orcid.org/0009-0008-6132-5198*
2. Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan, *ayeshakhawar14@gmail.com*, *https://orcid.org/0009-0003-0449-5960*
3. Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan, *kashafulnissabilal@gmail.com*, *https://orcid.org/0009-0004-9367-1352*
4. Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan, *ahmedsiddque8022@gmail.com*, *https://orcid.org/0009-0003-3843-0057*
5. Department of Entomology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, *shoaibshabbir1025@gmail.com*, *https://orcid.org/0009-0003-5083-189X*

Abstract

Alfalfa (Medicago sativa L.) is a perennial forage legume, renowned for its wider adaptability and environmental benefits. However, conventional breeding is stagnant and less adaptive because of its autotetraploid genetic complexity. This paper highlights the significant impact of contemporary breeding technologies on accelerating alfalfa breeding. Also focuses on integrating modern breeding technology with the latest biotechnological innovations to ensure the successful improvement of alfalfa. Additionally, genomic data facilitate the identification of genetic loci associated with important agronomic traits, including biomass yield, fodder quality, and abiotic stress tolerance, through marker-assisted selection (MAS), genome-wide association studies (GWAS), and genomic selection (GS). Additionally, we investigate the use of genome editing, specifically CRISPR/Cas9, for targeted genetic enhancement. Combining multi-omics methodologies. Crucially, we stress the importance of integrating GWAS, high-throughput technologies, and machine learning (ML) and artificial intelligence (AI) algorithms to optimize outputs. This paper promotes an innovative, integrated approach to alfalfa breeding that combines predictive modelling, CRISPR-Cas9, and pan-genomics. This pipeline is essential for speeding the development of improved alfalfa to satisfy future agricultural demands by going beyond incremental increases to a systems-level strategy.

Keywords: CRISPR/CAS, Multi-omics, Proteomics, Marker-assisted breeding, Genome-wide association sequencing

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1-Introduction:

Medicago sativa, a perennial crop known as Queen of Forages (Alfalfa), which can thrive for years under the right climate conditions, can even survive for up to ten years in arid environments. It belongs to the Leguminosae family (Fernandez et al., 2019; Suwignyo et al., 2023). Alfalfa breeders have mostly concentrated on increasing disease and pest resistance, fodder and forage nutritional value, winter, and frost tolerance to prevent production loss. Since the 1940s, various germplasms have been mixed, allowing selection for resistance to numerous diseases and pests (Solozhentseva et al., 2021). Recently, GWAS has become a potent technique for determining the genetic foundation of complex plant traits, such as yield-related attributes, disease resistance, and salt tolerance (Alseekh et al., 2021). After analyzing 291 alfalfa accessions, it was found that 21 candidate genes and 49 single-nucleotide polymorphisms (SNPs) were strongly linked to salt tolerance. Likewise, researchers have discovered 33 important SNPs linked to salt tolerance in seedlings and seed germination (He et al., 2025). Additionally, a multi-parent breeding population was used to forecast GWAS efficiency using machine learning. Furthermore, incorporating machine learning methods like deep learning, random forests, and support vector machines has increased crop breeding forecast accuracy (Sayed et al., 2022).

In the past decade, developments in omics and multi-omics techniques such as environics, genomics, transcriptomics, proteomics, and metabolomics have paved the way for faster breeding to adapt to withstand climatic change and increase food supply. Several experiments have been conducted to upgrade alfalfa; however, thorough characterization and correlations between desirable qualities require additional genetic and molecular study (Hrbáčková et al., 2020). This review discusses the role of modern breeding, including genomics and multi-omics, with the use of machine learning in accelerating alfalfa output and nutritional quality by combining GWAS with AI technology, which can revolutionize crop breeding technology with greater efficiency. To advance sustainability, this aids in the precise introduction of climate-resilient cultivars and predictive design.

2-Challenges in Conventional Breeding:

Alfalfa's autotetraploid nature, which complicates the breeding cycle, is one of the limitations of traditional breeding. Due to the emphasis on increasing yield, alfalfa has a slow breeding cycle and less adaptable cultivars. However, because conventional breeding techniques typically take a long time to generate cultivars that are utilized on farms, it is not even accomplished using this method (Shi et al., 2017a). Furthermore, costly, multi-year field experiments are necessary to assess important traits like yield, winter hardiness, and long-term persistence, which considerably slow down genetic advancement and the creation of new varieties (Shi et al., 2017b).

3-Modern Breeding:

Modern programs as an alternative breeding approach are moving towards genotype-based breeding approaches such as marker-assisted selection (MAS) and genomic selection, which promote faster breeding cycles with fewer phenotypic evaluations (Jannink et al., 2010). For this, a population of alfalfa must be genotyped for a set of markers, followed by phenotyping for the desired traits (Hawkins & Yu, 2018). Molecular markers in alfalfa breeding typically use RAPD, SSR, and ISSR Markers (Heiba et al., 2023). The benefit in alfalfa breeding programs may also be increased by other marker applications, such as paternity testing and diversity screening during cultivar synthesis (Tlahig et al., 2025).

Genetic Mapping and QTL Identification:

Case studies have analyzed the understanding of genetic regulation (QTLs) of features of agronomic importance in cultivated tetraploid alfalfa. Usually, AFLPs and SSRs are convenient, easy-to-use PCR markers. Due to their mass revelation, AFLPs aid in covering the whole genome. SSRs are codominant, locus-specific, and portable; they primarily come from M. truncatula EST databases (Malik et al., 2022). On the high-density genetic linkage maps created in our earlier study, 48 significant QTLs were found. Nine major QTLs were found for biomass yield (one), plant height (one), CP (two), ASH (one), P (two), K (one), and Mg (one) that explained more than 10% of the phenotypic variance. Based on the RNA-seq analysis under drought conditions, 31 potential genes were found in the nine major QTL intervals. 22 functional protein candidates were ultimately found after Blast-P was used to screen potential genes governing drought resistance (Jiang et al., 2022). Standard QTL mapping involves constructing a mapping population using genetic markers, constructing linkage mapping, phenotyping, Qtl analysis using software for result prediction (JIANG et al., 2022).

Genetic Transformation and CRISPR technology:

M. truncatula has produced a compact, deeply sequenced, and well-annotated genome, as well as ESTs and the Medicago truncatula Gene Expression (Meng et al., 2017). CRISPR/Cas9-based gene editing has been utilized to alter single or multiple targets in M. truncatula (Zhu et al., 2021). In past years, research has shown that MsGA3ox1 gene editing resulted in semidwarf and prostrate alfalfa with numerous secondary and primary branches, leaf and stem ratio, and crude protein levels (Zheng et al., 2022). Researchers have created a highly efficient multiplex gRNA genome editing in alfalfa, which allows the production of homozygous mutants with deletion of the four allele copies in the T0 generation. This method could be applied to genome editing with complex genomes, particularly legume species (Wolabu et al., 2020).

Research outlines a genetic transformation method for introducing DNA into economically significant winter-hardy alfalfa breeding lines using Agrobacterium infection. Out of around 1000 genotypes across 11 breeding lines, three particularly regenerative genotypes have been chosen. The capacity to generate stable transgenic material was evaluated as combinations of bacterial strains (C58, A281, LBA4404), expression vectors (pGA482, pGA643, pBibKan), and genotypes (11.9, 8.8, 1.5). Southern hybridization, recall utilizing assays, and nptII-specific PCR amplification were used to further select putative transgenic plantlets. According to the predicted transformation probability, strain LBA4404 with genotype 11.9 and the vector pGA482 has a transformation efficiency of above 60% and at least 10% of the calluses still have the capacity to develop into embryos (Petolescu et al., 2024).

Genome-wide association sequencing (GWAS) in Alfalfa:

Genome-wide association studies (GWAS) help precisely locate single-nucleotide polymorphisms (SNPs). With the development of sequencing and genetic analysis methods of GWAS, in several alfalfa populations, a set of candidate SNP markers was identified to be responsible for critical quantitative traits (Biazzi et al., 2017; T. Zhang et al., 2015). Using 336 genotypes, GWAS conducted several critical alfalfa variables, including fibre-related traits and digestibility, crude protein and mineral concentrations, and nine biomass-related traits (Jia et al., 2017; Z. Wang et al., 2016). Using half-sib offspring produced from three cultivars, many SNPs linked to forage quality were found (Z. Wang et al., 2020). GWAS uses high-density markers across the entire genome to find genetic loci related to desired attributes (Scheben et al., 2017). It has successfully been used to identify and analyze DNA markers related to agronomic properties in alfalfa and its close relative, M. truncatula. GBS markers were used to create high-density linkage maps of M. sativa and M. truncatula (Alqudah et al., 2020; Yu et al., 2020).

Although many sequencing approaches have been utilized to identify important markers related to biomass and cell wall biosynthesis in M. truncatula, they have focused solely on drought resistance (Li et al., 2014; Yu et al., 2020; F. Zhang et al., 2020; T. Zhang et al., 2015), salt tolerance (Yu et al., 2016), and forage quality, with few focusing on genetic and molecular regulatory mechanisms underlying flowering time traits in alfalfa (Biazzi et al., 2017; He et al., 2022; Lin et al., 2020, 2021; Sakiroglu & Brummer, 2017).

Genetic Approaches for Alfalfa Improvement:

Alfalfa genetics study demonstrates the widespread use of molecular approaches for both fundamental and applied plant improvement. Genetic techniques have been employed in two primary areas: genomics and transgenesis. In genomics, the identification of genes of interest and their regulatory components was made possible by molecular markers, structural genomics, and functional genomics. As an alternative, the transgenic method entails introducing particular and beneficial genes into alfalfa to enhance the desired characteristics (S. Kumar, 2011; Y. Zhang & Wang, 2025a).

Multi-Omics Approaches in Alfalfa:

Multiomics approaches combine several biological data from the same samples, including transcriptomics, proteomics, metabolomics, and genomics, to provide efficient case studies to evaluate the results. To find outcomes more precisely, these combined datasets are evaluated utilizing sophisticated computer techniques.

Pan-Genomics:

Pan-genomics was first proposed in 2005 and has grown significantly in the last twenty years because of the introduction of PacBio and ONT platforms. In contrast to short insertions/deletions and SNPs, structural differences (PAVs) and (SVs) found by pan analysis play an important role in the analysis of complex features. Structural differences discovered during the pan-genome era prompt a review of phenotypic grounds. SVs have previously been linked to environmental changes (Cook et al., 2012; Sutton et al., 2007), flowering period(Nitcher et al., 2013; Würschum et al., 2015), stress tolerance (Gabur et al., 2019), and plant domestication features (Tan et al., 2008; Zhou et al., 2013) and dehiscence (Z. Lin et al., 2012). ("Multi-omics revolution to promote plant breeding efficiency") Breeders can increase genetic structure to improve crop cultivation in changing climates, and scientists can investigate functional alleles for different variations using novel methodology made easier by the comprehensive composition of genome heterogeneity described by genomes and pan-genomes (Della Coletta et al., 2021; James et al., 2021).

Table no 1:

This table explains the comparison of pan-genomics reference genomes in alfalfa over the decade. INDELs, SVs, and SNPs were used in the study. Results have explained that the PGGB pipeline followed by Minigraph-Cactus (1.6 GB) and minigraph (1.4 GB), according to the comparison (Kaur et al., 2024).

Features	Mini graph	Minigraph-Cactus	PGGB	Reference
Nodes Count	918,744	56,279,782	62,708,282	(Kaur et al., 2024)
Edges/Links Count	1,292,003	76,572,277	87,025,931	(Kaur et al., 2024)
Segments/Sequence Count	307,012	920,868	62,708,282	(Kaur et al., 2024)
Total Length (bp)	1,369,702,218	1,577,591,934	2,931,709,605	(Kaur et al., 2024
Run Time (hh: mm: ss)[b]	6:39:59	7:29:44	38:56:23	(Kaur et al., 2024)
Total variants count	163,292	10,049,082	13,839,006	(Kaur et al., 2024)
SNPs	0	6,535,280	9,682,418	(Kaur et al., 2024)
SVs and INDELs	163,292	4,098,991	4,156,588	(Kaur et al., 2024)

Transcriptomics:

Transcriptomic research contributed to our understanding of alfalfa tolerance to aphids, stripes, and nematodes. Aphids are severe insect pests that significantly reduce alfalfa productivity. A transcriptome analysis was performed on two alfalfa cultivars with varying aphid resistance levels. Defensive mechanisms of both cultivars relied heavily on genes involved in salicylic acid production. The resistance of alfalfa to insects, primarily aphids, was primarily determined by the activating genes involved in linoleic acid, which is essential for jasmonic acid and flavonoid biosynthesis. Genes involved with jasmonic acid production also have potential for alfalfa resistance to strip. Furthermore, genes involved in degradation, metabolism, and flavonoid production also contributed to the resistance (Tu et al., 2018).

Table no 2:

This table represents the candidate genes revealed through transcriptomics after water stress induced by PEG-6000. According to the data, drought stress strongly upregulates important genes in the plant hormone signalling pathway, especially those associated with ABA. This implies that these genes are essential for alfalfa's drought response, most likely through controlling osmotic balance and water use (K. Wang et al., 2024). This is a great starting point for candidate genes, but not validated, as there is no phenotypic data based on results.

Gene ID	$\log_2$FC	Gene description	Reference
MS. gene020647	10.06	Transcription factor MYB36	(K. Wang et al., 2024)
MS. gene058475	9.51	WRKY transcription factor 40	(K. Wang et al., 2024)
MS. gene93372	6.04	Abscisic acid receptor PYL4	(K. Wang et al., 2024)
MS. gene072046	3.47	Serine/threonine-protein kinase SRK2A	(K. Wang et al., 2024)
MS. gene012975	4.21	ABA-responsive element-binding protein	(K. Wang et al., 2024)

Proteomics and Metabolomics:

Over the last 20 years, alfalfa has sparked significant interest in proteomics and metabolomics. A significant effort was made to locate novel proteins and metabolites (Table 1) involved in alfalfa development and stress response (Hrbáčková et al., 2020).

Table no 3.

Metabolites involved in alfalfa during deacclimation. Key metabolites found in alfalfa during deacclimation, which cause metabolic changes, are discussed by Li et al. (2022):

SR.NO	WL440_2 vs WL440_1	ZD_2 vs ZD_1	ZD_1 vs WL440_1	ZD_2 vs WL440_2	Reference
All	107	74	91	81	(Li et al., 2022)
Upregulated	27	42	25	54	(Li et al., 2022)
Downregulated	80	32	66	27	(Li et al., 2022)
Annotated in KEGG	23	14	19	15	(Li et al., 2022)

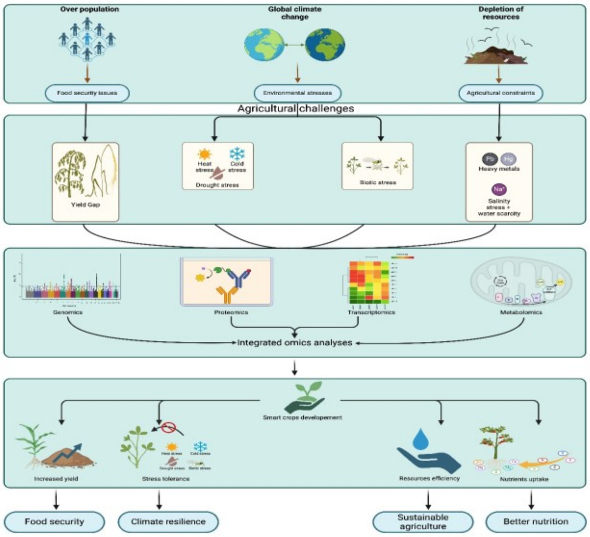

Figure 1. Described the Integration of multi-omics into modern breeding to promote sustainability in Alfalfa

High-Throughput Phenotyping in Alfalfa Breeding:

Remote sensing technologies, particularly high-throughput phenotyping, are becoming more commonly available, allowing for the screening of thousands of plants under varied conditions (White et al., 2012; Whitmire et al., 2021a). The development and use of high-throughput phenotyping technology, such as drones and ground-based sensors, can assist a variety of screening and breeding applications. These include assessing multiple accessions from germplasm collections for allele mining and quantifying slight differences in productivity between breeding lines (Furbank & Tester, 2011; Tattaris et al., 2016). Additional benefits of this and other sensor-based technologies include speedy data collection and processing, among other things (Cazenave et al., 2019). Using high-throughput sequencing technology, researchers discovered that salt stress affects many genes. Among the most affected were genes with known roles, such as DFR (DFR), transcription factor MYB59 (ERD), and IP5P2. The study discovered that salinity stress activated 86 transcription factors, including those from the GRAS, ARR, JUMONJI, and MYB families, which were selectively enhanced in the tolerant alfalfa cultivar (Postnikova et al., 2013).

The main issues in phenotyping of forage are the optimization of features that increase the productivity (Cheng et al., 2025).

(1) Interactions between genotypes and the environment

(2) Integrated HTP technologies

(3) Connecting Plant and Animal Sciences

(4) Sustainability and Environmental Impact

Role of Machine learning in Alfalfa Breeding:

Prediction of livestock feed is vital in almost everyone's daily lives, and numerous elements of data can be obtained. Combination with matching weather data can be utilized to train learning models for yield prediction and desired traits (Whitmire et al., 2021b). For this, we select the most regularly used ML models in the related area and the field, for the normal operation with the desired data set. We examined the effects of several feature selection techniques on machine learning (ML) models trained to forecast alfalfa production using yield data of various alfalfa varieties over several years in Kentucky and Georgia. Linear regression is one of the most frequent machine learning techniques that is commonly used as a baseline to compare the outcomes of other techniques in the field (Russell et al., 2016). Neural networks implement functions that can reduce the error between predictions and actual values (Rojas, 2013). Support vector machines (SVMs) are another strategy. However, this method can also locate nonlinear data (Sánchez et al., 2014). Both K-Nearest Neighbours and regression locate the data of productivity with great precision and compatibility (Jing et al., 2020; Whitmire et al., 2021a).Cross-validation was used in the development of linear regression, regression trees, support vector machines, neural networks, Bayesian regression, and k-nearest neighbours. The random forest and k-nearest neighbour techniques produced an average R value greater than 0.95 using these features. Even on basic datasets with a few variables, it demonstrates promise in crop yield prediction, and reporting accuracies in R and R2 provides an easy way to compare outcomes across different crops (Whitmire et al., 2021c). Recent research has focused on machine learning for crop prediction. Several machine learning techniques, including decision trees, support vector machines, and neural networks, have been examined by researchers to demonstrate how well they can forecast crop yields in various scenarios. These experiments highlight how ML may greatly improve yield prediction accuracy (Omar et al., 2025).

Discussion:

Various biotic and abiotic stressors, the integration of multi-omics data can improve the interpretation of gene functions and networks. Over the past few decades, several "omics" techniques have become effective technologies for plant systems. A new generation of omics, including proteomics, transcriptomics, and genomics, has been made possible by developments in next-generation sequencing (NGS). In agricultural research, however, metabolomics, ionomics, and phenomics have also been thoroughly studied. Growth, senescence, yield, and the reactions to biotic and abiotic stress in many crops have all been clarified by multi-omics approaches with high-throughput techniques (Shi et al., 2017c; Yang et al., 2021). The development of a molecular design paradigm for the "ideal alfalfa" requires the use of pangenomics, the clarification of self-incompatibility (SI) processes, the innovative de novo domestication method, and state-of-the-art intelligent breeding techniques (Y. Zhang & Wang, 2025b).

Compared to conventional breeding approaches, modern alfalfa breeding significantly accelerates the development of superior, resilient varieties by using genetic tools, which include DNA sequencing, gene editing, and HTP technology, to rapidly increase and select favourable breeding features. Using high-throughput phenotyping (HTP) is transforming alfalfa breeding by effectively screening huge breeding populations for important characteristics, including biomass, yield, and nutritional quality in a variety of settings. Another important and emerging technique known as Machine learning (ML) is increasingly being used to examine complex datasets.

Thus, we can find desired traits in alfalfa that can improve biomass formation, nutritional quality, blooming time, and resilience to different stresses by combining machine learning approaches with contemporary breeding technology.

Conclusion and Future Perspective:

To ensure faster breeding, new breeding techniques, including multi-omics, must be integrated for trait improvement. This integration is necessary to change alfalfa breeding from traditional breeding to intelligent breeding that guarantees a supercop that is climate resilient. This is crucial for creating a sustainable agriculture with food security since it is not only enhancing alfalfa breeding but also reexamining it in the context of a new era of predictive design.

To capture greater diversity, future studies will concentrate on increasing alfalfa breeding. We must integrate multi-omics data to functionally annotate these genes and their intricate relationships to close the gap between genotype and phenotype. This will make it possible to create more effective models and use multi-gene CRISPR techniques to add intricate features like nitrogen utilization efficiency. The primary goal for increased production should be multi-year field validation of modified cultivars, whose performance data will be transmitted back into the system, completing the loop and developing self-improving, predictive breeding.

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