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QTL mapping and candidate gene identification for fodder quality traits in Pearl millet

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Abstract

Background Pearl millet is an excellent forage crop with significant potential for forage production. Its fodder is rich in protein, calcium, phosphorus and other essential minerals while being low in undesirable components such as hydrocyanic acid and oxalic acid. Globally, the shortage of high-quality fodder poses challenges for maintaining animal health and productivity, ultimately impacting dairy farmers. Therefore, improving pearl millet for fodder traits should be a priority to meet the global demand for nutritious livestock feed.

Results Significant variability was observed for all forage quality related traits at both locations. A linkage map was constructed using 755 single-nucleotide polymorphisms (SNPs) markers, spanning a total length of 3080.44 cM. A total 8, 6 and 10 QTLs were identified for Ludhiana, Abohar and across the locations, respectively, for fodder quality. A common genomic interval with flanking markers S6_234379851- S6_64109715 was associated with IVOMD, CP and ME at all locations, with 10–34% phenotypic variance. Further, expression analysis identified BHLH 148, Resistance to phytophthora, Laccase 15, cytochrome P450, PLIM2c, GRF11, NEDD AXR1, NAC 92 and TF 089 as differentially expressed candidate genes in the leaf tissues of parental lines. A phylogenetic tree constructed using these genes revealed two clades identified with six paralogous events. Additionally, a phylogenetic tree of eight cereal species showed that the majority of shared similarity with the Pgl genes, suggestinga recent speciation event among them. Common genes, including cytochrome P450, PLIM2c, NEDD AXR1 and NAC domains were identified between QTL regions and expression analysis.

Conclusion The differentially expressed genes incorporating the regulatory elements governing the lignin pathway have direct or indirect effects on fodder digestibility and quality. Exploiting these factors can contribute to the direct improvement of fodder quality. The identified QTLs and candidate genes from this study could facilitate the development of gene based markers for fodder improvement.

Keywords Fodder, Digestibility, Gene expression, Cross taxa, QTL

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Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a major cereal crop widely cultivated in arid and semi-arid regions, primarily for its dual-purpose use as grain and fodder. It evolved under challenging conditions, endowing it with the capacity to thrive in adverse environments. It has one of the most efficient water-use systems among cereals, requiring significantly less water to achieve high biomass yield. Its extensive root system and C4 photosynthetic pathway enable efficient water absorption and retention and allow for optimal production even in high temperatures and drought conditions, positioning it as a model crop for drought adaptation [1]. This versatile crop serves as a primary source of sustenance and energy for impoverished people in developing nations and it is cultivated on approximately 30 million hectares globally [2]. It is an excellent forage crop with low anti-quality factors like hydrocyanic and oxalic acid [3]. Besides these factors, pearl millet is also referred to as a climate-resilient crop and is portrayed as a promising fodder in the face of changing climate [4]. In India alone, it occupies 6.7 million hectares, ranking as the fourth most cultivated crop following rice, wheat, and maize and contributing significantly to global production [5]. It is primarily cultivated as a fodder crop in many parts of the world such as the USA, Australia and South America. In America, forage hybrids have been cultivated in the southern region for many years, with landmark work accomplished there in developing genetic resources, identifying male sterility resources and deciphering the genetic basis of various traits [6].

In the future, the livestock sector is expected to play a significant role in agriculture in terms of added value. Consequently, it is growing at a much faster rate than other agricultural sectors [7]. As the global population continues to grow at a rapid pace, the demand for dairy products rises accordingly, placing additional pressure on the livestock sector to meet this demand. Unfortunately, future fodder availability is becoming increasingly uncertain due to climate change, land degradation, and competing demands on agricultural resources. Fodder shortages can significantly impact livestock production, threatening food security, livelihoods, and rural economies worldwide. As global populations grow and diets shift toward increased animal-based products [8, 9], the demand for high-quality fodder is expected to rise, placing even greater pressure on limited resources. Therefore, addressing the feeding needs of ruminants is essential to meet future demands.

The quality of fodder largely depends on its digestibility, which is determined by the three key cell wall components viz., cellulose, hemicellulose and lignin [10, 11]. These components are critical factors in fodder intake and digestibility [12]. Reduced fodder quality and availability can have significant impacts on livestock health and productivity. When access to high-quality fodder is limited, livestock may experience slower growth rates, reduced fertility, and lower milk yields. Consequently, meat and dairy production volumes could decline, affecting both supply and prices.

Overall, the cultivation of pearl millet as a fodder crop offers a promising pathway for sustainable livestock feed production under climate change pressures. By supporting soil health, optimizing water use, and delivering consistent nutritional value, pearl millet can play a critical role in safeguarding food and fodder security, thereby contributing to resilient agricultural system. Being an important climate change-ready fodder crop in arid and semi-arid regions, the genetic improvement for fodder quality-related traits is of paramount importance in pearl millet. The breeding for improved fodder hybrids/varieties with better quality is required to target the cell wall components and unravel the biochemical and genetic mechanism working behind it. Cell wall biosynthesis, controlled degradation and reassembly of cell wall polymers are dynamic processes involving multiple gene families [13, 14]. Currently, the regulatory genes governing fodder quality in pearl millet remain unclear, creating a critical knowledge gap that must be addressed to understand the genetics and molecular processes influencing fodder digestibility. Therefore, gaining a comprehensive understanding of these regulatory events could provide valuable insights into the factors determining fodder quality.

This study aims to map quantitative trait loci (QTLs) using a bi-parental mapping population and identify candidate genes linked to fodder quality by quantifying tissue-specific expression through cross-taxa genes. Despite the limited genomic resources directly associated with fodder quality in pearl millet, valuable insights can be drawn from the characterization of genes linked to fodder quality traits in major fodder crops such as maize and sorghum [11, 15, 16]. These insights provide a foundation for developing transformative genomics resources in pearl millet. Therefore, the findings from this study, in the form of QTLs and candidate genes regulating fodder quality, will contribute to the development of improved lines with high fodder quality and biomass, thereby contributing to the growing demand for high-quality fodder in arid and semi-arid regions.

Materials and methods

Plant population and field experiment

A total of 45 lines (Table S1) representing global variation for fodder related traits and parents of 23 bi-parental populations (one common parent shared between two populations) were evaluated in three replications, a three-row plot in an alpha lattice design during the rainy season

2020 at Ludhiana for agronomy, yield, biomass and forage quality related traits. These genotypes were assessed in a pairwise manner. Based on the extent of contrast for the mentioned traits, the best mapping population (Jakhrana S8-28-2-P4 x RIB 335/74-P1) was selected for multilocation phenotyping to map fodder quality related QTLs.

The bi-parental mapping population was developed by crossing Jakhrana S8-28-2-P4 (a landrace from Rajasthan, with low fodder quality) with RIB 335/74-P1 (originating from Durgapur, Jaipur, Rajasthan and has high fodder quality). This population comprised 274 lines and was generation advanced to the F_{10} using the Single Seed Descent method at ICRISAT used for QTL mapping. The field experiment was conducted in an alpha lattice design with two replications at two locations in Punjab viz., Forage, Millets and Nutrition farms of Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana (Latitude: 30.9041° N, longitude: 75.8066° E) and J.C. Bakshi Regional Research Station PAU, Abohar (Latitude: 30.1734° N, longitude: 74.2080° E) in the rainy season of 2021. These locations represent two distinct agroclimatic zones (Ludhiana- Central Plain Zone and Abohar- Western Plain Zone). The experiment was carried out with a total of 273 lines + 2 parents and 1 check (PAC-981) with a row-to-row spacing of 30 cm and plant-to-plant distance of 10 cm according to the standard pearl millet fodder cultivation practices. Each plot was sown in triple rows having a 2m length. The expression analysis experiment was conducted at the Forage, millets and nutrition farm of Punjab Agricultural University, where the mapping population parents (Jakhrana S8-28-2-P4 and RIB 335/74-P1) and PAC-981 as a standard check were sown in three replications.

Analysis of fodder quality

In vitro organic matter digestibility was calculated according to Menke et al. [17]. Fodder samples were sun-dried for one week, oven dried and finely ground. From each sample, 375 mg was weighed and placed into 100 ml calibrated glass syringes, ensuring no material adhered to the syringe walls. Petroleum jelly was applied to the piston before insertion. Each sample was prepared in triplicate and incubated at 39 °C. To each syringe, 30 ml of the strained rumen liquor buffer solution was added, and the contents were gently mixed by shaking. Syringes were kept in a water bath at 39 °C, with swirling at regular intervals for the initial few hours, reading of Gas volume was taken at 8 and 24 h. After incubation, the contents were centrifuged, which was further used for ash and NDF estimation. IVOMD was then calculated according to [18].

For acid detergent fibre, one gram of the sample was placed in a beaker and mixed with 100 ml of acid detergent solution. For neutral detergent fibre, 0.5 g of finely ground sample was placed in a beaker, and 50 ml of neutral detergent solution was added and the mixture was then refluxed for one hour. Each mixture was then filtered through pre-weighed sintered glass crucibles (G-l), washed with hot water, and rinsed with acetone. The residue was dried overnight in a hot air oven at 80 °C. ADF and NDF content were calculated as the weight difference between the empty crucible and the crucible with residue, expressed as a percentage on a dry matter basis [19]. Crude protein, the Macro-Kjeldahl method was used to estimate the N content [20]. CP (%) content was calculated by multiplying the nitrogen by 6.25. Metabolizable Energy was calculated by the equation given by Menke et al. [17]. as follows:

$$ME\ (kg) = 1.24 + 0.146\,G\left(\frac{ml}{200}mg\ DM\right)$$

$$+ 0.007\,CP + 0.0244\,EE$$

Where,

ME = Metabolizable energy, MJ/kg DM.
G = Net gas production, ml/200 mg DM.
CP = Crude protein, g/kg.
EE = Ether extract, g/kg.

Statistical analysis

The recorded data was analysed in R software using the metan and agricolae packages for the Analysis of variance [21], broad sense heritability [22], mean, range, coefficient of variance, and correlation of coefficient [22] analysis.

Genomic DNA extraction, genotyping and QTL mapping

Genomic DNA was extracted by the CTAB method [23]. The RIL mapping population segregating for fodder quality-related traits was genotyped via a genotyping by sequencing (GBS) approach, with RILs and parents sequenced using Illumina Nextseq 500TM at ICRISAT, Hyderabad. The FASTX Toolkit (version 0.0.13) was then used to separate samples from combined data based on barcodes and FastQC was used for Quality Check. Parameters such as base quality score distribution, sequence quality score distribution, average base content per read and GC distribution were assessed. Reads were aligned to the reference genome using the MEM algorithm of BWA (version 0.7.5). Variants were called with the UGBS-GATK pipeline (version v3.6, https://gatk.broadinstitute.org/hc/en-us). Variant screening was performed using vcf tools. Further, Linkage map analysis was performed using JoinMap (version 3.0) with the regression mapping algorithm, followed by rippling with a window size of 1.0. Markers were grouped into 7 Linkage groups. Phenotypic and genotypic data generated

from the RIL population were then used for QTL analysis using composite interval mapping (CIM) in QTL cartographer software.

Expression analysis and in Silico protein analysis

The gene associated with fodder quality, previously characterized in species viz., *Sorghum bicolor*, *Medicago sativa*, *Triticum aestivum* and *Zea mays* were retrieved from the NCBI database. These sequences were analysed using BLASTn (at e-value 10^{-10}) against the pearl millet genome [24] to identify homologs. Corresponding protein sequences were obtained with the Expasy translate tool (https://web.expasy.org/translate/), and conserved domains were identified via Motif Search (https://www.genome.jp/tools/motif/) [25]. The identified fodder qual ity-related genes were mapped to their respective chromosomes based on the data from the pearl millet genome database (https://cegresources.icrisat.org/datapublic/PearlMillet_Genome/). Gene structures, including exons, introns and UTRs were obtained from the gene structure display server (https://gsds.cbi.pku.edu.cn). Additio nally, properties like molecular weight (MW), isoelectric point (pI), and grand average of hydropathy (GRAVY) were analysed using the Expasy's ProtParam tool [26], while phosphorylation sites and subcellular localization were determined with Expasy's NetPhosK3 [27] and WOLFPSORT program (https://wolfpsort.org/) [28], respectively.

In Silico prediction of cis-regulatory elements and phylogenetic analysis

The Plant PAN software [29] was used to predict *cis*-acting elements in the 2000 bp genomic sequences upstream of the start codon in targeted genes. A maximum likelihood tree was constructed using protein sequences from fodder quality-related genes in pearl millet and other cereals like *Setaria italica*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum*, *Hordeum vulguare*, *Setaria viridus* and *Zea mays* with MEGA software [30], applying Poisson correction, pairwise deletion, and bootstrap value (1000 replicates) permutation.

qRT-PCR analysis

The tissue samples for RNA isolation were collected at the vegetative, reproductive and mature grain stages. Flag leaf and root tissues were harvested at the vegetative stage, then again at the reproductive stage, and mature grain tissue at maturity. Each sample was immediately frozen in liquid nitrogen and stored at − 80 °C. RNA was extracted from the tissues using Takara RNAiso plus extraction kit and 2.5 µl of RNA (5 µg concentration) was converted to cDNA with a first-strand cDNA synthesis kit (Thermo Scientific) for qRT-PCR analysis of pearl millet fodder quality genes. The cDNA template was diluted with nuclease-free water (1:10).

Gene expression analysis was conducted with 16 gene-specific primers (Table S2), and one reference gene, elongation factor 1-alpha (EF-1α) [31] for normalization. Using 96-well PCR plates, the SYBR Green PCR master mix (Applied Biosystems, CA, USA) was used for gene expression analysis according to the manufacturer's instructions. Amplification was performed on an ABI step one real-time PCR system (Applied Biosystems, USA) with the following thermal cycling conditions; cycle 1 at 95 °C for 5 min, followed by 40 cycles at a temperature of 95 °C for 45s and 55 °C for 45s min (variable based on primers gradient temperatures) and 72^0C for 45s. The amplicon dissociation curves were recorded with a fluorescent lamp after the 40th cycle by heating from 58 to 95 °C within 20 min. Three biological replicates were taken in addition to two technical replicates each time. The relative expression values of 16 genes in five tissues were calculated using Stepone Plus software. The Cq values recorded from real-time PCR instruments were imported into a spreadsheet. The mean Cq values and standard deviations for both the target genes and internal control genes were determined. The fold change in the target genes was calculated for each sample using the Eq. 2 -ΔΔCq [32].

QTLs annotation and identification of common genes

QTL intervals were scanned to retrieve the genes by using reference genome assembly [24]. These genes were then screened to filter out those related to fodder quality, and the appropriate genes were selected. We also identified common genes between the key candidate genes from the expression analysis experiment and the selected genes from the QTL intervals.

Results

Variability in the mapping population and correlation studies

The analysis of variance revealed highly significant genotypic variances for all fodder-related traits at both locations. Significant differences ($p < 0.01$) were observed among lines within the RIL population for all investigated traits (Tables 1 and 2, 3). The genotypes ability to exhibit differential performance across environments was evident from the significant genotype and environment interactions observed for all traits except ME. Moreover, the mapping population exhibited substantial variation for fodder quality traits, with the widest range observed for IVOMD (41.49–69.60, 43.50-75.51, and 43.49–70.48%) at Ludhiana, Abohar, and across environments, respectively, followed by NDF (48.39–71.82, 42.99–90.94, and 46.72–80.54%). ME displayed the narrowest range (7.34–10.73, 7.85–11.49, and 7.73–10.84 MJ/kg) at these

Table 1 Genetic parameters for traits related to fodder quality in a mapping population, observed across Ludhiana (LDH), Abohar (ABH) and pooled over the locations

Trait		Mean	Range	CV%	Heritability
IVOMD	Ludhiana	56.62 ± 0.21	41.49–69.60	0.96	0.98
	Abohar	59.56 ± 0.21	43.50-75.51	0.85	0.99
	Pooled	58.07 ± 0.8	43.49–70.48	0.90	0.15
ADF	Ludhiana	40.23 ± 0.18	30.36–57.10	1.04	0.99
	Abohar	39.75 ± 0.18	29.44–57.11	2.08	0.96
	Pooled	39.99 ± 0.13	31.17–54.60	1.64	0.50
NDF	Ludhiana	61.85 ± 0.22	48.39–71.82	2.07	0.93
	Abohar	57.04 ± 0.25	42.99–90.94	1.05	0.99
	Pooled	59.42 ± 0.18	46.73–80.54	1.67	0.31
ME	Ludhiana	9.21 ± 0.03	7.34–10.73	5.77	0.42
	Abohar	9.48 ± 0.03	7.85–11.49	4.17	0.67
	Pooled	9.34 ± 0.02	7.73–10.84	5.01	0.53
CP	Ludhiana	9.01 ± 0.04	6.52–11.31	7.05	0.40
	Abohar	9.15 ± 0.04	6.88–11.80	6.05	0.63
	Pooled	9.08 ± 0.03	6.96–11.25	6.56	0.47

IVOMD- *In vitro* Organic Matter Digestibility, ADF-Acid Detergent Fibre (ADF), NDF-Neutral Detergent Fibre, ME- Metabolizable Energy, CP-Crude Protein

respective locations. Additionally, the genotype x environment variance was lower than the genotypic variance, indicating predominant genetic control with minimal environmental influence on these traits. The mean performance of all traits was higher in the parents and the check at Ludhiana, while the population exhibited better performance at Abohar. High heritability was observed for IVOMD (0.98 and 0.99), NDF (0.93 and 0.99), and ADF (0.99 and 0.96), with moderate heritability for CP (0.40 and 0.63) and ME (0.42 and 0.67) at both Ludhiana and Abohar. Across locations, heritability was generally moderate for all traits except for IVOMD (0.15), which showed low heritability (Table 1). IVOMD was positively correlated with CP (0.63) and ME (0.65), while negative correlations were observed with NDF (-0.62) and ADF (-0.64) (Table 4), which is desirable for high quality fodder since NDF and ADF are anti-quality parameters.

Table 4 Correlation of different fodder quality traits

	IVOMD	ADF	NDF	ME	CP
IVOMD	1	-0.64***	-0.62***	0.65***	0.63***
ADF		1	0.63***	-0.41***	-0.41***
NDF			1	-0.44***	-0.44***
ME				1	0.97***
CP					1

IVOMD- *In vitro* Organic Matter Digestibility, ADF-Acid Detergent Fibre (ADF), NDF-Neutral Detergent Fibre, ME- Metabolizable Energy, CP-Crude Protein

Fodder quality analysis

The estimation of fodder quality-related traits viz., IVOMD, NDF, ADF, CP and ME was conducted in the parental lines of the RIL mapping population, Jakhrana S8-28-2-P4 and RIB 335/74-P1 along with the standard check cultivar, PAC-981, at three growth stages: active vegetative stage, reproductive stage and mature grain. Analysis showed that Jakhrana was an inferior parent for fodder digestibility, while RIB was superior. The check cultivar performed better than both parents during the active vegetative stage and reproductive stages, however, at the mature grain stage, fodder digestibility fluctuated, with values observed to be lower than those of both parents (Fig. 1).

Gene expression profiles in different tissues in contrasting genotypes

The expression levels of genes governing fodder quality, identified across different taxa were quantified by qRT-PCR in various tissues taken in a spatiotemporal manner to understand their role in cell wall formation and lignification. The gene expression levels at different developmental stages of three genotypes Jakhrana, RIB and

Table 2 Individual ANOVA for fodder quality related traits

Sources	DF	Ludh_IVOMD	AbH_IVOMD	Ludh_ADF	AbH_ADF	Ludh_NDF	AbH_NDF	Ludh_ME	AbH_ME	Ludh_CP	AbH_CP
Genotype	276	56.33***	57.2***	41.29***	43.30***	65.37***	83.79***	0.69***	0.77***	1.13***	1.31***
Replication	1	155	126.57	130	133.87	84.8	131.31	119	119.01	224	183
Block	44	0.436	0.17	0.168	0.67	1.68	0.28	0.12	0.093	0.891	0.21

IVOMD- *In vitro* Organic Matter Digestibility, ADF-Acid Detergent Fibre (ADF), NDF-Neutral Detergent Fibre, ME- Metabolizable Energy, CP-Crude Protein

* Significant at 0.05, ** Significant at 0.01, ***Significant at 0.001

Table 3 Pooled ANOVA for fodder quality related traits

Source	DF	IVOMD_pooled	ADF_pooled	NDF_pooled	ME_pooled	CP_pooled
Replication (Environment)	2	140.90**	131.89**	108.07**	118.86**	203.33**
Genotype	276	104.02**	64.20**	87.32**	1.25**	2.00**
Environment	1	2079.05**	56.59**	4106.26**	26.96**	6.88**
Gen: Env	276	9.59**	20.39**	61.84**	0.21	0.44**
Error	552	0.28	0.43	0.97	0.21	0.35

IVOMD- *In vitro* Organic Matter Digestibility, ADF-Acid Detergent Fibre (ADF), NDF-Neutral Detergent Fibre, ME- Metabolizable Energy, CP-Crude Protein

* Significant at 0.05, ** Significant at 0.01, ***Significant at 0.001

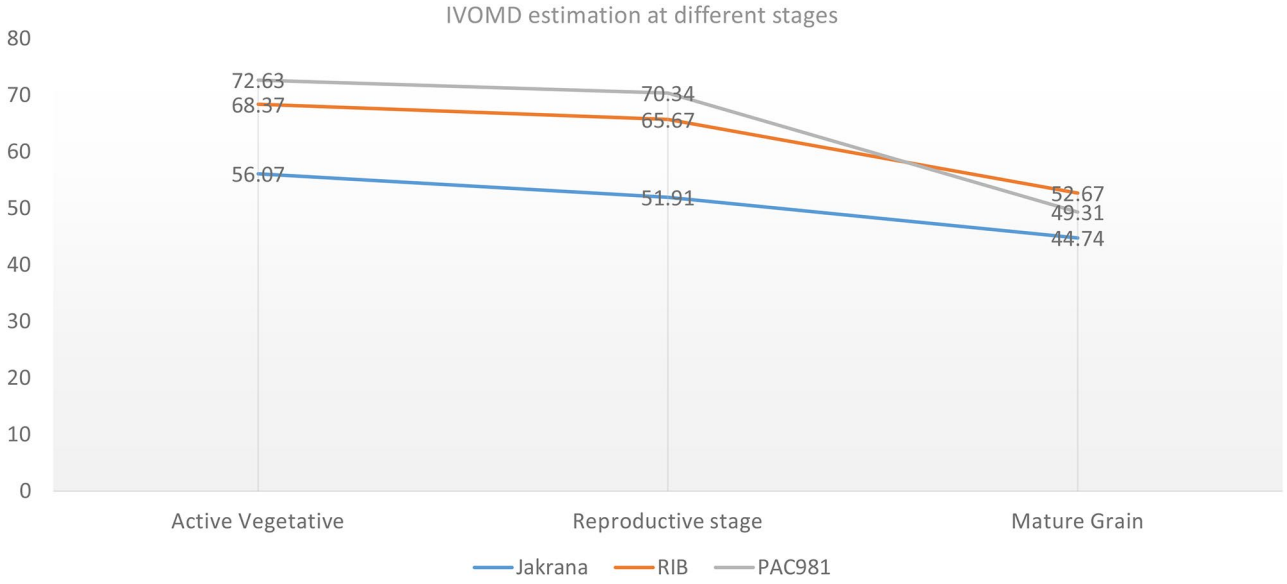

Fig. 1 Fodder digestibility analysis

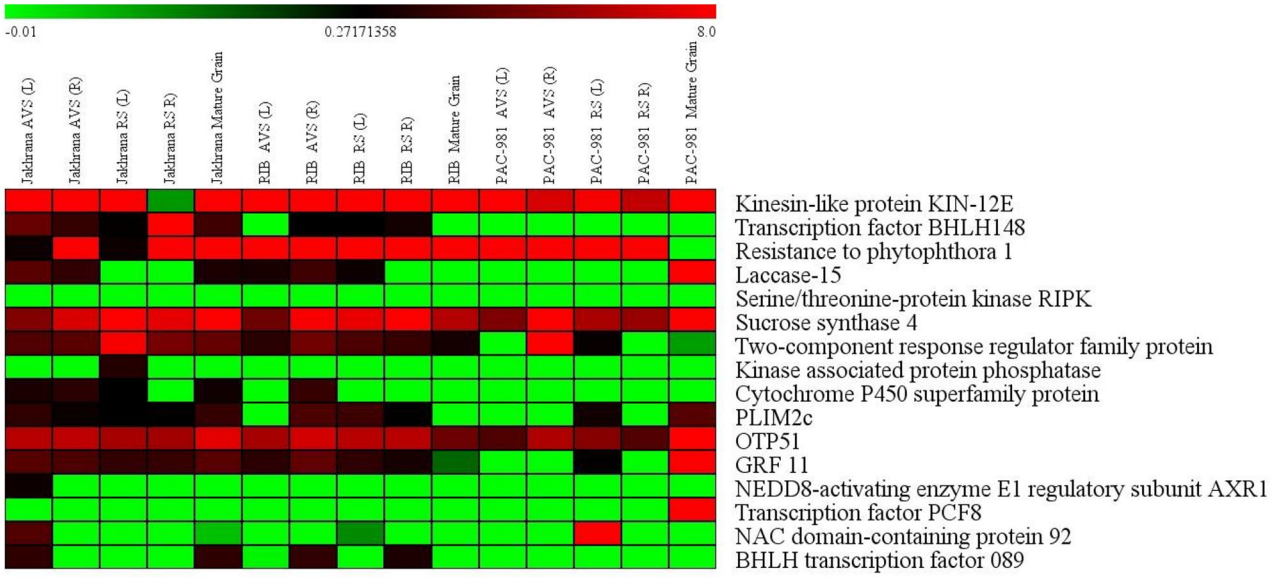

Fig. 2 Heat map of gene expression of fodder quality related genes in parents and check

PAC-981(Check) were visualized using Multiexperiment viewer v4.9.0 (Fig. 2). Gene expression analysis revealed varying patterns, as in the case of AVS (L) most key candidate genes showed higher expression in Jakhrana. However, at the RS (L) stage, RIB exhibited higher expression of all these key candidate genes compared to Jakhrana. Six genes demonstrated a contrasting expression pattern, showing downregulation in Jakhrana and upregulation in RIB. These genes included G3 (*Resistance to phytophthora 1*), G4 (*laccase-15*), G10 (*PLIM 2c*), G12 (*GRF 11*), G15 (*NAC domain-containing protein 92*), G16 (*BHLH transcription factor 089*). No key gene with differential expression has been observed in AVS (R), RS (R) and MG tissues sampled at various stages. Of the genes analysed (Table 5), nine showed differential expression between parent genotypes, suggesting their pivotal role as key candidates for fodder digestibility. The variable expression patterns of these nine genes were displayed using Circos plots for AVS (L) (Fig. 3) and RS (L) in (Fig. 4).

Construction of linkage map and QTL mapping

In the present investigation, QTL analysis was conducted to identify genomic regions associated with fodder quality traits. The mapping population was genotyped using the GBS approach, resulting in a data range of 61.52–738.8 Mb per sample, with an average of 264.55 Mb/

Table 5 Cross taxa candidate genes used for the expression analysis

S.No	Gene	Transcript	Function	Crop
1	Sorghum bicolor kinesin-like protein KIN-12E	Pgl_GLEAN_10024329	Involved in signal cascade for nitrogen metabolism in plants, during secondary growth of fiber cells the kinesin protein is reported to be involved in the deposition of cellulose	Sorghum
2	Sorghum bicolor transcription factor BHLH148	Pgl_GLEAN_10019519	Regulate the biosynthesis of secondary walls, cellulose and lignin biosynthesis	Sorghum
3	Resistance to phytophthora 1	Pgl_GLEAN_10021401	Barrier against pathogens and herbivory and characterized to be involve in positive regulation of hydrogen peroxidase biosynthetic process which involves in lignification	Sorghum
4	Sorghum bicolor laccase-15	Pgl_GLEAN_10006570	Lignin synthesis, Lignin degradation and detoxification of lignin-derived products	Sorghum
5	Sorghum bicolor serine/threonine-protein kinase RIPK		Major role in the signal cascade for nitrogen metabolism in plants	Sorghum
6	Sorghum bicolor sucrose synthase 4	Pgl_GLEAN_10008136	Integral component of the cellulose synthesis mechanism	Sorghum
7	Zea mays Putative two-component response regulator family protein	Pgl_GLEAN_10003307	Vital for organizing the response to changes in environmental conditions, nutrients, oxygen, light, and osmotic pressure	Maize
8	Zea mays kinase associated protein phosphatase	Pgl_GLEAN_10035097	Cell wall formation	Maize
9	Zea mays putative cytochrome P450 superfamily protein	Pgl_GLEAN_10035752	Catalyses the hydroxylation reaction of the aromatic rings of p-coumaric acid and convert it to caffeic acid. In lignin pathway C3H provides watershed between the non-methoxylated p-hydroxyphenyl (H) branch and guaiacyl (G)/syringyl (S) branch	Maize
10	PLIM2c	Pgl_GLEAN_10029302	Regulation of cell wall synthesis	Sorghum
11	OTP51	Pgl_GLEAN_10035040	PPR proteins targets the organelles and reported to be targeting the both mitochondria and plastid, plays vital role in coordination of gene expression in both organelles	Sorghum
12	GRF 11	Pgl_GLEAN_10003181	Regulation of cell wall synthesis	Sorghum
13	Zea mays NEDD8-activating enzyme E1 regulatory subunit AXR1	Pgl_GLEAN_10030016	Play crucial role in multiple cell and organ development processes, supporting cell structure, stability, and transportation of cell wall components in plants and involved in leaf morphogenesis.	Maize
14	Sorghum bicolor transcription factor PCF8	Pgl_GLEAN_10034338	Higher transcripts during fiber initiation and elongation in G. hirsutum	Maize
15	Zea mays NAC domain-containing protein 92	Pgl_GLEAN_10031107	Role in secondary wall biosynthesis and deposition in different tissues	Maize
16	Zea mays BHLH transcription factor 089	Pgl_GLEAN_10012884	Regulate the biosynthesis of secondary walls, cellulose and lignin biosynthesis	Maize

sample. A total of 781,968 raw single nucleotide polymorphisms (SNPs) were detected between the parents, of which 767,000 SNPs were biallelic. After applying a missing data filtration criterion of 0.8%, 95,000 SNPs were retained. Subsequently, 755 markers were selected through filtration based on a distortion *p*-value threshold of 0.01, using Chi-square analysis for the RIL population, resulting in the formation of 7 linkage groups (LG). The highest number of polymorphic SNP markers (222) was observed on LG4, while the lowest number (68) was on LG6. The total length of the genetic map was 3080.44 cM, with LG4 exhibiting the longest map length (748.08 cM) and LG3 the shortest (290.34 cM). LG3 showed the densest distribution of SNPs with a 2.93 cM distance interval, while LG2 had the lowest density at 5.64 cM.

In the QTL analysis for fodder quality traits at Ludhiana (Table 6; Fig. 7), two QTLs (qIVOMD_Ludh6.1 and qIVOMD_Ludh6.2) were identified on LG6 for IVOMD, with LOD scores of 6.6, 3.4 and phenotypic variances of 38%, 12%, respectively. For NDF, one QTL (qNDF_Ludh3) was mapped on LG3 with a LOD of 3.3 and a phenotypic variance of 9%. For ME, three QTLs were identified on LG6, LG5, and LG4 with LOD scores of 3.4, 3.1, and 3.0 and phenotypic variances of 26%, 13%, and 11%, respectively—all associated with the donor parent (better parent). Two QTLs for CP were mapped on LG6 and LG2 with LOD scores of 3.4, and 3.6 and phenotypic variances of 21%, and 11%, respectively, both from the donor parent.

In Abohar, two QTLs qIVOMD_Abh6 and qIVOMD_Abh4 were identified on LG6 and LG4, respectively, for IVOMD with phenotypic variances of 24% and 9%, both originating from the donor parent. For ADF, qADF_Abh4 was detected on LG4 with a phenotypic variance of 13%, attributed to the recipient parent. QTLs qNDF_Abh6 and qNDF_Abh4 were identified on LG6 and LG4,

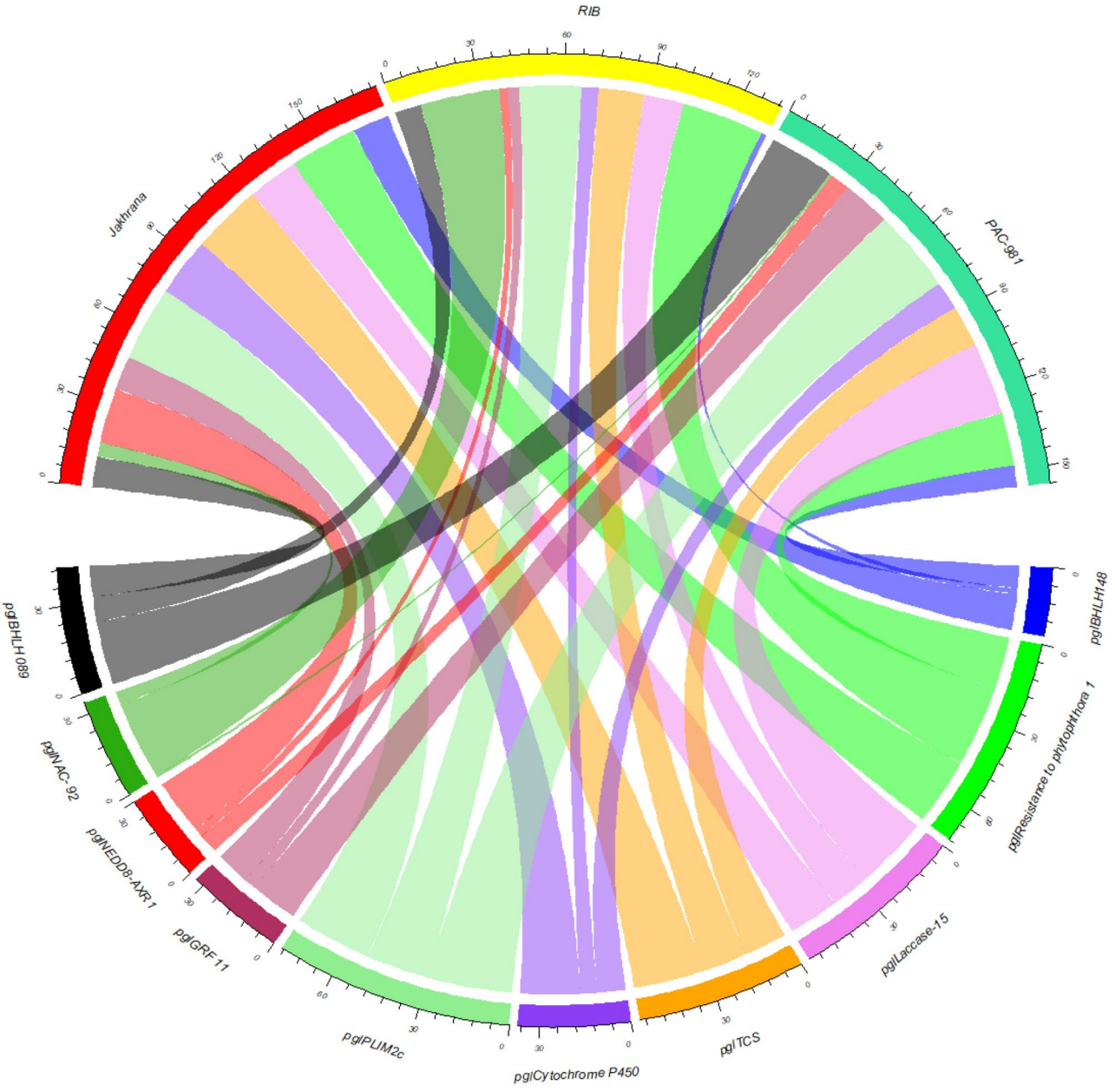

Fig. 3 CIRCOS of key candidate genes in Active Vegetative Stage (Leaf Tissue)

respectively, with phenotypic variances of 38% and 32%, both originating from the recipient parent. Additionally, qME_Abh6 was detected for ME on LG6 with a LOD score of 3.0 and a phenotypic variance of 22%, stemming from the donor parent. Across locations, qIVOMD_Ov6 was identified on LG6 for IVOMD with a phenotypic variance of 33%, also originating from the donor parent. For ADF, two QTLs were identified on LG6 and LG4 and for NDF, two QTLs were found on LG6 and LG4, all attributed to the recipient parent. Finally, several QTLs on LG4, LG5, and LG6 were associated with CP, originating from the donor parent with phenotypic variances ranging from 10 to 21%.

In Silico identification of fodder quality-related genes

A total of 51 genes associated with fodder quality traits (IVOMD, ADF, NDF, CP, and ME) were collected from various plant species—comprising 10 from *Zea mays*, 14 from *Sorghum bicolor*, and 27 from *Medicago sativa*—through retrieval from NCBI, MaizeGDB, and plantGDB databases. Subsequently, a BLAST analysis was conducted against the pearl millet genome, leading to the identification of 16 homologous genes. These homologs

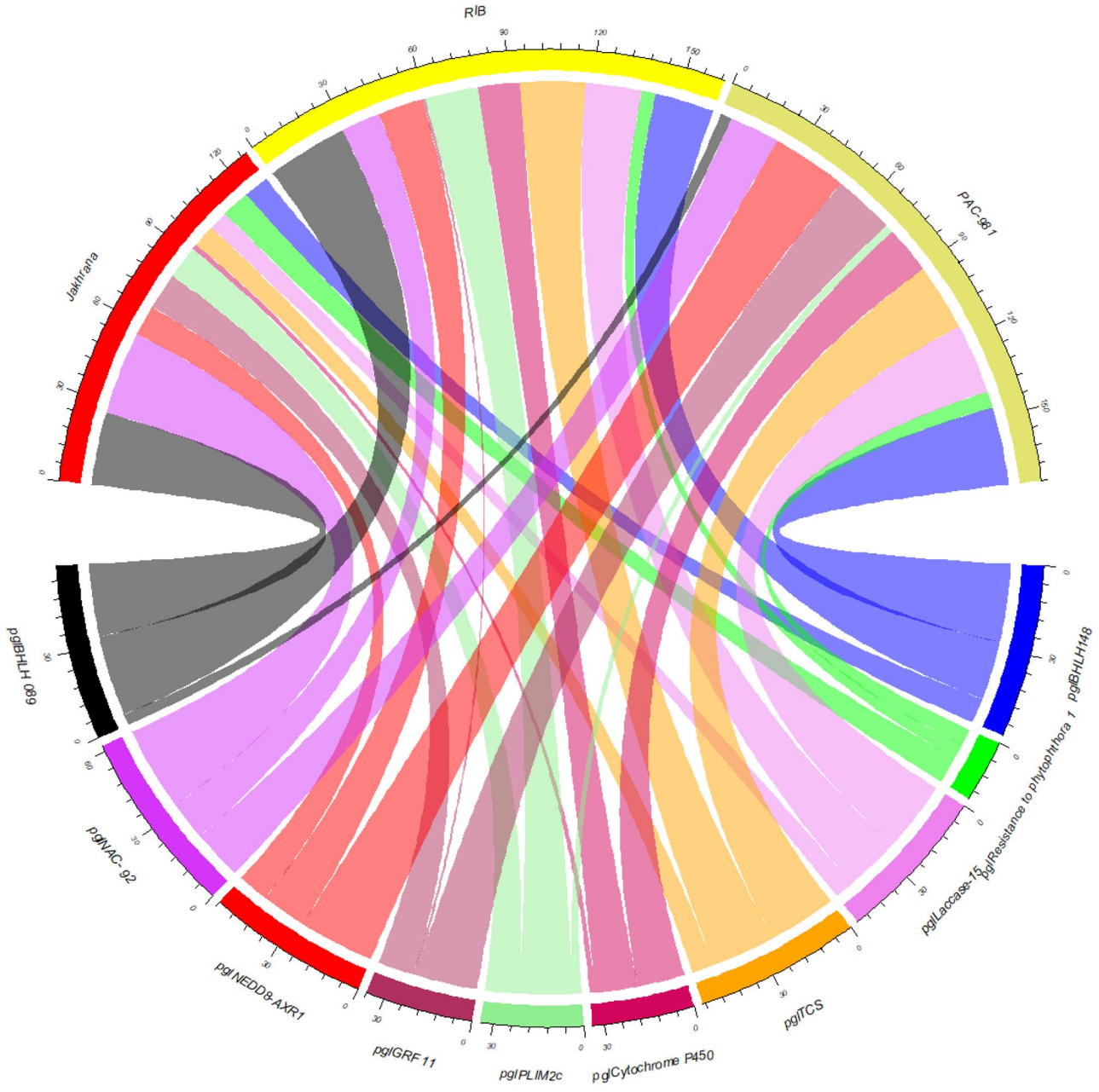

Fig. 4 CIRCOS of key candidate genes in Reproductive Stage (Leaf Tissue)

were found to be distributed across multiple chromosomes in pearl millet, with chromosome 7 harbouring the highest count of six genes, followed by chromosome 5 with four genes, and chromosome 3 with three genes. Chromosomes 1 and 2 each contain two genes. Gene structures analysis revealed that all identified genes exhibited a combination of introns and exons, with the intron range spanning from 1 (for genes such as *pgl_OTP 51*, *pgl_BHLH 148*, *pgl_PCF8*) to 29 (for *pgl_KIN 12-E*). Furthermore, homologs of these identified genes were located in *Setaria italica*, *Sorghum bicolor*, *Oryza sativa*, *Zea mays*, *Hordeum vulgare*, *Triticum aestivum*, and *Setaria viridis*, which were subsequently used to construct the phylogenetic relationships.

Sequence and conserved motif analysis of fodder quality genes

The number of amino acids in the proteins ranged between 600 (*Pgl_PLIM2c*) and 5187 (*Pgl_KIN-12E*); and the pI ranged from 4.78 (*Pgl_KIN-12E*) to 5.11 (*Pgl_Resistance to phytophthora 1*). Out of 16 proteins, 9 were found acidic and the remaining were basic. The molecular weight varied between 49358.58 (*Pgl_PLIM2c*) and 419206.9 (*Pgl_KIN-12E*) Dalton's. All proteins were

Table 6 List of QTLs mapped for fodder quality at Ludhiana, Abohar and across the location

S. No.	QTLs	LG	Position	Left Marker	Right Marker	Superior Parent	LOD	R^2	Additive effect
InVitro organic matter digestibility (%)									
1	qIVOMD_Ludh6.1	6	114.54	S6_234379851	S6_64109715	RIB	6.6	0.38	-3.47
2	qIVOMD_Ludh6.2	6	156.02	S6_64569364	S6_61583354	RIB	3.4	0.12	1.49
3	qIVOMD_Abh6	6	108.54	S6_234379851	S6_64109715	RIB	3.0	0.24	-3.2
4	qIVOMD_Abh4	4	506.32	S4_125384777	S4_172551157	RIB	3.1	0.09	-5.0
5	qIVOMD_Ov6	6	111.54	S6_234379851	S6_64109715	RIB	3.8	0.33	-3.02
Acid detergent fibre (%)									
1	qADF_Abh4	4	506.32	S4_125384777	S4_172551157	JAKHRANA	5.0	0.13	6.4
Neutral detergent fibre (%)									
1	qNDF_Ludh3	3	233.08	S3_41321931	S3_57233963	JAKHRANA	3.3	0.098	1.81
2	qNDF_Abh6	6	110.54	S6_234379851	S6_64109715	JAKHRANA	5.8	0.38	5.6
3	qNDF_Abh4	4	505.69	S4_158264731	S4_174558391	JAKHRANA	3.9	0.32	6.2
4	qNDF_Ov6	6	110.54	S6_234379851	S6_64109715	JAKHRANA	3.2	0.32	1.72
5	qNDF_Ov4	4	507.32	S4_125384777	S4_172551157	JAKHRANA	3.1	0.16	2.51
Metabolizable energy (MJ/kg)									
1	qME_Ludh6	6	111.54	S6_234379851	S6_64109715	RIB	3.4	0.26	-0.22
2	qME_Ludh5	5	372.31	S5_71645760	S5_38564451	RIB	3.1	0.13	-0.16
3	qME_Ludh4	4	325.36	S4_7928148	S4_31417052	RIB	3.0	0.11	-0.13
4	qME_Abh6	6	111.54	S6_234379851	S6_64109715	RIB	3.0	0.22	-0.29
5	qME_Ov6	6	113.54	S6_234379851	S6_64109715	RIB	3.2	0.21	-0.25
6	qME_Ov5.1	5	371.31	S5_71645760	S5_38564451	RIB	3.5	0.15	-0.21
7	qME_Ov5.2	5	376.17	S5_70400731	S5_44101022	RIB	3.2	0.11	-0.21
8	qME_Ov4	4	326.36	S4_7928148	S4_31417052	RIB	3.2	0.1	-0.15
Crude protein (g/kg)									
1	qCP_Ludh6	6	11,154	S6_234379851	S6_64109715	RIB	3.4	0.21	-0.26
2	qCP_Ludh2	2	357.88	S2_219479257	S2_232799177	RIB	3.6	0.11	-0.17
3	qCP_Ov6	6	111.54	S6_234379851	S6_64109715	RIB	3.0	0.21	-0.31
4	qCP_Ov5	5	376.17	S5_70400731	S5_44101022	RIB	3.1	0.12	-0.27
5	qCP_Ov4	4	325.36	S4_7928148	S4_31417052	RIB	3.0	0.11	-0.20

identified as hydrophobic. The aliphatic index ranged from 15.9 (*Pgl_PCF8*) to 33.66 (*Pgl_KIN-12E*). Transmembrane analysis indicated that *pgl_laccase*_15, *pgl_KAPP*, *pgl_cytochrome_P450* each contained one transmembrane helice, while and *pgl_resistancetophythophthora1* contained three.

Subcellular localization analysis showed that among the 16 putative candidate genes, 5 were localised in the nucleus, 6 in the chloroplast, 1 in the plasma membrane and 2 each in the mitochondria and cytoplasm. Phosphorylation analysis revealed that these proteins are phosphorylated at serine, threonine and tyrosine residues. The common kinases involved in phosphorylation included CKI (casein kinase 1), PKC (protein Kinase C), cdc2 (cyclin-dependent protein kinase), GSK3 (Glycogen synthase kinase-3), PKA (protein kinase A), CkII (casein kinase 2), DNAPK (DNA-dependent protein kinase) and P38MAPK (mitogen-activated protein kinase).

Promoter analysis

The upstream sequences of putative genes were analysed using the PLANTPAN software, revealing various regulatory elements associated with plant development, including growth, secondary cell wall biosynthesis, and stress response. Across the genes analysed, transcription factors critical for cell wall syntheses, such as MYB (associated with lignin, cellulose, and hemicellulose synthesis), WRKY, ERF, BHLH (involved in cellulose or lignin biosynthesis), LIM, and GRF (related to fiber digestibility), were identified. Notably, MYB, a key element essential for secondary cell wall synthesis, displayed diverse roles in plants and was found in the promoter regions of all studied genes. For instance, the promoter region of *pgl_resistance to phytophthora* gene contained elements associated to fiber (LIM and GRF), reflecting its role in biotic stress response, where increased fiber content is positively correlated with enhanced resistance to biotic stress. The promoter regions of all genes exhibited various stress-responsive elements including STRE, as-1, MBS, and light-responsive elements such as G-box, SP1 GATA-motif, and GT1-motif, genes 5, 6, 8, 13, 14, and 15 displayed a higher possession of stress-related elements, with counts of 11, 13, 8, 10, 8, and 10, respectively (Fig. 6). Additional stress-responsive elements identified in these genes were ABRE, SP1 and DRE1. Specifically, the *pgl_KIN-12E* gene contained 19 ABRE elements,

while all genes displayed at least one of each of these stress responsive elements.

Phylogenetic analysis

A phylogenetic tree was constructed using homologous sequences of 16 putative candidate genes associated with fodder quality traits in pearl millet, retrieved from cross taxa species. The analysis revealed two distinct clades and identified six duplication events. Three of these involved tandem duplications (*PCF8/resistance_to_phytophthora*, *TCS/PLIM2c*, *and OTP51/GRF11*), while the remaining three were segmental duplications (*KIN-12E/BHLH148*, *NAC92/KAPP*, *and NEDD-AXR-1/Sucrose_synthase_4*) (Fig. 7).

This phylogenetic tree, encompassing multiple cereal species viz., *Pennisetum glaucum*, *Setaria italica*, *Hordeum vulgare*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum*, *Zea mays*, and *Setaria viridis*) was constructed to investigate evolutionary relationships among these species. The tree showed clear segregation into two clades. Clade A comprised five genes: *Sucrose synthase 4*, *KIN-12E*, *RIPK*, *OTP51*, and *NEDD-AXR1*. Clade B comprised *PLIM2c*, *BHLH 089*, *BHLH 148*, *KAPP*, *GRF 11*, *Resistance to phytophthora*, *Laccase-15*, *Cytochrome P450*, *PCF8*, and *NAC-92*.

Most species showed similarity with the *Pgl* genes, indicating a close relationship. Orthologous events were identified between species for various genes, such as *PglNAC92/Si.01NAC92/SvTKW42179*, *PglPCF8/Si.03PCF8/SvTKW29636*, *PglTCS/Si.04TCS/SvTKW19877*, *PglcytochromeP450/all* species. Other notable orthologous relationships included *Pgllaccase15/HORVU7Hr1G002740.1/SvTKW24870*, *Pglresistancetophytophthora1*, *PglGRF11*, *PglKAPP*, *PglbHLH14*, *PglPLIM2c*, *PglKIN-12E*, *Pglsucrosesynthase* orthologous with orthologoues in *S. italica* and *S. viridis.* Additionally, *PglbHLH089/Si.02bHLH089* and *PglNEDDAXR1* exhibited orthology with sorghum, maize, rice and wheat, with *PglOTP51/Si.01OTP51*, *PglRIPK* showing orthologous relationships across multiple cereals, including sorghum, maize, rice, barley, foxtail millet and its wild ancestor (Fig. 8).

QTLs interval annotation and identification of common genes

The QTL intervals were further explored to identify the genes governing fodder quality. In this analysis, a total of 4,573 genes were identified across all intervals. A common QTL region (S6_234379851-S6_64109715) was found to be associated with multiple fodder quality traits, identifying 1,962 genes, the highest among the intervals. This was followed by S4_125384777-S4_172551157 (1,046 genes), S4_7928148-S4_31417052 (631 genes), S5_71645760-S5_38564451 (499 genes), and S5_70400731-S5_44101022 (435 genes), the lowest number of genes. Furthermore, these intervals were filtered to screen for fodder quality-related traits. Out of the 4,573 genes, we observed 259 genes associated with fodder quality, with 103, 55, 35, 34, and 32 genes, respectively (Table 7). These genes are involved in cell wall biosynthesis and lignification, including *cellulose synthase*, *LIM binding domains*, *NAC binding domains*, *serine/threonine protein kinases and phosphatases*, *NEDD8*, *cytochrome P450*, and *PPR* genes.

To validate these genes, common genes between expression analysis and selected genes in QTL intervals were identified. In the S6_234379851-S6_64109715 interval, 27 genes were identified as key candidate genes similar to those found in expression analysis, such *as Cytochrome P450*, *NEDD8 domain*, and *NAC domain*. In the S4_125384777-S4_172551157 interval, 24 genes were identified, including *LIM binding domains*, *Cytochrome P450*, and *NAC domain*. In the S4_7928148-S4_31417052 interval, 17 genes were identified, including *LIM binding domains*, *Cytochrome P450*, and *NAC domain*. In the S5_71645760-S5_38564451 interval, 13 genes were identified, including *Cytochrome P450*, and in the S5_70400731-S5_44101022 interval, 13 genes were identified, including *Cytochrome P450*.

Discussion

Livestock plays a crucial role in supporting the nutritional security and economic stability of millions of farmers in developing nations. Over the past four decades, the global increase in human population has raised concerns about food security. Efforts have been made to boost food production to meet this growing demand, especially

Table 7 Genes identified from QTL intervals

S. No	QTL Interval Start	End	Trait	Total Genes in interval	Selected genes	Common genes between QTL interval and key candidate genes in expression analysis
1	S4_7928148	S4_31417052	ME, CP	631	35	17 Genes LIM bind domains, Cytochrome P450, NAC domain
2	S4_125384777	S4_172551157	NDF	1046	55	24 Genes LIM bind domains, Cytochrome P450, NAC domain
3	S5_70400731	S5_44101022	ME, CP	435	32	13 Genes Cytochrome P450
4	S5_71645760	S5_38564451	ME	499	34	13 Gene Cytochrome P450
5	S6_234379851	S6_64109715	ME, NDF, IVOMD, CP	1962	103	27 Genes Cytochrome P450, NEDD8 domain, NAC domain

since the advent of the green revolution. However, more than 800 million people still suffer from malnutrition, primarily in developing countries where limited production restrict food availability [33]. In the future, population growth will likely worsen these challenges by reducing available arable land for crop cultivation, which could also lead to fodder shortages for livestock. To address this issue, it is essential to increase crop productivity per unit area by developing high-yielding and high quality fodder varieties/hybrids of pearl millet. This improvement can be achieved by exploiting the genetic potential within the gene pool. Currently, there are no genomic resources available globally that could be used specifically for improving fodder quality in pearl millet. Therefore, the development of genomic resources for fodder quality-related traits in pearl millet is imperative.

Variability analysis of mapping population

The quality of fodder in pearl millet is highly important as it directly influences its nutritional value and its suitability as a fodder crop. In the present study, keeping in mind the complexity of phenotyping for fodder quality, we conducted the experiment at two locations to ensure efficient and reliable results. This is the first study on fodder quality in pearl millet that incorporates an RIL population with 274 lines. An analysis of variance revealed highly significant genotypic variances for fodder quality traits across all locations. The population displayed a broad range of variation for various traits, both at individual and across the locations. Similarly, a substantial amount of variability was also reported by Govintharaj et al. [34]. for fodder yield, IVOMD and CP in pearl millet. Furthermore, genotype and environment interactions were significant for all traits except metabolizable energy, Govintharaj et al. [34]. also reported significant genotype and environment differences and even observed the influence of environment on Metabolizable energy. The genotype by environment variance was recorded as lower than the genotypic variance, suggesting that these traits are predominantly under genetic control and minimally influenced by the genotype by environment interaction. Similar findings were also reported by Singhal et al. [35]. and Mahendrakar et al. [36]. for grain micronutrients (zinc and iron) in pearl millet using the RIL population.

The mean performance for fodder quality was recorded to be higher for parent genotypes at Ludhiana, while the population mean was observed to be higher at Abohar. These results highlight the importance of a suitable environment for harnessing the full potential of genotypes. Abohar, with its semi-arid climate and history as a traditional belt for pearl millet cultivation, provided conditions that allowed the RIL population in this study to express its whole spectrum of variation to the full extent. The high heritability observed for most fodder-related traits suggests a strong genetic influence with minimal environmental variation, making these traits more reliably inheritable. This aligns with findings from [37], which also reported high heritability for crude protein in pearl millet germplasm. Additionally, several studies examining variability in agronomic traits such as plant height, panicle length, flowering duration, and grain yield in pearl millet—using diverse mapping populations—have similarly reported high heritability [36, 38, 39]. Further, IVOMD was observed to be positively correlated with the majority of traits, whereas negative correlations with Neutral and Acid detergent fiber were observed, which is desirable for fodder quality. Similar results were also reported by Bind et al. [40]. in pearl millet, where they observed a highly positive correlation of leaves/plant, branches/plant and stem girth with both green and dry fodder yield. This association between targeted traits, due to linkage and pleiotropic effects is very beneficial for the simultaneous improvement of multiple traits.

Therefore, our findings highlight the potential of phenotypic selection to leverage existing variation within the population, facilitating the mapping of fodder quality-related traits and the development of improved quality fodder varieties/hybrids with high biomass. This goal could be achieved through the selective introgression of desirable genomic regions into high biomass backgrounds, facilitated by the development of genomics tools for marker and genomic-assisted breeding. Such genomic resources could drive a paradigm shift in pearl millet fodder breeding.

Construction of linkage and QTL mapping

In pearl millet [41], laid the groundwork by establishing the first linkage map (303 cM) using 181 restriction fragment length polymorphism markers with an F_2 mapping population. Subsequently [42], developed a longer map (617.6 cM) based on an F_2 mapping population representing 91 loci. Following these foundational studies, several other investigations constructed genetic-linkage maps using various mapping populations and marker systems [43–46]. In this present study, a high density SNP markers linkage map was generated. This map would facilitate precise mapping of agriculturally and economically significant traits with enhanced resolution and accuracy.

In the present investigation, QTL mapping for fodder quality traits identified 8, 6 and 10 QTLs at Ludhiana, Abohar and across the locations, respectively (Fig. 5). These QTLs were detected on different genomic regions, however, the interval with flanking markers S6_234379851- S6_64109715 was consistently associated with IVOMD, CP and ME at Ludhiana, Abohar and across the locations (Fig. 9), indicating that these traits are tightly linked and correlate well with each

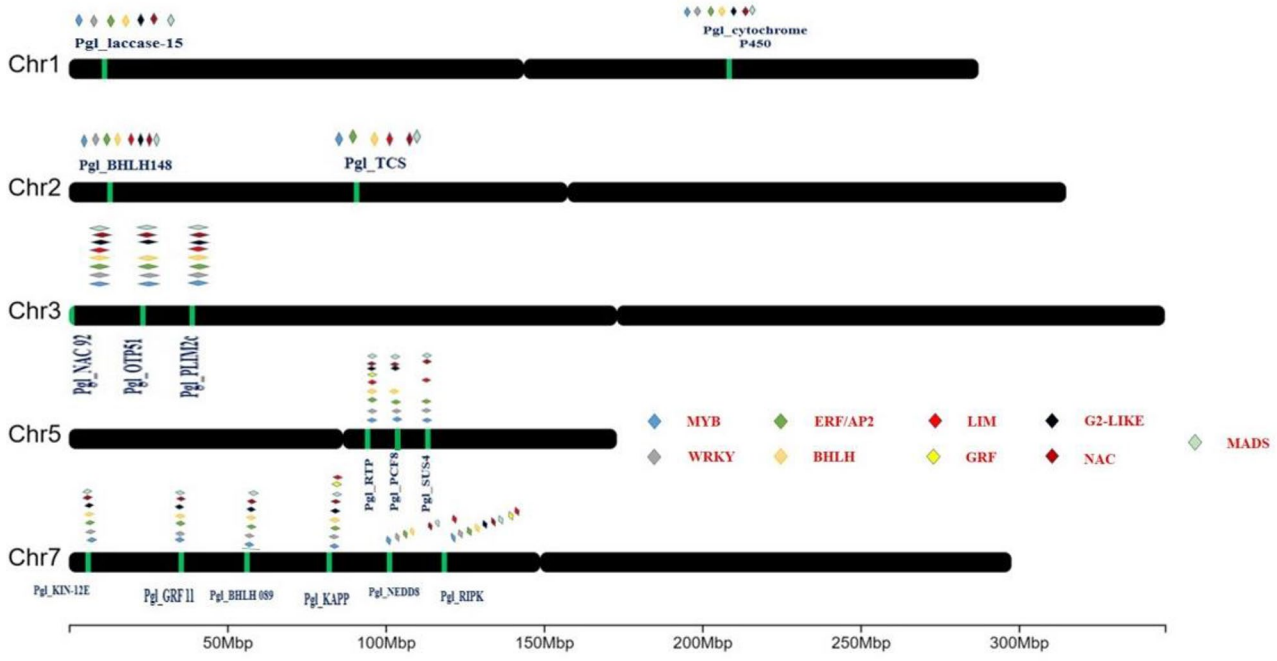

Fig. 5 In silico promoter analysis

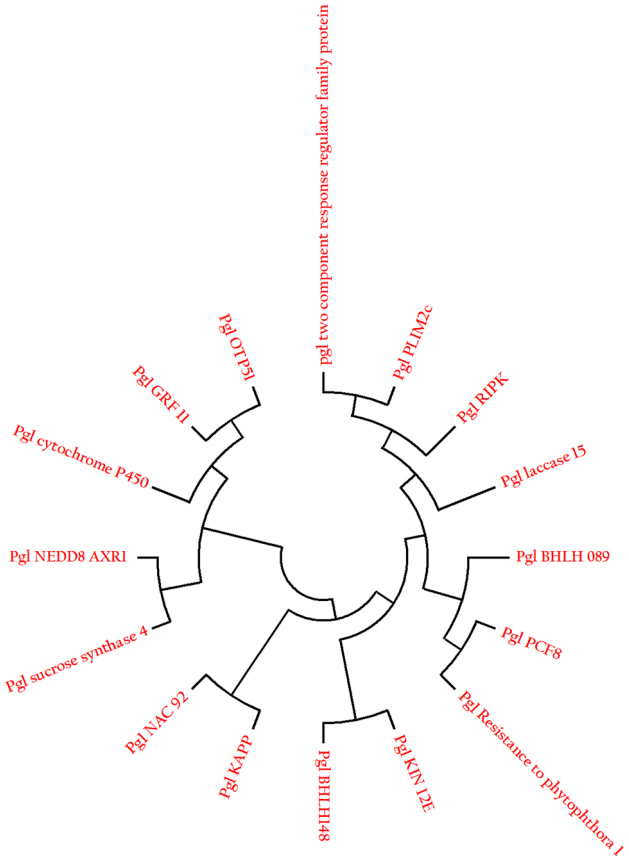

Fig. 6 phylogenetic tree of fodder quality related genes in Pearl millet

other. In previous mapping studies [47], identified a QTL for IVOMD on LG 2 (flanking marker Xpsmp2066–Xpsm380) with a phenotypic variance of 15.2% and a positive effect. In wheat [48], reported IVOMD QTLs with negative effects for straw fodder with a phenotypic variance of 2-4.5%. In sorghum [49], also reported IVOMD QTLs on the same linkage group (LG1) for two years, with most QTLs showing negative effects and phenotypic variance between 2 and 7.5%. Additionally, one QTL for ME was detected on LG 1 at Abohar, similar to findings by Nepolean et al. [47], who identified a QTL for ME on LG1 with flanking markers Xbm1RA10a– Xpsm761 in pearl millet. Furthermore, ADF, and NDF were mapped in close proximity on the linkage map. Therefore, simultaneous selection against these QTLs could reduce fiber content in fodders to improve their digestibility, as a 1% increase in IVOMD can lead to a 6–8% boost in animal productivity [50].

The environmental effect on these QTLs was found to be minimal in this study. Similar findings were reported for CP and IVOMD by Govintharaj et al. [51]. in pearl millet and [49] in sorghum, where these traits were mapped in close vicinity during multi-environment evaluations. Thus, these QTLs represent multiple traits that could yield valuable outcomes by enabling the simultaneous improvement and selection of high-quality lines for fodder quality.

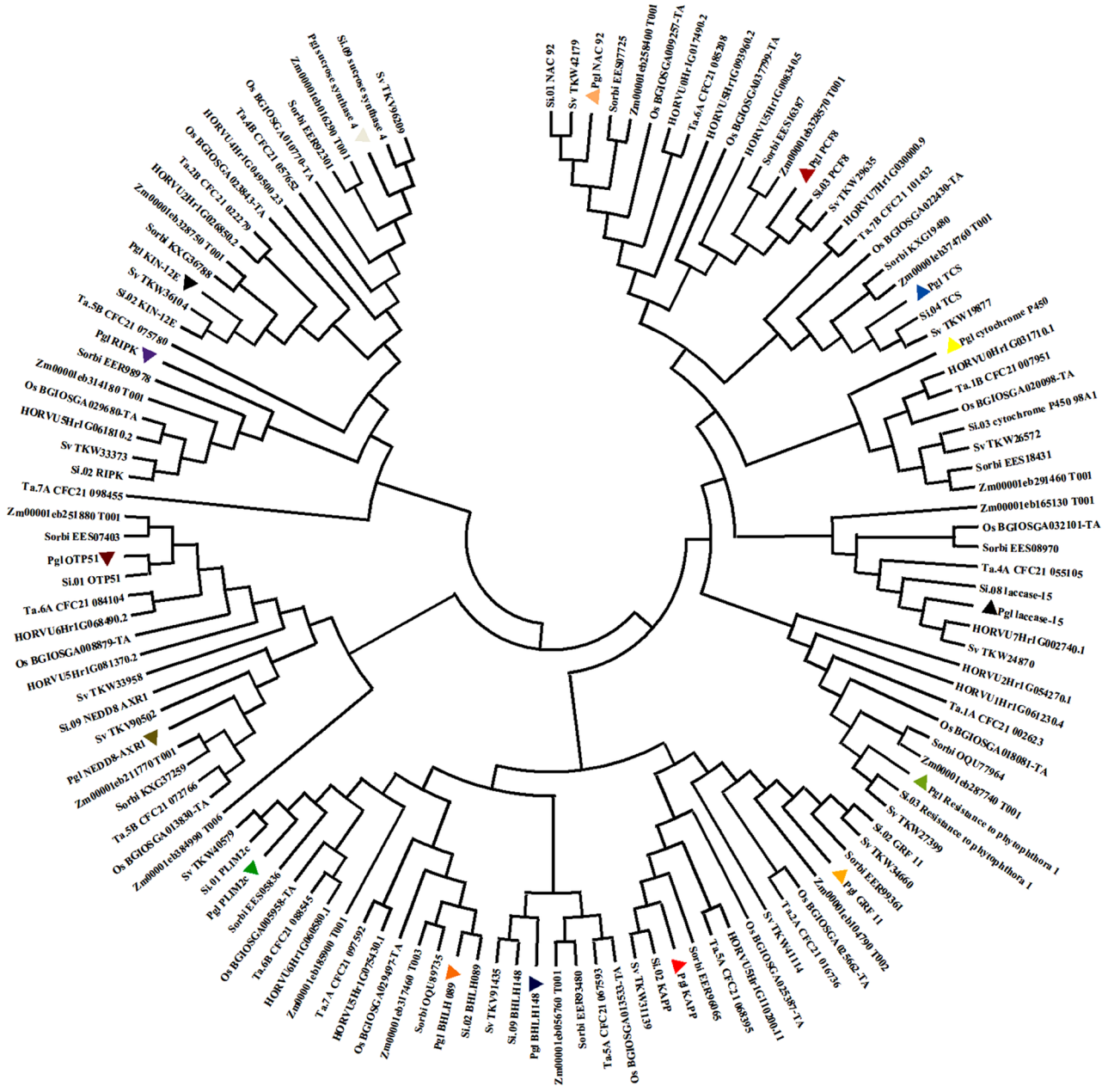

Fig. 7 phylogenetic tree of fodder quality related genes in Pearl millet and cross taxa species

Identification of candidate genes for fodder quality

Several genes governing fodder quality-related traits have been identified and characterized in major fodder crops such as maize and sorghum [11, 15, 16]. The cell wall is composed of three key components viz., Lignin, cellulose, and hemicellulose [52] which play a crucial role in fodder quality, particularly in digestibility. These components are synthesized by genes regulating various elements of cell wall biosynthesis. The process of secondary cell wall biosynthesis is complex, involving numerous transcription factors and enzymes such as NAD and MYB [53, 54] WRKY, ERF and bHLH [55]. Thus, elements that regulate cell wall synthesis also significantly impact fodder quality and biomass.

Comparative expression analysis of these genes in diverse parental lines could facilitate the identification of candidate genes responsible for regulating fodder digestibility. Additionally, these genes were carefully examined for their sequences using various bioinformatics tools. The results provided valuable insights into the role of these genes in cell wall biosynthesis. Promoter regions were found to contain transcription factors (TFs) associated with cell wall biosynthesis, and some TFs related to biotic and abiotic stress responses were also identified.

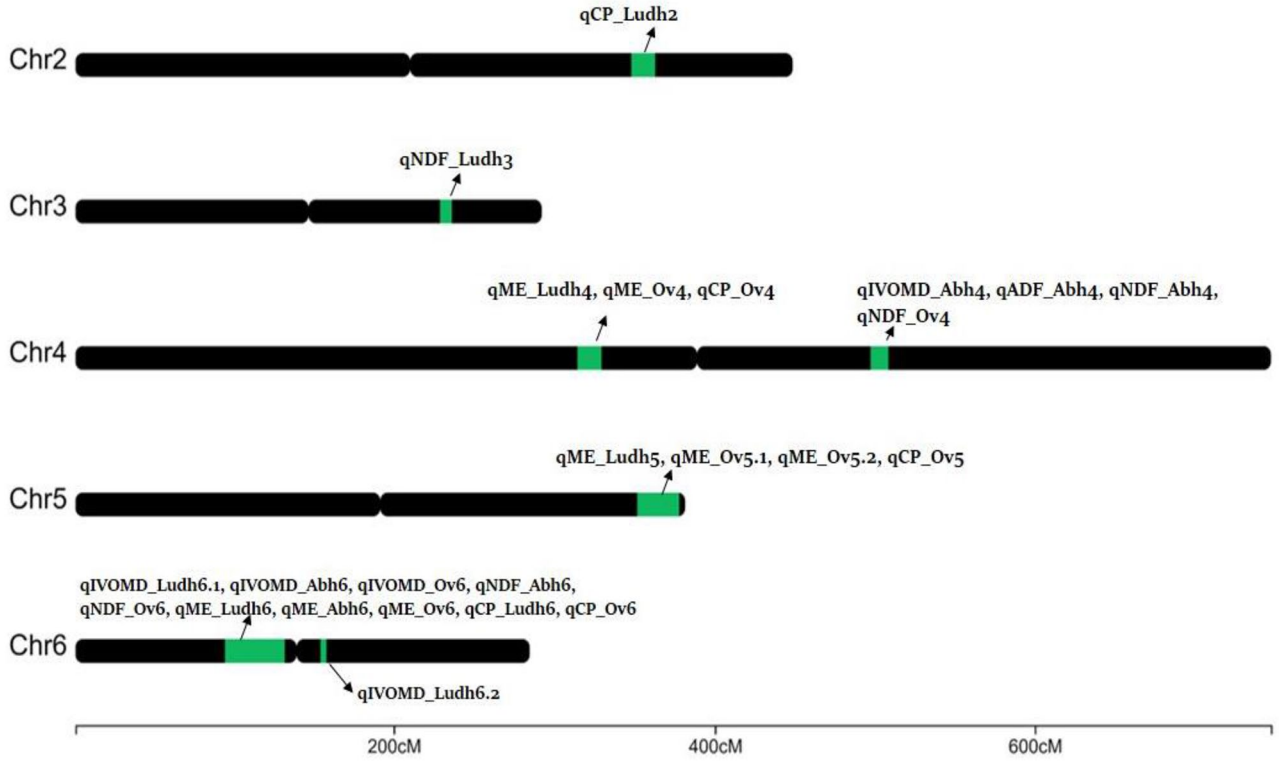

Fig. 8 Chromosomal positions of the QTLs identified for fodder quality related traits on pearl millet Linkage Map

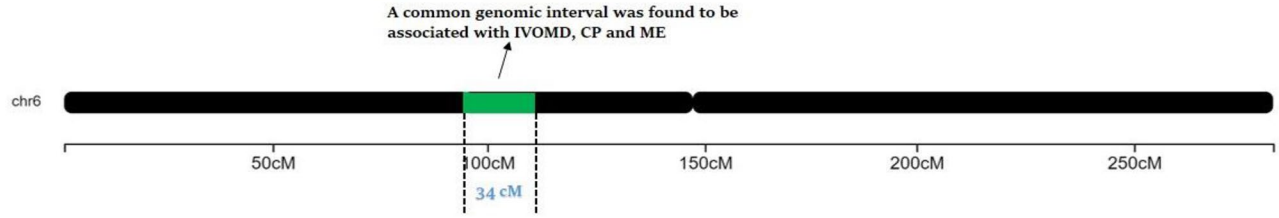

Fig. 9 Common QTL interval associated with IVOMD, CP and ME

In expression analysis, nine out of sixteen genes showed differential expression between parental lines in leaf tissues. These genes include *BHLH 148*, *Resistance to phytophthora*, *Laccase 15*, *cytochrome P450*, *PLIM2c*, *GRF11*, *NEDD AXR1*, *NAC 92* and *TF 089*. A common trend observed was variable expression in leaf tissue at the active vegetative stage (AVS) and Reproductive Stage (RS). In AVS, most genes were highly expressed in Jakhrana, while several were downregulated in RIB. However, at RS, these patterns reversed, with down-regulation in Jakhrana and increased expression in RIB. Many of the differentially expressed genes are associated with the synthesis of secondary cell wall components. This might explain why Jakhrana has a thicker stem and broader leaves during AVS, which correlates with lower digestibility. With the up regulation of these genes in RIB at RS, this line also develops thicker stems and broader leaves, affecting plant structure and stability but reducing fodder quality. The observed variability in gene expression across tissues may be due to distinct genetic control in different tissues as reported in alfalfa [56].

Furthermore, the expression for most genes is similar between RIB and PAC-981(both are superior in quality in comparison to Jakhrana). In the mature grain stage, nearly all genes exhibit higher expression in PAC-981 compared to both parent lines. This might result from PAC-981's prolonged green foliage and rapid cell wall synthesis as it matures, with the lower stem becoming harder while the upper part remains leafy and green. This delayed flowering and the extended green stage could explain the higher expression of cell- wall related genes in PAC-981.

In gene ontology analysis, genes with different expression levels were found to be involved in various biological pathways related to cell development, including lignin biosynthesis, organ development, and leaf

morphogenesis. *Laccase-15*, for instance is involved in the lignin biosynthesis pathway which contributes to lignin formation in cells. Four specific genes (*BHLH 148*, *BHLH089*, *Resistance to phytophthora* and *NAC 92*) are involved in responding to hydrogen peroxide, a molecule crucial for monolignol generation in the cytosol and subsequent polymerization in the apoplast [57]. Several Cu/Zn superoxide dismutases, localized in the apoplast, also support hydrogen peroxidase generation, which promotes lignification [58, 59], and up gradation of lignin in cells [60]. The *P450 cytochrome* enzyme aids in converting p-coumaric acid to caffeic acid, providing a shared platform for lignin's three units. NAC domain-containing proteins are essential for secondary cell wall biosynthesis and deposition in various tissues of Arabidopsis [61–64]. Additionally, *P450 cytochrome*, *NEDD8-AXR1*, *PLIM2c* and *NAC-92* play crucial roles in multiple cell and organ development processes, supporting cell structure, stability, and transportation of cell wall components in plants and involved in leaf morphogenesis.

The differentially expressed genes between the parents are primarily associated with the lignin pathway. As in secondary cell wall biosynthesis, the shift from primary to secondary cell wall synthesis involves the reorganization of microtubules. This reorganization alters the enzyme and substrate machinery, leading to lignin biosynthesis [65]. Our findings are in agreement with the developmental-specific regulation of cell wall biosynthesis in plants. Indicating that the regulatory elements in the lignin pathway directly or indirectly affect fodder quality. Targeting these factors could improve fodder quality, though selecting only low lignin lines may reduce biomass [67]. Notably, the Jakhrana genotype, which has higher expression of all relevant genes and is high-yielding but of lower quality, highlights the correlation between cell wall composition and biomass. The rate of flux through the secondary cell wall biosynthesis pathway is highly influenced by the variable concentration of transcripts of the genes related to its synthesis [65]. Therefore, based on differential gene expression across genotypes, these nine genes have been identified as candidates for cell wall component synthesis and transport in plants, influencing fodder digestibility.

Phylogenetic and promoter analysis

In phylogenetic analysis, homologs of targeted genes from eight species were used to construct a phylogenetic tree. Genes from pearl millet showed high similarity with those from *Setaria italica* and *Setaria Viridis*, consistent with prior reports of syntenic relationships for growth-regulating genes between *P. glaucum* and *setaria italica* [68]. *S. viridis*, the wild ancestor of *S. italica*, shares a validated homology, as confirmed by studies using isozyme, chloroplast, ribosomal markers, and chromosomal fluorescent in situ hybridization techniques [69–72]. Additionally, most genes showed, contemporary delineation in maize and sorghum, similar findings were also reported by Zheng et al. [73]. in seven pairs of syntenic genes from maize and sorghum for Root system architecture. Interestingly, the *Cytochrome P450* gene diverged earliest in pearl millet compared to the other species. Meanwhile, the *RIPK* showed recent differentiation, this was the only gene reported to have very low homology with the *Setaria italica* and *Setaria viridus.* In barley, paralogous events were observed for the *NAC 92*, Resistance to phytophthora1 and *OTP51*. However, barley orthologous were absent for *GRF11*, *bHLH148*, *PLIM2c* and *NEDD AXR1*, likely due to distinct selection pressures on these genes.

The promoter analysis revealed several transcription factors related to cell wall synthesis, such as *MYB*, *WRKY*, *ERF*, *bHLH*, *LIM* and *GRF* in the promoter region of genes. Additionally, various stress-responsive elements were also identified, indicating a link between cell wall components and resistance to abiotic and biotic stress. The role of TFs in both secondary cell wall and stress resistance was reported in various investigations and all these transcription factors were reported to play roles in both secondary cell wall biosynthesis and stress resistance [11, 74, 75].

QTL annotation and validation

In the QTL annotation, genes related to fodder quality traits are identified in the targeted intervals. These genes are reported to be involved in various cell wall-related functions and have a direct impact on the quality of fodder. Approximately 28% of identified genes were found similar to key candidate genes identified by differential expression between parents of the RIL population, such as *LIM domains*, *Cytochrome P450*, *NEDD8 domains*, and *NAC domains*. Genes such as *peroxidase* and *cellulose synthase* were also identified in these intervals. As *cellulose synthase* plays a major role in the biosynthesis of the cell wall [13, 76] and *peroxidase* contributes to lignification, which is negatively correlated with the digestibility of fodder [77], therefore diversity of genes related to cell wall found in these intervals endorsed the authenticity of QTL mapping. Among the 259 selected genes, 141 were observed to belong to the two gene families viz., *Cytochrome P450* (73) and *serine/threonine kinase and phosphatase* (68) families. As discussed above, *Cytochrome P450* plays a significant role in various processes, and *serine/threonine-protein kinases* (*RIPK*) have a major role in the signal cascade for nitrogen metabolism in plants. Nitrogen is a building block of amino acids that form proteins [78]. The amount of nitrogen is a major determining factor of crude protein in fodder. In *S. bicolor*, a gene encoding a *serine/threonine-protein kinase*

(*RIPK*) was found to be associated with crude protein, biomass, and digestibility by Li et al. [15]. The genes identified from both experiments depict their involvement in the regulation of fodder quality. Therefore, these gene-based markers would be incorporated into fodder quality improvement programs in pearl millet to meet global fodder demand.

This is the foundation study conducted to map and identify candidate genes for fodder quality-related traits in pearl millet, which succeeded in mapping and validating the genomic regions governing fodder quality. Also, findings from gene expression analysis along with the correlation with the morphological and biochemical aspect of fodder in parents and checks have authenticated the involvement of key candidate genes in regulating the fodder quality. Therefore, genomic resources developed in this study would be further used for crop improvement by rapid introgression of desirable genomic regions into elite backgrounds.

Conclusion

Pearl millet is a climate resilient, future-ready crop and an ideal option for fodder production amid ongoing climate change challenges globally. In this investigation, we identified candidate genes within various forage quality QTL intervals as well as from differentially expressed genes through QTL mapping and gene expression studies. These include genes such as *cytochrome P450*, *PLIM2c*, *NEDD AXR1* and *NAC domains*, *which regulate the* lignin pathway and have a direct impact on fodder quality. The candidate genes identified in this study could be utilized for fodder improvement using marker-assisted selection (MAS)/genomic selection (GS) and could be targeted for gene editing. This is the first report providing a functional understanding of fodder quality and digestibility-related genes in pearl millet. The genomic resources developed in this study may aid in the development of a breeder-friendly marker system for fodder quality improvement in pearl millet breeding pipelines globally.

Supplementary Information

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Supplementary Material 1

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Author contributions
H.S.D: Formal analysis, software, Visualization, Investigation, Writing original draft, methodology. R.B: Methodology, Resources, Formal analysis, Review & Editing, methodology. J.S.L: Formal analysis. Y.V: Formal analysis. R.K.S: Conceptualization, Research Coordination, Methodology, Resources, Review & Editing.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

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