Multivariate Analysis of Morphological and Biochemical Traits Revealed Some Promising Pigmented Rice Genotypes of North Western Himalayas for Future Breeding Program

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SHORT COMMUNICATION

Multivariate Analysis of Morphological and Biochemical Traits Revealed Some Promising Pigmented Rice Genotypes of North Western Himalayas for Future Breeding Program

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Abstract

Here, 140 pigmented rice genotypes from the North Western Himalayas were evaluated with the objective of addressing the concerns of nutritional insecurity faced by the population having rice as a staple food. Using an augmented block design, we assessed morphological traits alongside biochemical traits like total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. The principal component analysis identified genotypes SR-2 and NBPGR-16 as promising for plant height and panicle length, while GS-608 excelled in grain yield and antioxidant activity. High TPC and TFC were found in Black Rice, SKUA-533-1, and ZAG-V4, with GS-596 and Chanab showing strong antioxidant activity. Furthermore, the results revealed substantial variability, indicating the potential for targeted breeding to enhance yield and nutritional quality. This study highlights promising pigmented genotypes with superior agronomic performance and health-promoting properties, providing a foundation for breeding programs aimed at improving food security and public health through biofortified rice.

Keywords: Pigmented rice, Genetic Variability, Flavonoids, Phenols, Antioxidants.

Introduction

Rice (*Oryza sativa* L.) is indispensable for global food security, serving as a staple food for over half the world's population, particularly in Asia, Africa, and Latin America. With an estimated global population increase to 9.7 billion by 2050 and amid evolving climate challenges, enhancing rice yield and quality has become a critical priority. Although rice breeders have made strides in yield improvement, progress is constrained by limited genetic diversity within cultivated rice (Xing *et al*., 2010). To bridge this knowledge gap, it is essential to explore untapped genetic variability in diverse rice germplasm (Devi *et al*., 2017).

Beyond yield, the nutritional quality of rice is gaining importance. White rice contains significantly fewer bioactive compounds compared to pigmented rice varieties (Unpublished data). Black rice exhibits the highest levels of flavonoids, followed by red rice, while white rice has the lowest content. Additionally, the antioxidant capacity of red and black rice is notably high, primarily due to the presence of catechin in red rice and quercetin in black rice (Chen *et al*., 2022). These compounds have been traditionally valued for therapeutic purposes. This study advances prior research by providing a comprehensive analysis of 140 diverse rice genotypes, both pigmented and non-pigmented, from Kashmir Valley and Northeast India. While previous studies, such as those by Kaur *et al*. (2018), addressed limited genotypic diversity of North Western Himalayas, especially Kashmir valley, our work encompasses a broader genetic spectrum, including landraces, pigmented, aromatic, and some released varieties from the North-Western Himalayas. This expanded genetic framework highlights the untapped potential of pigmented rice for biofortification and lays a foundation for identifying key quantitative trait loci (QTLs). These findings will guide the development of targeted breeding strategies, such as crossing high-yielding varieties with nutritionally superior pigmented genotypes, to bridge existing gaps in nutritional quality and yield stability across diverse agro-climatic zones.

This study was conducted over two consecutive kharif seasons, from 15th June to 8th October 2022 and from 7th June to 15th October 2023, at the experimental field of

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MRCFC Khudwani, SKUAST-Kashmir, India. A total of 144 pigmented rice genotypes, including four checks from Kashmir Valley and Northeast India, were evaluated. The genotypes were planted in an augmented block design with seven blocks and four check varieties, following recommended agronomic practices with 20 x 10 cm spacing. Five plants were randomly selected and tagged from each line of individual genotypes for observations at different growth stages. Traits measured included grain yield, 1000 seed weight, spikelets per panicle, number of tillers, plant height (cm), panicle length (cm), days to 50% flowering, phenols, flavonoids and antioxidant content. Data analysis was done using ANOVA in the R package 'augmented RCBD' to assess variability, heritability, and genetic parameters. Further, trait variability was evaluated through range, mean, and coefficient of variation, with correlations and principal component analysis employed to explore trait associations. For biochemical parameters, total phenolic content (TPC) and total flavonoid content (TFC) estimation were carried out using the method proposed by Shen *et al*. (2008) with slight modifications. DPPH method (2, 2-diphenyl-1-picrylhydrazyl) was used for antioxidant activity estimation (Oki *et al*., 2005). Further, the population structure analysis was done using the Bayesian clustering method in STRUCTURE version 2.3.4 (Pritchard *et al*. 2000) by analysis of SSR-based marker data. The length of the burn-in period and Markov Chain Monte Carlo (MCMC) were set at 100,000 iterations (Evanno *et al*. 2005). To obtain an accurate estimation of the number of populations, three runs were performed for each K-value, ranging from 1 to 10. Further, Delta K (Figure 1b) values were calculated and the appropriate K value was estimated by implementing Evanno *et al*. (2005) method using the STRUCTURE Harvester program (available at http://taylor0.biology.ucla.edu/structureHarvester/).

The study revealed significant phenotypic and genotypic variability across multiple rice genotypes, which highlights promising opportunities for enhancing key traits. Phenotypic analysis showed a wide range of variability, with traits like days to 50% flowering, plant height, tiller number per plant, grain yield, spikelets per panicle, panicle length, and 1000 seed weight displaying notable variation. ANOVA results (Table 1) demonstrated significant differences for both adjusted and unadjusted sums of squares, underscoring the presence of substantial genetic diversity, consistent with findings by Bairwa *et al*., 2023; El-Agoury *et al*., 2024. This aligns with prior studies suggesting that phenotypic variability is crucial for selection in crop breeding (Naseem *et al*., 2014).

In terms of genetic parameters (Table 2), the study found that phenotypic coefficient of variation (PCV) values exceeded genotypic coefficient of variation (GCV) across all traits, indicating a notable environmental influence. Heritability in the broad sense, combined with genetic advance, was particularly high for traits such as grain yield and spikelets per panicle, suggesting that these traits could be reliably improved through selection. Traits with high heritability and genetic advance indicate a strong genetic basis, making them suitable targets for breeding. However, for traits with lower heritability, such as the number of effective tillers, environmental factors likely play a more substantial role, complicating the reliability of direct selection.

In the biochemical analysis (Figure 1a), pigmented rice genotypes (black, red, purple, brown) showed high levels of bioactive compounds, with black rice exhibiting the highest phenolic (upto 1563.59 mg GAE/100 g) and flavonoid contents (523.69 mg RE/100 g), along with antioxidant activity of 94.68%. These results align with previous observations that pigmented rice tends to have higher bioactive content compared to non-pigmented varieties, suggesting their potential for enhancing nutritional value. Notably, even non-pigmented white rice genotypes demonstrated significant variation in phenolic and flavonoid contents, with certain genotypes surpassing

Table 1: ANOVA for augmented block design for seven traits in 140 pigmented rice genotypes

Source	*Df*	*50F*	*PH*	*PL*	*NT*	*SPP*	*GY*	*1000 SW*
Treatment (ignoring Blocks)	143	64.0 4**	328.89 **	7.46**	3.70 **	783.41 **	151.41 **	18.38 **
Treatment (eliminating Blocks)	143	44.46**	246.39**	6.52**	3.27^{ns}	606.27**	125.94**	17.42**
Block (eliminating Treatments)	6	1.17^{ns}	9.82^{ns}	0.54^{ns}	0.31^{ns}	313.49^{ns}	0.58^{ns}	0.39^{ns}
Block (ignoring Treatments)	6	467.86**	1976.04**	23.08**	10.51**	4535.33**	607.48**	23.16**
Checks	3	680.92**	2294.65**	57.11**	29.14**	2690.30**	433.34**	74.12**
Test entries vs. Checks	1	1.09^{ns}	00.0 **	45.87 **	1.87^{ns}	6.34^{ns}	418.14**	421.74 **
Test entries	139	51.18 **	288.83**	6.12**	3.17^{ns}	747.84**	143.40**	14.27**
Error	18	1.61	6.20	1.01	1.84	206.86	0.59	0.38

PH= Plant Height, PL= Panicle Length, NT= Number of tillers per plant, SPP= Spikelets per Panicle, DF= Days to 50% flowering, GF= Grain yield, SY= Seed yield, 1000 SW= 1000 seed weight

Table 2: Descriptive and genetic variability analysis for seven traits in 140 pigmented rice genotypes

Trait	*Mean*	*Range*		*Coefficient of variation*		*h2(%)*	*GA*	*GA%*
		Min	*Max*	*GCV (%)*	*PCV (%)*			
50F	95.77	79.94	113.44	7.35 (Low)	7.47 (Low)	96.86 (High)	14.30	14.93 (Medium)
PH	114.43	76.84	147.50	14.69 (Medium)	14.85 (Medium)	97.85 (High)	34.31	29.98 (High)
PL	21.17	14.82	27.15	10.68 (Medium)	11.68 (Medium)	83.49 (High)	4.26	20.12 (High)
NT	11.69	7.33	17.88	9.85 (Low)	15.23 (Medium)	41.83 (Medium)	1.54	13.14 (Medium)
SPP	117.83	51.38	188.67	19.74 (Medium)	23.21 (High)	72.34 (High)	40.81	34.63 (High)
GY	25.78	5.57	57.27	46.35 (High)	46.45 (High)	99.59 (High)	24.60	95.43 (High)
1000 SW	24.49	8.69	34.75	15.22 (Medium)	15.43 (Medium)	97.33 (High)	7.59	30.97 (High)

PH= Plant Height, PL= Panicle Length, NT= Number of tillers per plant, SPP= Spikelets per Panicle, DF= Days to 50% flowering, GF= Grain yield, SY= Seed yield, 1000 SW= 1000 seed weight, (PCV %) Phenotypic coefficient of variation percentage, (GCV %) Genotypic coefficient of variation percentage, (h2%) Heritability percentage, GA Genetic Advance, GA% Genetic Advance as percentage mean.

pigmented ones. This suggests that some white rice varieties may also serve as valuable nutritional resources (Figure 1a). Correlation analysis revealed a strong positive association between flavonoid content and antioxidant activity, indicating that flavonoids significantly contribute to antioxidant properties. Morphological traits, especially grain yield, positively correlated with panicle length, days to 50% flowering, number of effective tillers, 1000 seed weight, and spikelets per panicle, supporting the study indicated that these traits are critical for yield improvement (Anisuzzaman *et al*., 2023). Conversely, a negative correlation was observed between plant height and tiller number, highlighting potential trade-offs between plant architecture and yield traits. Principal component analysis (PCA) identified three significant components explaining 61.35% of the total variance. The first two components, which captured nearly 50% of this variance, were strongly associated with plant height, panicle length, and antioxidant activity, suggesting these traits as key differentiators among genotypes. The genotype-by-trait (GT) biplot (Figure 2) further supported this, as genotypes with high PC scores for yield and biochemical traits clustered together. In conclusion, based on multivariate analysis and genetic evaluation, we identified several potential rice genotypes with exceptional performance as GS-596, Chanab, Zag-V4, and NBPGR-11-1, that showed high antioxidant activity. For flavonoid content, Black Rice was the top performer, followed by SKUA-544, Zag-V3, Zag-V4, and NBPGR-21, while ZAG-V5, ZAG-V4, black rice, GS-596, and Jehlum excelled in phenolic content. From PCA results we found SR-2, NBPGR-16, NBPGR-2, NBPGR-34, and NBPGR-5 contributed most towards plant height, panicle length, and 50% flowering. Further traits like grain yield and antioxidant activity having dominant contributions in PC2 were represented by GS-608, GS-480, GS-484, GS-474, and GS-621. For PC3, phenolic and flavonoid content were key traits, with black rice, SKUA-533-1, ZAG-V4, ZAG-V15, and SKUA-556 as leading genotypes. These genotypes hold strong potential for breeding programs aimed at improving rice varieties for both nutritional quality and agronomic performance.

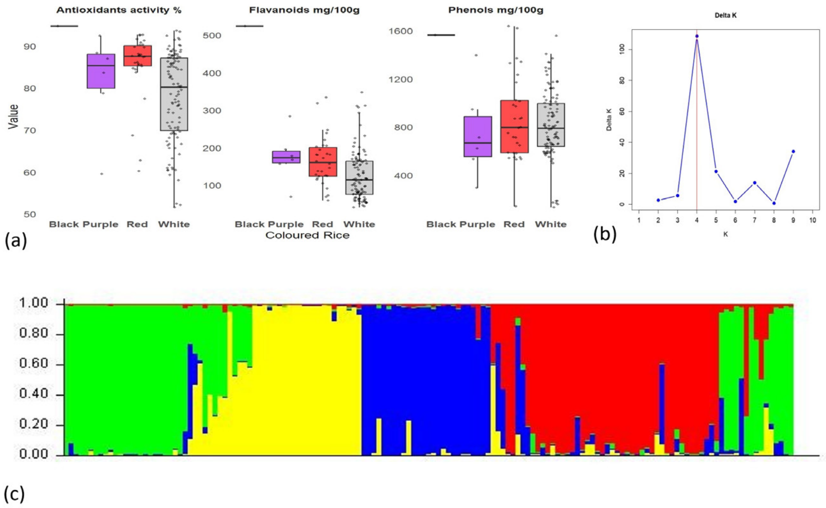

Figure 1: (a): Distribution of phenols, flavonoids and antioxidant activity in colored rice germplasm., (b): Delta K showing the number of populations, (c): Population structure of 147 rice genotypes.

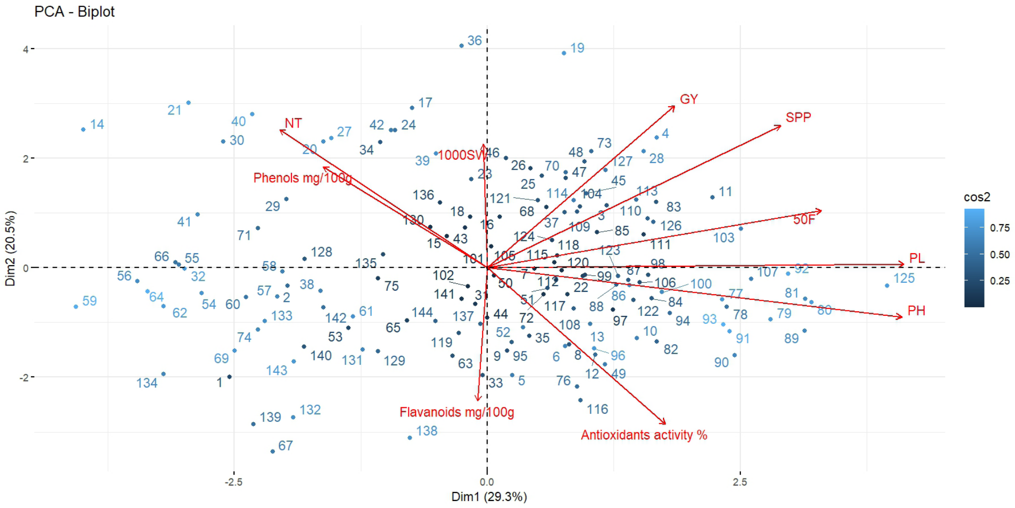

Figure 2: Genotype by trait (GT) biplot based on the individual genotypic data explaining the contribution of 10 traits to the total variation. The biplot was based on singular value decomposition of trait-standardized data ("Scaling = 1, Centering = 2") and trait-focused singular value partition ("SVP = 2")

The population structure analysis grouped the population into four sub-populations (Figure 1c), reflecting their diverse genetic backgrounds. Landraces predominantly belonged to the green cluster, indicating high genetic uniformity with minimal admixture. Indian collections were largely assigned to the yellow cluster, with some genotypes showing admixture, suggesting historical gene flow. Advanced breeding lines exhibited significant admixture, highlighting their derivation from diverse parental sources. Similarly, released varieties displayed contributions from all clusters, reflecting efforts to combine genetic resources for improved traits. Collections from international sources primarily clustered in the red group, indicating their distinct genetic composition. This emphasized the importance of utilizing diverse genetic resources, particularly the landraces and exotic collections, in breeding programs to enhance genetic diversity and adaptability. Preserving unique landraces and leveraging exotic collections can contribute to sustainable crop improvement efforts.

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Competing Interests

The authors declare they have no conflicts of interest.

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Non-coding RNAs and their role in plants: prospective omics-tools for improving growth, development and stress tolerance in field crops

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REVIEW

Non-coding RNAs and their role in plants: prospective omics-tools for improving growth, development and stress tolerance in field crops

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Abstract

Plants, as sessile organisms, must adapt to dynamic environmental changes through a range of response strategies that confer phenotypic flexibility. Breakthroughs in next-generation sequencing technologies have led to significant improvements in our understanding of the genomic and molecular mechanisms underlying plant growth, development and stress responses. Non-coding RNAs (ncRNAs), have emerged as pivotal regulators in these processes. This article reviews the roles of regulatory ncRNAs in plant stress responses and development, highlighting their intricate molecular interactions. It presents a comprehensive atlas of differentially regulated ncRNAs across key crop genomes, enhancing our understanding of their roles in stress responses, growth, and development. The atlas presented herein offers a foundation for further research in agronomically important crops, paving the way for crop improvement through genetic engineering and sustainable agricultural practices. Additionally, we discuss the role of ncRNAs that have already been functionally characterized in growth, development and stress tolerance, providing insights into their potential for developing stress-resistant and high-yielding crops.

Keywords Non-coding RNAs (ncRNAs) · Plant stress · Gene regulation · Crop improvement · Cereals

Introduction

Plants need to meticulously coordinate their responses to different environmental stresses with their growth, development, and reproductive processes. They activate specific signalling pathways and adjust their gene expression to survive and adapt. The regulatory mechanism of gene expression is the major unanswered query in the intriguing domain of plant stress response, and these are uncovered using novel breakthrough technologies like multiplexing highly selective chemical probes for profiling 'active protein in intact cells' (Activity Based Protein Profiling) [1]. The elements in the regulatory system are crucial in reprogramming at the transcriptional, post-transcriptional, and epigenomic levels so that plants adapt to biotic and abiotic stresses [2, 3]. Several biotechnological methods have been utilized to alter the genetic makeup of significant crops for economic purposes, creating genetically modified plants with enhanced resistance against biotic and abiotic stressors [4, 5]. However, the utilization of genetically modified plants harbouring trans- or cis-genes has faced significant scepticism due to the potential risk of the transfer of these 'genes' across natural sexual barriers, and the so-called 'precautionary principle' [6, 7]. Modern biotechnological approaches and technologies based on multi-omics, robotics, nanotechnology, and plant-microbial interactions are being integrated to overcome these challenges [8–11]. One of the most promising molecular biology-based resources includes the major-effect multiple trait coding 'pleiotropic genes' because these can target multiple stresses simultaneously [12]. However, recent advancements have highlighted the importance of 'intergenic' regions between protein-coding genes, which are 'non-coding regulatory RNAs'. These RNAs are frequently known as non-coding RNAs (ncRNAs). These ncRNA transcripts were historically dismissed as transcriptional noise due to their inability to code for proteins, and their often poorly conserved sequences arising from regions of the genome once thought to be inactive, known as intergenic regions, transposons, pseudogenes and repetitive sequences [13].

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Long non-coding RNAs (lncRNAs) function as intermediaries between RNA molecules and proteins. Depending on their interactions, lncRNAs can act as transcriptional activators, enhancing gene expression, or as repressors, inhibiting it. Plant lncRNAs function in both cis and trans contexts [13]. Cis-acting lncRNAs function near their sites of synthesis. They act directly on local nucleotide sequences or chromosomal regions associated with one or more adjacent genes. Conversely, trans-acting lncRNAs migrate from their point of synthesis and can impact multiple genes, even across considerable distances, including those located on different chromosomes. Furthermore, lncRNAs can also serve as precursors for small RNAs (sRNAs). Certain lncRNAs can form double-stranded RNA duplexes with Natural Antisense Transcripts (NAT), generating sRNAs that perform regulatory functions. Additionally, lncRNAs can serve as miRNA decoys, binding to miRNAs and preventing them from interacting with their target mRNAs. This interference reduces miRNA activity and relieves the repression of the target gene. Moreover, lncRNAs often act through various mechanisms, such as protein-protein interactions and post-translational modifications via epigenetic regulatory mechanisms, including methylation of DNA, histone modification, and chromatin remodelling.

The review highlights a comprehensive analysis of the roles of non-coding RNAs (ncRNAs) in regulating plant growth, development, and stress responses. The review attempts to present a comprehensive genome-wide atlas of ncRNAs in field crops (cereals, pulses and legumes). Furthermore, the article aims to enlist and provide useful information about the prospects of using ‘functionally validated ncRNAs’ to enhance crop resilience and as biomarkers for stress tolerance.

Non-coding RNAs functions in plant growth and development: an overview

miRNAs play a key role in modulating gene expression across multiple aspects of plant growth and development. The study of plant developmental stages revealed a complex network of miRNAs and their target genes. miRNA-target modules include miR156-SPL, miR159-MYELOBLASTOSIS (MYB), miR172-APETALA 2 (AP2), and miR156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE [14]. The miR156-SPL module in Arabidopsis operates as a negative regulator during the stages of germination, vegetative growth, and reproductive development. Reduced miR156 levels lead to increased SPL expression, thereby accelerating these transitions. Conversely, the miR172-AP2 module facilitates these developmental transitions [15]. Elevated miR172 levels decrease AP2 expression, promoting the transition. Numerous studies have shown that miRNAs function in conjunction with various hormones, including abscisic acid (ABA) and gibberellic acid (GA), to control the germination and dormancy of plants. For example, miR159 plays a critical role in modulating seed dormancy and germination by maintaining a balance between ABA and GA hormone levels [16]. It achieves this by targeting MYB transcription factors, MYB33, MYB101, which positively regulate abscisic acid signalling during seed germination and dormancy, as shown in (Fig.1). Moreover, different miRNAs targeting multiple members belonging to the same gene family contribute to a wide range of biological functions. As an instance, miR160 negatively controls the expression of AUXIN RESPONSE FACTORS (ARFs) in rice and Arabidopsis affecting seed germination [17]. Likewise, miR167 promotes root development by regulating the expression of ARF6 and ARF8 [18]. Polarity in leaves is regulated by targeting multiple ARF genes through the generation of small interfering RNAs (siRNAs) by miR165/166 [19]. These observations point out the specialized regulatory functions of miRNAs during various developmental phases. They achieve this through their involvement in specific signalling pathways. miRNAs can integrate their actions to modulate a particular biological function. Within a miRNA family, different isoforms can participate in analogous biological functions by targeting either the same genes or various genes [20]. An example is the involvement of the miR159a.1-MYB and miR159a-p5-Tetraketide alpha-pyrone reductase 1 (TKPR1) modules in male meiosis, with substantial expression observed in pollen and embryo sacs. Together the complex regulatory network of miRNA-target modules forms the molecular basis of plant growth and development as shown in (Fig.1).

Besides miRNAs, siRNAs have demonstrated significant involvement in plant development [21]. For example, the ARF family members are targeted by the phased TAS3-tasiRNAs which originate from the miR390-AGO7 complex. This regulatory network is essential to many aspects of plant development and is conserved across plant species. It affects the genesis of embryos, root architecture, shoot apical meristem (SAM) development, leaf morphology, flower development, and phytohormone interactions during developmental transitions [22]. miR828 activates TAS4-tasiRNAs, which target MYB genes involved in anthocyanin production in Arabidopsis. This includes the genes PAP1, PAP2, and MYB113 [23] as shown in (Fig.1). Remarkably, two homologous MYB genes that correspond to TAS4 orthologs in both cotton and *Arabidopsis* are involved for regulating fibre development in cotton. miR828 targets one of these MYB genes to generate 21-nt phasiRNAs. Furthermore, miR828 produces phasiRNAs and cis- and trans-acting siRNAs that also contribute to the regulation of trichome (hair) formation [24]. While there has been less research on natural antisense siRNAs (natsiRNAs) and heterochromatic siRNAs

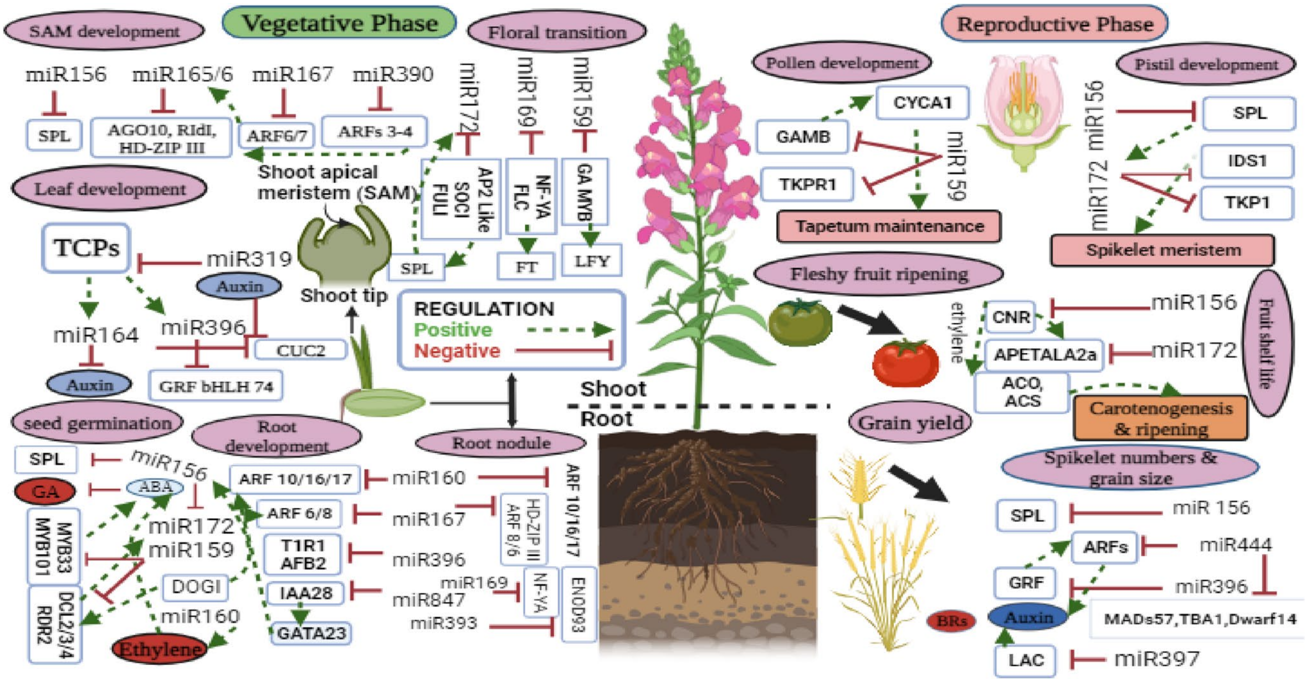

Fig. 1 Regulation of plant growth, development and stress responses by miRNAs. This figure illustrates various miRNA-target modules that are crucial for plant growth and development, as well as their roles in mediating responses to abiotic and biotic stresses. The diagram highlights how different miRNA regulate these processes and adapt plant physiological mechanisms to environmental challenges

(hcsiRNAs) in plant development, they have been studied in specific contexts. For example, cell-specific atsiRNAs have been shown to regulate the KOKOPELLI (KPL) and ARIADNE14 (ARI14) genes during the double fertilization process in Arabidopsis. These findings underscore the diverse and complex roles of siRNAs in governing various aspects of plant development.

Long non-coding RNAs (lncRNAs) are implicated in plant development through diverse regulatory pathways. There are over 1543 experimentally verified lncRNAs from 77 species in the EVLncRNAs database. It has provided valuable insights into the realm of long non-coding RNAs (lncRNAs). 428 lncRNAs from 44 plant species, including rice and *Arabidopsis*, have been catalogued in this database [25]. In mammals, lncRNAs are recognized for their function in gene expression regulation through chromatin remodelling. Interestingly, certain plant lncRNAs, such as COLDAIR, also function via this regulatory pathway. Successful reproduction is closely tied to the precise timing of floral transition and robust flower development in plants. Regulation of flowering time encompasses internal signals, such as plant hormones, and external cues, such as day length and temperature. The prolonged cold exposure during winter, known as vernalization, leads to the downregulation of the major flowering repressor, FLOWERING LOCUS C (FLC), thus promoting flowering in spring in Arabidopsis. A number of long non-coding RNAs (lncRNAs), such as COLDAIR, COOLAIR, ANTISENSE LONG (ASL), and COLDWRAP, are essential for precisely regulating the expression level of the Flowering locus (FLC) [26]. As an example, following vernalisation, COLDAIR is engaged in suppressing FLC. It interacts with the PRC2 complex's CURLY LEAF (CLF) protein, with the strongest association occurring after 20 days in the cold [27]. PRC2 must be recruited to the FLC locus by COLDAIR in order to facilitate the insertion of the repressive H3K27me3 chromatin modification.

Many long non-coding RNAs (lncRNAs) have been identified as important regulators of various aspects of flower and reproductive development. These include LINC-AP2, Photoperiod-sensitive genic male sterility T (PMS1T), and Early flowering-completely dominant (Ef-cd) and Long-day specific male-fertility-associated RNA (LDMAR) [26]. An example of this relationship can be observed in LINC-AP2, an intergenic lincRNA that is positioned close to the flower development governing gene APETALA2 (AP2). Interestingly, when AP2 expression is reduced by Turnip crinkle virus (TCV) infection, LINC-AP2 expression increases conversely. This notable elevation of LINC-AP2 expression has been associated with the formation of abnormal floral structures [28]. Moreover, there is a long intergenic rice lncRNA known as XLOC-057324, which exhibits high expression levels in reproductive organs. A comprehensive analysis involving T-DNA insertion mutants suggests that this lncRNA plays a role in the regulation of flowering and plant fertility [29].

Insights from overexpression and loss-of-function studies into the role of non-coding RNAs in plant growth and development

Non-coding RNAs (ncRNAs) play critical roles in regulating plant growth and development across various stages. In *Arabidopsis thaliana*, ncRNAs are involved in processes like seed germination, lateral root development, and vegetative phase transition. For instance, disruption of the miR156 → SPL13 pathway delays seedling development [30]. miR156 and miR172 also interact to control post-germination growth, with miR156 overexpression delaying the juvenile-to-adult phase transition [31]. Fertility, too is influenced by miR156/7 targeting SPL genes, where mutations lead to sterility [32]. In *Nicotiana tabacum*, miR156 plays a key role in regulating the juvenile-to-adult phase transition. Overexpression of miR156 extends the juvenile phase, while knockdown of miR156 accelerates the transition to the adult phase. This miRNA also influences various vegetative traits, including leaf shape, trichome density, stomata number, and chlorophyll content, which collectively differentiate the juvenile and adult phases in tobacco [33] (Fig.1 and Table 1). In *Oryza sativa*, miR156 is involved in regulating leaf development and phase change. Overexpression of miR156 accelerates leaf maturation and promotes rapid tiller initiation, while also affecting temporal gene expression, including other miRNAs. miR156 regulates leaf development in an age-dependent manner, with higher expression in older leaves [34]. Additionally, miR156 controls panicle architecture by targeting genes like LAX1, LAX2, RCN2, and OsRA2, which are involved in axillary meristem development and pedicel length regulation. Genetic interactions between these genes and miR156 influence panicle traits, with a buffering mechanism suggested between miR156 and RCN2 [35]. In *Triticum aestivum*, miR156 plays a crucial role in regulating plant architecture, including tillering and spikelet formation, by repressing SPL genes and interacting with strigolactone (SL) signalling. Overexpression of miR156 increases tiller number but severely disrupts spikelet formation. Specifically, miR156 represses TaTB1 and TaBA1 through TaSPL3/17. Additionally, the SL signalling repressor TaD53 interacts with miR156-regulated TaSPLs to suppress TaTB1 and TaBA1, impacting both tillering and spikelet development [36]. miR156 also influences ovary development and carpel formation by regulating SPL genes and meristematic activity, with overexpression causing abnormal flower and fruit morphology [37] (Fig.1)(Table 1). In mulberry (Morus spp.), miR156 regulates the MnSPL/mno-miR172 pathway to control the vegetative phase transition. Overexpression of miR156 in transgenic *Populus* extends the juvenile phase. In mulberry, miR156 represses mno-miR172 and modulates the expression of nine MnSPLs, which are direct targets of miR156. MnSPLs activate transcription of mno-miR172, but miR156 represses it to regulate developmental transitions [38].

Root development is modulated by miR160, which regulates ARF genes and influences root elongation and lateral root formation [39], while miR164 regulates lateral root emergence by targeting NAC1 [40]. In *Medicago truncatula*, miR160 regulates root growth and nodulation by targeting ARF genes. Overexpression of miR160 causes root growth defects, disorganization of the root apical meristem (RAM), gravitropism issues, and reduced nodule numbers. miR160 expression varies during root and nodule development, with distinct profiles for mtr-miR160d and mtr-miR160c. Activation of miR160 during early symbiotic stages was not observed in nodulation signaling or autoregulation mutants [41] (Fig.1 and Table 1).

In terms of grain development, the miR396 family, which targets Growth-Regulating Factor (GRF) genes such as GRF1, GRF6, and GRF9, plays a significant role in wheat grain filling. Seventeen miR396 members have been identified, including five unique haplotypes, such as miR396a and miR396n, which are absent in other species. These haplotypes demonstrate distinct Gene Ontology (GO) enrichment functions and are integral to grain development, with polyploidization driving their diversification and enhancing the functional networks involved in grain filling [42] (Fig.1 and Table 1). Mutations in MIR396e and MIR396f lead to larger grains and altered plant structure, enhancing grain yield by promoting leaf elongation and gibberellin biosynthesis [43]. Additionally, miR160 negatively regulates OsARF18, affecting rice growth, development, and auxin signalling, with overexpression of OsARF18-resistant versions leading to various growth defects [44]. miR396 regulates flower and fruit growth by targeting SlGRFs, and overexpression in transgenic lines results in larger flowers and fruits [45]. Finally, miR396 regulates grain size through OsGRF8, with target mimicry of OsmiR396 increasing grain size and improving yield. OsmiR408, regulated by OsGRF8, also plays an essential role in grain size regulation [46] (Fig.1 and Table 1).

In shoot apical meristem (SAM) development, AGO10 sequesters miR165/166, ensuring proper SAM establishment [47]. In *Larix leptolepis*, overexpression of miR166a reduces somatic embryo (SE) formation, affects shoot apical meristem (SAM) development, and enhances rooting and lateral root formation. miR166a down-regulates LaHDZ31-34 in transgenic lines and upregulates WOX expression, suggesting an indirect role in SAM development. This indicates that miR166a influences both rooting and SAM formation [48]

Table 1 Characterization of non-coding RNAs through overexpression and loss-of-function in plant growth and development

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
A. thaliana	Seed germination, Early growth	SPL13, SNZ (targets of miR156 and miR172)	• Disruption of the miR156 → SPL13 pathway leads to delayed seedling development • miR156 and miR172 pathways interact to regulate post-germination development	[30–32, 39, 40, 47, 64, 65]
	Lateral root development	NAC1 (NAC domain transcription factor)	• miR164 overexpression reduces lateral root emergence, while its knockdown increases it • miR164 regulates NAC1 by targeting its mRNA for cleavage • Auxin-induced miR164 expression modulates NAC1 levels to restrict lateral root development as a homeostatic mechanism	
	Vegetative phase change	SPL genes (targets of miR156)	• miR156 overexpression delays the transition to the adult phase by repressing SPL gene expression • Leaf primordia ablation increases miR156 expression, promoting the juvenile phase and delaying vegetative phase	
	Shoot Apical Meristem (SAM) development	PHABULOSA (PHB), REVOLUTA (REV), miR165/166 targets)	• AGO10 sequesters miR165/166 activity to regulate SAM development by repressing their activity in specific regions • In AGO10 mutants, sequestration restores SAM function by limiting miR165/166 activity	
	Leaf development	Growth-Regulating Factors (GRFs), AS1/AS2, RDR6, SGS3, AGO7	• miR396 overexpression in 35S transgenic lines reduces leaf size, and in rdr6 mutants causes lobed leaves • Mutations in as2 and rdr6 enhance the miR396 phenotype, producing lotus- or pin-like organs	
	Fertility, Anther development	SPL8 and other SPL genes	• miR156/7 targets multiple SPL genes in anther development • miR156 overexpression in spl8 mutants causes complete sterility • SPL8 is required for sporogenic tissue development in anthers and its loss causes semi-sterility	
	Root system development	ARF10, ARF16, ARF17, Auxin Signaling	• miR160 regulates root development by repressing ARF genes ARF10, ARF16, ARF17 • miR160 repression of ARF17 promotes primary root elongation and enhances lateral/adventitious roots • DRB1 and DRB2 regulate miR160 production for proper root development	

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
	Vegetative phase transition, Leaf development	CIB4, FT (Flowering Time), ARP6, PIE1	• miR396 negatively regulates leaf size, vegetative phase transition, and promotes early flowering • miR396 overexpression in 35S/b and cib4-1 lines delays flowering, while miR396ab mutants flower earlier • miR396 expression is repressed in arp6 and pie1 mutants, altering leaf and flowering patterns • miR396 is regulated by SWR1-C-mediated epigenetic mechanisms • miR396 expression negatively correlates with FT gene expression	
Nicotiana tabacum	Juvenile-to-adult phase transition	miR156, regulating SPL genes	• miR156 overexpression prolongs the juvenile phase, while its knockdown promotes the adult phase transition • miR156 regulates key vegetative traits (leaf shape, trichome density, stomata number, chlorophyll content)	[33]
Oryza sativa	Leaf development	SPL genes (SQUAMOSA-like transcription factors)	• miR156 overexpression leads to precocious leaf maturation, rapid tiller initiation, and attenuation of temporal gene expression changes • miR156 regulates leaf development in an age-dependent manner, with higher expression in older leaves	[34], [35], [52], [43], [44], [46]
	Panicle architecture regulation	LAX1, LAX2, RCN2, OsRA2	• miR156 regulates panicle architecture by targeting genes involved in axillary meristem development and pedicel length • Genetic interactions between miR156, LAX1, LAX2, RCN2, and OsRA2 regulate panicle traits • Tight genetic linkage between miR156 and RCN2 suggests a buffering mechanism for panicle architecture	
	Growth and development, Tiller formation, Auxin signaling	OsARF6, OsARF12, OsARF17, OsARF25 (Auxin Response Factors)	• miR167 regulates auxin signaling by targeting OsARF6, OsARF12, OsARF17, and OsARF25 • miR167 overexpression in transgenic rice reduces OsARF expression, leading to reduced stature and decreased tiller number • miR167 is essential for proper OsARF expression, which mediates auxin responses critical for rice growth and development	

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
	Grain size, Plant architecture	Gibberellin (GA) biosynthesis, CYP96B4	• miR396e and miR396f mutations increase grain size, grain yield and alter plant architecture • miR396ef mutations promote leaf elongation by increasing mevalonic acid levels, a GA precursor, enhancing GA biosynthesis	
	Growth and development, Auxin signaling	OsARF18, Auxin Signaling	• miR160 negatively regulates OsARF18, crucial for rice growth, development, and auxin signaling • Overexpression of OsARF18-resistant versions in transgenic plants causes defects like dwarf stature, rolled leaves, and small seeds • miR160, derived from OsMIR160a and OsMIR160b, fine-tunes auxin signaling by targeting OsARF18	
	Grain size and yield	OsGRF8 (Growth-Regulating Factor), OsMIR408, Hormone signaling pathways	• OsMIR396 regulates grain size by modulating OsGRF8 expression • Silencing OsmiR396 through target mimicry (MIM396) increases grain size in both japonica and indica subspecies, as well as in plants with excessive tillers and high panicle density • OsMIR408 expression is regulated by OsGRF8 and silencing OsMIR408 leads to smaller grains	
Triticum aestivum	Plant architecture, Tillering, Spikelet formation	TaSPL3, TaSPL17, TaTB1, TaBA1, TaD53	• miR156 regulates plant architecture, including tillering and spikelet formation, via repression of SPL genes and interaction with strigolactone (SL) signaling • miR156 overexpression increases tiller number but causes severe defects in spikelet formation • SL signaling repressor TaD53 interacts with miR156-regulated TaSPLs to repress TaTB1 and TaBA1, affecting tillering and spikelet development	[36], [42]
	Grain development	GRF1, GRF6, GRF9 (Growth-Regulating Factor genes), miR396 Haplotypes	• The miR396 family regulates grain filling by targeting GRF genes GRF1, GRF6, GRF9 • A total of 17 miR396 members were identified in wheat, including 5 unique haplotypes (miR396a and miR396n) not found in other species • Polyploidization has driven diversification of the miR396 family, enhancing functional networks involved in grain filling	

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
Zea mays	Leaf polarity establishment, Adaxial-abaxial patterning	miR166, miR390, tas3-derived ta-siRNAs	• miR166 accumulation is regulated by transcription of its precursor loci and modulated by tas3-derived ta-siRNAs, which impact leaf polarity • Tas3-derived ta-siRNAs restrict miR166 expression to the adaxial side of the leaf, aiding in abaxial side establishment • miR390, an upstream determinant, triggers the biogenesis of tas3 ta-siRNAs and regulates spatial accumulation of miR166	[50]
Glycine max	Shoot Apical Meristem (SAM) development, Leaf development	miR166, miR4422a, miR390	• High-throughput sequencing and microarray analysis identified 32 conserved miRNA families and 8 novel miRNAs in SAM and mature leaf tissues • miR166 plays different roles in leaf development • miR4422a was localized to the nucleus, indicating a potential nuclear function • Expression pattern of miR390 suggested its involvement in regulating SAM development	[51], [53], [54], [55], [55]
	Root nodule formation, Lateral root development	GmARF8a, GmARF8b, Auxin Signaling	• miR167 positively regulates lateral root and nodule formation by repressing GmARF8a and GmARF8b auxin response factors • miR167c expression is upregulated in soybean root vasculature, pericycle, cortex after inoculation with *Bradyrhizobium japonicum* • miR167 promotes lateral root formation and positively regulates nodule numbers by repressing GmARF8a and GmARF8b	
	Stress-induced flowering	AtNF-YA2, FLOWERING LOCUS C (FLC), FT, LFY	• miR169d-overexpressing plants flower early, independent of photoperiod, ambient temperature, ageing, and gibberellin pathways • miR169d represses AtNF-YA2, reducing FLC expression and triggering early flowering • Stress-induced flowering is mediated by a novel signaling pathway involving miR169	
	Juvenile development, Vegetative phase transition	SPL transcription factors (SPL family)	• gma-miR156a regulates vegetative development and the juvenile-to-adult phase transition • Overexpression of gma-miR156a promotes juvenile development by downregulating SPL family genes	
	Reproductive phase, Flowering initiation	AP2-like transcription factors	• gma-miR172a is highly expressed under inductive flowering conditions, playing a pivotal role in the reproductive phase transition by downregulating AP2-like genes • Overexpression of gma-miR172a accelerates flowering by regulating AP2-like genes	

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
Hordeum vulgare	Lodicule development, Floret opening, Cleistogamy	Cly1 (APETALA2 family transcription factor)	• miR172 and Cly1 co-localize in the lodicule primordium • A mutation in the miR172 target site of Cly1 reduces CLY1 protein levels but not transcript levels • miR172a's role is in cleistogamy regulation and Cly1 regulates floret opening by controlling lodicule development	[56]
Medicago truncatula	Root development, Nodulation	ARF10, ARF16, ARF17, mtr-miR160 variants	• miR160 regulates root growth and nodulation by targeting ARF genes • miR160 overexpression leads to root growth defects, disorganization of the root apical meristem (RAM), gravitropism defects, and reduced nodule numbers	[41]
Brassica species	Flowering time regulation, Floral organ identity	AP2, AP2-like genes	• Overexpression of miR172 variants accelerates flowering time but causes floral organ defects in some lines • Overexpression of miR172b, miR172d, and miR172e accelerates flowering, with miR172e causing marginal earliness • miR172e also led to altered floral organ formation compared to miR172b and miR172d, indicating divergence in the target spectrum	[57]
Solanum lycopersicum	Flower development, Floral organ identity	APETALA2-like transcription factors (AP2-like genes)	• Loss-of-function and hypomorphic mutations in miR172c and miR172d cause floral organ abnormalities (petals and stamens convert to sepaloids) • CRISPR-Cas9 co-targeting of SlMIR172c and SlMIR172d creates mutants (slmir172c-dCR) with graded floral abnormalities • Sly-miR172d regulates floral organ identity and number by repressing APETALA2-like targets	[58], [60], [45], [61], [37],
	Fruit morphology regulation	SlGAMYB2, SlGA3ox2 (GA biosynthesis pathway)	• Sly-miR159 regulates fruit size and shape by repressing SlGAMYB2, which modulates GA biosynthesis • Overexpression of SlGAMYB2 results in larger fruits with a reduced length/width ratio, while its loss leads to smaller, more elongated fruits	

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
	Flower and fruit growth regulation	SlGRFs (Growth Regulating Factors)	• miR396 regulates growth and development of flowers, sepals, and fruit by targeting SlGRFs • Overexpression of STTM396a/396a-88 in tomato leads to downregulation of miR396a and miR396b, resulting in upregulation of SlGRFs • Transgenic lines showed larger flowers, sepals, and fruits, with sepal length increasing by 75–81% and fruit weight increasing by 39–45%	
	Ovary development, Fruit set	SlGAMYB1, SlGAMYB2, Auxin signaling, Gibberellin (GA) signaling	• Overexpression of SlMIR159 causes precocious fruit initiation and parthenocarpy (seedless fruits) • Silencing SlGAMYB1/2 in these plants disrupts ovule development pathways, including AINTEGUMENTA-like genes and the miR167/SlARF8a module • The miR159/GAMYB1/2 pathway modulates auxin and gibberellin responses during ovule and ovary development, contributing to fruit initiation	
	Ovary and early fruit development	SPL genes, LeT6/TKN2, GOBLET	• miR156 regulates meristematic tissues in the ovary, influencing carpel development and organ formation • Overexpression of AtMIR156b causes abnormal flower and fruit morphology, including extra carpels and ectopic organ formation • miR156 plays a role in meristem maintenance and early fleshy fruit development	
Fortunella hindsii (Hong Kong Kumquat	Pollen development, Seedlessness, Fruit size	DUO1, YUC2/YUC6, SS4, STP8, Auxin Signaling, Starch Metabolism	• miR159a overexpression or DUO1 knockout causes small, seedless fruits and pollen abortion due to arrested pollen mitotic I and abnormal starch metabolism • DUO1 is the key target of miR159a for regulating male sterility in citrus • The miR159a-DUO1 module modulates auxin signaling and starch metabolism, crucial for proper pollen development and male sterility in citrus	[62]
Actinidia deliciosa	Floral organ identity, Floral development, Dormancy	AP2 (APETALA2-like transcription factor)	• miR172 negatively regulates AP2 to specify floral organ identity • In the 'Pukekohe dwarf' mutant, AP2 transcript accumulation is increased and miR172 is absent in developing whorls, resulting in multiple perianth whorls and petaloid features	[59]

Table 1 (continued)

Plant species	Developmental stage	Target gene(s)/pathway(s)	Characterized ncRNAs through overexpression or loss of function	Reference
Mulberry (Morus spp.)	Vegetative phase transition	MnSPL (SQUAMOSA-promoter binding-like genes) and miR172	• miR156 regulates the MnSPL/mno-miR172 pathway, controlling the vegetative phase transition in mulberry • miR156 represses mno-miR172 and modulates the expression of nine MnSPLs, which are direct targets of miR156 • MnSPLs activate transcription of mno-miR172, but miR156 represses it to control developmental transitions	[38]
Larix leptolepis	Somatic embryogenesis, Shoot Apical Meristem (SAM) development, Rooting	LaHDZ31-34, WOX, CLV	• Overexpression of miR166a reduces normal SE formation, affects SAM development, and increases rooting and lateral root formation • miR166a down-regulates LaHDZ31-34 in transgenic lines and upregulates WOX expression threefold, suggesting an indirect role in SAM development	[48]
Malus domestica	Spur-type growth, Shoot Apical Meristem development	miR164, miR166, miR171, miR159, miR167, miR396, Hormonal Pathways	• miRNA164, miRNA166, miRNA171 regulate shoot apical meristem growth • miRNA159, miRNA167, miRNA396 regulate cell division and internode length	[49]
Vitis vinifera	GA-induced parthenocarpy	VvGAMYB, GA–DELLA (SLR1)	• VvmiR159s (a, b, c) regulate floral development in response to GA by targeting VvGAMYB • VvGAMYB expression is inversely correlated with VvmiR159s, with the lowest levels at this critical stage • DELLA proteins (SLR1) mediate GA signaling through the GA–DELLA–VvmiR159c–VvGAMYB module	[63]

(Fig.1 and Table 1). In *Malus domestica*, overexpression of miR166a affects spur-type growth, shoot apical meristem (SAM) development, and increases rooting and lateral root formation. miR166a down-regulates LaHDZ31-34 and upregulates WOX expression, suggesting an indirect role in SAM development [49] (Fig.1 and Table 1). Leaf polarity and adaxial-abaxial patterning are regulated by a complex interplay between miR166, miR390, and tas3-derived ta-siRNAs in maize. Accumulation of miR166 is controlled by the transcription of its precursor loci and further modulated by tas3-derived ta-siRNAs, which influence leaf polarity. Tas3-derived ta-siRNAs restrict miR166 expression to the adaxial side of the leaf, facilitating the establishment of the abaxial side. MiR390 acts as an upstream determinant, initiating the biogenesis of tas3 ta-siRNAs and governing the spatial accumulation of miR166. While miR390 accumulates in the adaxial region, miR166 expression is spatially regulated at both the precursor transcription and ta-siRNA biogenesis stages [50]. In *Glycine max*, miRNAs regulate key developmental processes. miR166 and miR4422a influence shoot apical meristem and leaf development, with miR166 showing distinct spatial patterns and miR4422a localizing to the nucleus [51] (Fig.1 and Table 1).

miR167 is crucial for proper auxin response and rice growth. It regulates auxin signalling by targeting OsARF6, OsARF12, OsARF17, and OsARF25, and its overexpression results in reduced plant stature and tiller number [52]. miR167 promotes lateral root and nodule formation by repressing GmARF8a and GmARF8b, especially after *Bradyrhizobium japonicum* inoculation [53]. miR169d triggers early flowering by repressing AtNF-YA2 and FLC expression, independent of environmental factors [54]. gma-miR156a promotes juvenile development by downregulating SPL genes, while gma-miR172a accelerates flowering by regulating AP2-like transcription factors during the reproductive phase [55] (Fig.1 and Table 1).

In *Hordeum vulgare*, miR172 regulates lodicule development and floret opening by targeting the Cly1 gene. miR172a is the most abundant isomer in immature spikes. A mutation in miR172's target site reduces CLY1 protein levels without affecting transcript levels. miR172 and Cly1 co-localize in the lodicule primordium, suggesting interaction. A Hv-miR172a mutant leads to small lodicules and failed growth, demonstrating its role in cleistogamy regulation [56]. Overexpression of miR172 variants in *Brassica* species accelerates flowering but causes floral organ defects. Five miR172 clusters (a–e) were identified, with Brassica species showing higher retention of miR172 compared to its target gene AP2. Overexpression of miR172b, miR172d, and miR172e accelerates flowering, with miR172e causing slight earliness in *B. juncea* and altering floral organ formation, indicating target gene divergence [57]. In *Solanum lycopersicum*, miR172 regulates floral organ identity, with mutations in miR172c and miR172d causing abnormalities in petals and stamens. Seven miR172 genes produce four species, with miR172c and miR172d being most abundant during flower development. Co-targeting these genes via CRISPR-Cas9 results in graded floral abnormalities [58]. In *Actinidia deliciosa*, miR172 negatively regulates AP2 to specify floral organ identity. In the 'Pukekohe dwarf' mutant, increased AP2 transcript levels and absence of miR172 in developing whorls cause multiple perianth whorls and petaloid features. AP2 in kiwifruit is homologous to AP2/AP2-like genes in other plants, with splice variants detected, including one lacking the miR172 target site. Additionally, AP2 regulates dormancy, accumulating in axillary buds before and after budbreak [59] (Fig.1 and Table 1).

For fruit morphology, miR159 regulates fruit growth by repressing SlGAMYB2, which modulates the GA biosynthesis pathway, influencing fruit size and shape. Overexpression of SlGAMYB2 leads to larger fruits, while loss of function results in smaller, elongated fruits [60]. miR159 also affects ovary development and fruit set by modulating auxin and gibberellin pathways, while overexpression leads to parthenocarpy [61]. In *Fortunella hindsii*, overexpression of csi-miR159a or DUO1 knockout results in seedless, small fruits and pollen abortion due to arrested pollen development and abnormal starch metabolism. Cross-pollination experiments confirm DUO1 as the key target of miR159a in regulating male sterility. DAP-seq and RNA-seq identify YUC2/YUC6, SS4, and STP8 as downstream targets of DUO1, involved in auxin signalling, starch metabolism, and sugar transport. The miR159a-DUO1 module plays a crucial role in pollen development and male sterility in citrus [62]. In *Vitis vinifera*, VvmiR159s regulate floral development in response to GA by targeting Vv-GAMYB. VvmiR159c shows peak expression before flowering, with its levels inversely correlated to VvGAMYB. The GA–DELLA–VvmiR159c–VvGAMYB module mediates parthenocarpy, offering insights for seedless grape breeding [63] (Fig.1 and Table 1).

Epigenetic regulation of ncRNAs has also been shown to play an important role. For example, the epigenetic regulation of miR396 impacts both vegetative phase transitions and flowering time, emphasizing the complex regulation of plant development by ncRNAs [64] (Fig.1 and Table 1). Additionally, miR396 negatively regulates leaf size and promotes early flowering by targeting growth-regulating factors (GRFs) [65].

Role of ncRNAs in plant stress responses

Extensive research has delved into the regulatory functions of non-coding RNAs in diverse stress scenarios within the plant kingdom. Stress signals activate many regulatory ncRNAs, which interact with target transcripts to coordinate important stress-responsive pathways [66]. A study used miRNA chips covering almost all known miRNAs in *Arabidopsis* to analyse the expression of 117 miRNAs in salinity, drought, and low temperatures [67]. Seventeen stress-responsive miRNAs were detected. Their expression patterns were validated through analysis, which included studying the cis-regulatory elements in their promoter sequences. Sunkar *et al.* developed a library of small RNAs from *Arabidopsis* seedlings. The seedlings were subjected to a range of abiotic stresses, such as high salinity, dehydration, cold and abscisic acid [68]. They discovered several novel miRNAs that are responsive to abiotic stress. For instance, treatments with ABA, cold, dehydration, and salinity elevated the expression of miR393. Under usual stress conditions, there was an insignificant upregulation of miR402 and miR397b. Cold stress was the sole treatment that selectively induced miR319c; the other treatments did not. On the other hand, miR389a was downregulated in response to all stresses. These results imply that stress-induced miRNAs either target positive regulators of processes that are impeded by stress or negative regulators of stress responses. A few recently discovered miRNAs showed expression patterns specific to particular tissues or developmental stages (see Fig. 1).

A comprehensive analysis of differentially modulated non-coding RNAs in stress responses of field crops

Rice (*Oryza sativa*)

According to a study profiling microRNA expression in drought-stressed rice (*Oryza sativa*) using oligonucleotide microarrays, miR-169g is the only member of the miR-169 family that exhibits a significant increase in response to drought. Remarkably, this upregulation of miR-169g was most pronounced in the roots (Table 2) [69]. Likewise, Zhou et al., [70] used a microarray platform to thoroughly investigate microRNA expression in rice affected by drought stress throughout different developmental stages, ranging from tillering to inflorescence formation. 16 microRNAs were shown to be highly downregulated in response to drought stress in their study (miR529miR1035, miR396, miR159, miR168, miR171, miR39, miR319, miR170, miR1088, miR408, miR172, miR896, miR1030, miR156, miR1050). In contrast, 14 microRNAs (miR901, miR1125, miR169, miR851, miR319, miR845, miR171, miR854, miR896, miR474, miR395 miR903, miR1026, and miR159) considerably upregulated during the drought stress (see Table 2, Fig. 2). These findings underscore the complex and dynamic nature of miRNA responses to drought stress in rice and offer insightful information about the regulatory systems that allow plants adapt to environmental challenges [70]

In another investigation, RNA-seq studies of rice leaf subjected to drought stress uncovered distinct expression patterns of noncoding RNAs. This analysis identified 98 long noncoding RNAs (lncRNAs) and their associated antisense transcripts, some of which responded to drought stress. These lncRNAs exhibited significant regulation under drought conditions, with their expression levels inversely correlated with those of putative target genes. A total of 98 longer than 1 kb drought-responsive lncRNAs had been identified, including their corresponding antisense transcripts. Of these, 31 were upregulated, and 67 were downregulated. Two natural antisense transcripts (NATs) were identified that showed inverse correlations with their target genes. Specifically, NAT Os02g0250700-01 exhibited an inverse correlation with its target gene Late embryogenesis abundant protein (LEA), and NAT Os02g0180800-01 exhibited an inverse correlation with its target gene cinnamonoyl-CoA reductase) [71] (Table 2, Fig. 2).

Furthermore, Dongxiang wild rice (DXWR), renowned for its exceptional drought resistance, is highly valued as a key genetic resource for developing drought-resistant rice varieties. A study discovered that 1092 lncRNAs expressed differentially when exposed to drought stress. MSTRG69391 was the most significantly upregulated lncRNA, followed by MSTRG41712 and MSTRG68635. On the contrary, MSTRG65848, along with MSTRG27834 and MSTRG46301, showed the most significant downregulation among all lncRNA (Table 1). The results of these investigations improve our knowledge of the biology of lncRNAs. Additionally, they suggest potential candidates regulators that could possibly be used to genetically improve rice cultivars' resilience to drought [72].

Salinity is a major abiotic stress, affecting approximately 6% of the global arable land. According to a study, rice miRNAs responsive to increased salt are miR-169g and miR-169n (o) [73]. Within a miRNA cluster, miR-169n and miR-169o are situated 3,707 base pairs apart. The study also revealed that these miR-169 members specifically target and cleave NF-YA gene Os03g29760, which encodes a CCAAT-box binding transcription factor that regulates the transcription of various genes (Table 1, Fig. 2). Furthermore, several members of the ath-miR-169 family were found to be upregulated in response to high salinity.

Cold stress is a significant abiotic factor that adversely affects rice yields by damaging tissue and impeding growth. A recent study identified 18 rice miRNAs responsive to cold stress through microarray analysis. Among the 18 identified cold-responsive miRNAs, most exhibited downregulation, with notable changes observed in the miR-167 and miR-319 families. The expression patterns of these miRNAs varied over time: some, such as miR-166k and miR-166m, were initially upregulated but returned to baseline, while others, like miR-1868 and

Table 2 Differentially modulated non-coding RNAs in stress responses of important field crops

Crop	Regulated ncRNAs	Stress condition	Reference
Rice	miR-169 g	Drought	[69]
	miR901, miR169, miR1125, miR1126, miR156, miR851, miR474, miR170, miR171, miR172, miR396, miR397, miR408, miR896, miR1030, miR1035, miR1050, miR1088, and, miR159, miR159, miR854, miR319, miR171, miR319, miR395, miR845, miR896, miR168, miR903, miR1026 and miR529	Drought	[70]
	NAT Os02g0180800-01 and NAT Os02g0250700-01	Drought	[71]
	MSTRG41712, MSTRG27834, MSTRG46301, MSTRG68635, MSTRG65848 and MSTRG69391,	Drought	[72]
	miR-169 g and miR-169n (o)	Salinity	[73]
	miR319b, miR167e, miR167g, miR167i, miR167d miR319a,, miR167f, miR167j, miR167h	Cold	[74]
	miR604, miR606, miR601, miR603, miR602	Cadmium stress	[75]
	miR528-ASCORBATE OXIDASE (AO)	Virus defence	[77]
	Identified 1197 differentially expressed genes (DEGs), 131 lncRNAs (DELs), and 52 miRNAs (DEMs) Significant expression changes in response to glyphosate, suggesting that SPL12, osa-miR156a, and certain lncRNAs play a role in glyphosate tolerance	Herbicide stress	[78]
Wheat	miR2118, miR5049, miR408, miR396, miR160, miR1858, miR172, miR395, miR166, miR159, miR472, miR477, miR482, and miR16	Drought	[80]
	miR2012, miR396, miR827, miR159, miR159, miR2013, miR2006, miR393, miR444, miR2005, miR2001, miR827, miR2001, miR17, miR2008, miR156, miR164, and miR2011	*Erysiphe graminis*	[79]
	miR160, miR172, miR166, miR827, miR159, miR156, miR2005, miR169, and miR168	Heat stress	[79]
	Identified 5,309 long non-coding RNAs, 1,574 fusion genes, and 739 transcription factors	Water logging	[81]
Maize	7245 lncRNAs were identified, including 637 nitrogen-responsive lncRNAs	Nitrogen response	[82]
	miR164-MYB, miR156-SPL, miR159-MYB, miR160-ARF, and miR164-NAC	Drought	[83]
	Identified 6,099 long non-coding RNAs (lncRNAs), with 3,190 differentially expressed	Water logging	[84]
Barley	Hvu-miR156, Hvu-miR166, Hvu-miR171, and Hvu-miR408	Drought	[85]
	CNT0018772 *CNT0031477*	Salinity	[86]
	50 miRNAs responsive to aluminium stress	Metal stress	[87]
Chickpea	4446 differentially expressed lncRNAs	Salinity	[88]
	miRNAs: miR5213, miR5232, miR2111, miR2118 and miR530	*Fusarium oxysporum*	[89]
	miR167, miR168, miR171, miR390, miR2118, nov-miR8 and nov-miR2	Salinity stress	[90]
Soybean	miR-Seq15, miR-Seq13, miR166f, miR169f-3p, miR397ab, miR-Seq11, miR1513c, and miR166-5p	Drought stress	[92]
	miR166f, miR397ab, miR-Seq13, miR169-3p miR482bd-3p, miR1513c, miR166a-5p, miR4415b and miR-Seq15ab	Rust stress	
	Identified 20 conserved miRNA families (gma-miR156 a,b,c,d,e, gma-miR159b,c, gma-miR160, gma-miR162, gma-miR164, gma-miR166ab, gma-miR167a,b,c, gma-miR168, gma-miR169b,c, gma-miR171a, gma-miR172a,b, gma-miR319a,b,c, gma-miR390a,b, gma-miR393, gma-miR396a,b, gma-miR397) Identified 35 novel miRNA families (gma-miR1507, gma-miR1508, gma-miR1509, gma-miR1510, gma-miR1511, gma-miR1512, gma-miR1513, gma-miR1514a, gma-miR1514b, gma-miR1515, gma-miR1516, gma-miR1517, gma-miR1518, gma-miR1519, gma-miR1520a, gma-miR1520b, gma-miR1520c, gma-miR1520d, gma-miR1521, gma-miR1522, gma-miR1523, gma-miR1524, gma-miR1525, gma-miR1526, gma-miR1527, gma-miR1528, gma-miR1529, gma-miR1530, gma-miR1531, gma-miR1532, gma-miR1533, gma-miR1534, gma-miR1535, gma-miR1536, gma-miR171b, gma-miR482)	*Bradyrhizobium japonicum* inoculation / nodulation	[91]
Common bean	49 novel miRNAs and 120 known miRNAs were identified	Drought	[94]

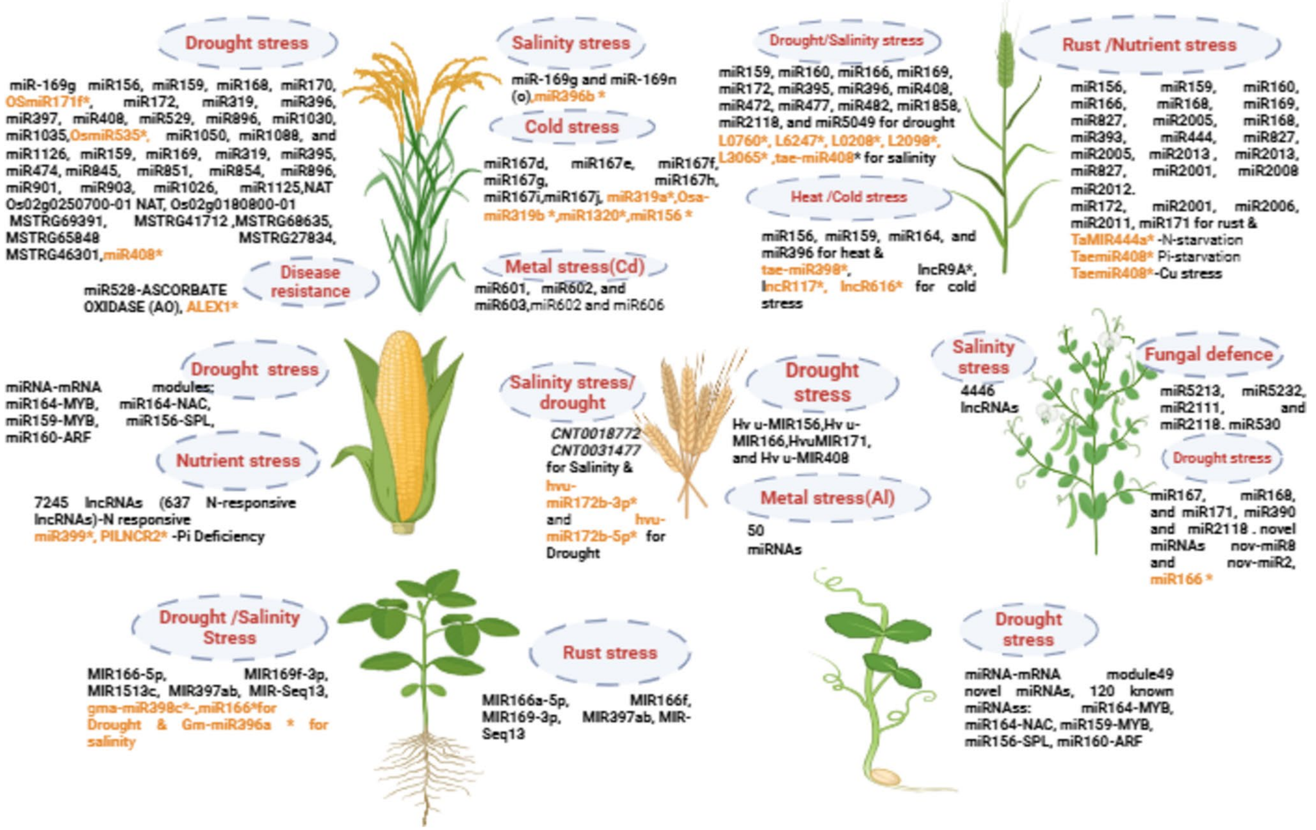

Fig. 2 Atlas of ncRNAs showing their functional diversity in field crops (cereals, pulses and legumes): Rice, wheat, maize, barley, chickpea, soyabean and common bean, respectively (from left to right). The tagged ncRNAs (* brown colored) have been functionally validated, primarily using overexpression and knockout/ knockdown studies

miR-1850, showed transient upregulation followed by downregulation. miR-1435 and miR-535 demonstrated sustained positive induction at 24 hours. Downregulated miRNAs, including miR-167a and miR-319a, experienced significant reductions after 12 hours, with others showing declines after 6 hours. Early downregulation was observed in miR-444a, miR-1320, miR-1876, miR-171a and miR-156k after 1 hour. Overall, these findings highlight the dynamic response of rice miRNAs to cold stress, shedding light on the regulatory processes that underlie rice's ability to adapt to cold stress (Table 2, Fig. 2) [74].

Additionally, to explore novel miRNAs regulated by heavy metal stress, a study created a small RNA library from rice seedlings subjected to toxic concentrations of cadmium (Cd^{2+}). Sequencing and analysis of the library revealed 19 novel miRNAs belonging to six different families. The study emphasized the distinct expression patterns of rice miRNAs in response to Cd exposure in both leaves and roots. Specifically, miR603, miR602, and miR601 were found to be upregulated in the roots, whereas miR602 and miR606 were downregulated in the leaves. Furthermore, miR604 exhibited reduced levels in the roots (Table 2, Fig. 2) [75].

Beyond regulating stress responses through miRNA-target modules in plants, evidence supports their involvement in biotic stress responses triggered by bacteria, fungi, viruses, and insects [76]. The miR528-Ascorbate oxidase (AO) module is critical in boosting antiviral responses in rice viral defence. After infection with rice stripe virus (RSV), miR528, in conjunction with AGO18, enhances ascorbate oxidase (AO) activity. This increase in AO activity results in higher levels of basal reactive oxygen species (ROS), which bolsters the plant's defence mechanisms against the virus (Table 2) [77].

Glyphosate has become an integrated component of weed management in crops. An interesting study explored the molecular responses of rice to glyphosate stress, focusing on miRNAs, lncRNAs, and mRNAs [78]. The non-transgenic glyphosate-tolerant germplasm CA21 was treated with glyphosate, and high-throughput sequencing identified 1197 differentially expressed genes (DEGs), 131 lncRNAs (DELs), and 52 miRNAs (DEMs). The study found that SPL12 was a target of osa-miR156a_L+1, and a lncRNA-miRNA-mRNA regulatory network was established. The results showed significant expression changes in response to glyphosate, suggesting that SPL12, osa-miR156a, and certain lncRNAs play a role in glyphosate tolerance. These findings contributed to the development of glyphosate-tolerant rice varieties.

Wheat (*Triticum aestivum* L.)

In an early study small RNA libraries were created from wheat leaves to investigate the role of miRNAs in regulating wheat's response to powdery mildew infection and heat stress [79]. The study identified miRNAs with distinct expression patterns in response to powdery mildew, with some specific to susceptible or resistant lines and others affecting both. Validation through northern blotting supports the sequencing results and underscores the role of these miRNAs in wheat's defense mechanisms. Solexa high-throughput sequencing was employed to discover 153 miRNAs, which were identified in 51 known and 81 unknown families. Of these, it was found that 12 miRNAs responded to heat stress, and 24 miRNAs responded to powdery mildew infection. According to the study, novel wheat miRNAs may regulate 149 target genes (Table 2, Fig 2). Northern blot analysis confirmed that miR156 was downregulated in both genotypes, miR164 in JD8-Pm30 only, and miR393 in JD8-Pm30 but not JD8 [79]. In the same study, for heat stress analysis, miR172 decreased significantly while, miR168, miR2005, miR159, miR160, miR156, miR169, miR827and miR166 were upregulated, with miR168 showing the highest increase of 2.9-fold. Northern blot analysis validated the expression patterns of 9 miRNAs in TAM107 and the heat-susceptible Chinese Spring (CS) genotype. Furthermore, 9 miRNAs were found to be co-regulated by both powdery mildew infection and heat stress, with miR827 and miR2005 upregulated in response to both stressors, suggesting their potential role in managing both abiotic and biotic stress in wheat [79]

Additionally, a study investigated drought stress-responsive miRNAs in bread wheat (*Triticum aestivum* cv. Sivas 111/33) using miRNA microarray screening. The analysis revealed that distinct expression patterns of these miRNAs and their target transcripts were identified between wheat cultivars that are drought-tolerant and those that are drought-sensitive. Notable drought-responsive miRNAs included miR396, miR1858, miR160, miR169, miR172, miR395, miR166, miR2118, miR159, miR472, miR477, miR482, miR408, and miR5049. Regulatory network analysis highlighted that miR395 targets multiple transcripts, while miR159 and miR319 share several target genes (Table 2, Fig. 2) [80]

Waterlogging impacts wheat production, and PacBio SMRT combined with Illumina sequencing has been used to study its genetic regulation. The analysis of two wheat cultivars, XM55 and YM158, identified 5309 long non-coding RNAs, 1574 fusion genes, and 739 transcription factors. Illumina sequencing of waterlogged and control plants revealed 6829 differentially expressed genes (DEGs), with photosynthesis-related genes downregulated, and steroid biosynthesis and plant hormone signaling genes upregulated. The two cultivars exhibited different genetic responses to waterlogging, offering insights into the molecular breeding of waterlogging-tolerant wheat [81].

Maize (*Zea mays*)

In maize, a critical crop cultivated in various environments, research into responses to stresses such as nitrogen (N) deficiency and drought has focused on regulatory molecules including lncRNAs and miRNAs. A study on N-deficiency stress investigated intergenic and intronic lncRNAs in maize B73 leaves at the V7 growth stage using deep sequencing. This analysis identified 7245 lncRNAs, with 637 being responsive to nitrogen deficiency and displaying distinct expression profiles. Expression network modeling revealed that these nitrogen-responsive lncRNAs were primarily grouped into one of three co-expressed modules (Table 1). This enriched module contained genes primarily involved in NADH dehydrogenase activity, oxidative phosphorylation, and nitrogen compound metabolism [82].

In a study analyzing miRNome in two maize inbred lines with different drought tolerances, 11 miRNAs uniquely responded to drought in the drought-tolerant line H082183, while 34 miRNAs were specific to the drought-sensitive line Lv28 in leaf tissues. In root tissues, 19 miRNAs in H082183 and 23 miRNAs in Lv28 uniquely responded to drought. Expression analysis of miRNA-mRNA modules showed negative regulatory interactions for miR160-ARF, miR164-MYB, miR156-SPL miR164-NAC and miR159-MYB. The miR164-MYB and miR164-NAC modules in H082183 regulated drought response in an ABA-dependent manner, whereas miR156-SPL and miR160-ARF modules in Lv28 were associated with the suppression of metabolic processes in drought-exposed leaves (Table 2, Fig. 2) [83].

A recent study on hypoxia-related regulatory network in maize under waterlogging identified 6099 long non-coding RNAs (lncRNAs), with 3,190 differentially expressed, along with protein-coding genes involved in key metabolic and hypoxia response pathways, such as glycolysis and methionine metabolism. The study also highlighted enriched transcription factor families (AP2-EREBP, bZIP, NAC, bHLH, MYB) and identified co-expression of lncRNAs with genes linked to waterlogging tolerance [84].

Barley (*Hordeum vulgare*)

Barley shows considerable genetic variation in how it responds to different abiotic stresses. A study on barley's response to dehydration identified 28 novel miRNAs across 18 families, with differential expression observed in leaf and root tissues under stress conditions [85]. Key microRNAs (miRNAs) like Hvu-miR408, Hvu-miR156, Hvu-miR171, and Hvu-miR166 were found to exhibit significant responses to dehydration stress (Table 2, Fig. 2). To investigate their role further, a modified 5′ RLM-RACE (RNA Ligase-Mediated Rapid Amplification of cDNA Ends) technique was employed, which allowed for the retrieval of seven distinct cleaved miRNA transcripts from leaves under drought stress. Complementary in silico analysis predicted a total of 15 potential EST (Expressed Sequence Tag) targets that might be regulated by these miRNAs. Expression analysis revealed a positive correlation between these miRNAs and the suppression of their target mRNA transcripts under dehydration stress [85].

Salinity is a major stressor that limits plant productivity globally. Barley, however, shows notable adaptability to salt stress. A study examining four barley genotypes (Hasat, Beysehir 99, Konevi 98, and Tarm 92) under 150 mM salt stress during a 3-day germination period revealed that lncRNA CNT0018772 was down-regulated in both roots and shoots of all genotypes, indicating a common stress response [86]. Conversely, lncRNA CNT0031477 was down-regulated in most genotypes but up-regulated in Tarm 92. This unique expression pattern in Tarm 92 may contribute to its enhanced salt tolerance (Table 2).

Although generally more sensitive to aluminum (Al) toxicity than other cereals, Barley shows significant genotypic variation in Al tolerance. A well-known mechanism of Al tolerance in barley is Al exclusion, facilitated by the Al-activated citrate transporter 1 (HvAACT1). However, recent research found that the expression of HvAACT1 and the secretion of citrate and other organic acids did not fully explain the difference in Al tolerance between the Al-tolerant wild barley XZ29 and the Al-sensitive cultivated barley Golden Promise. To investigate further, a study constructed eight small RNA libraries from the roots of barley genotypes under both control and Al-treated conditions. Deep sequencing of these libraries identified 342 miRNAs in total, with 296 being common to both genotypes. Comparative analysis identified 50 miRNAs responsive to Al stress (Table 2, Fig. 2), including some uniquely expressed in XZ29 and potentially linked to Al tolerance [87].

Chickpea (*Cicer arietinum* L.)

Chickpea (*Cicer arietinum* L.) is an important legume known for its high protein and fiber content. A recent study focused on identifying long non-coding RNAs (lncRNAs) induced by salt stress in chickpea roots and exploring their regulatory roles. The research uncovered 3452 novel lncRNAs across all eight chickpea chromosomes. Comparing salt-tolerant cultivars (ICCV 10, JG 11) to salt-sensitive cultivars (DCP 92-3, Pusa 256), differential expression analysis revealed 4446 lncRNAs with modified expression under different salt treatments. Of these, 373 lncRNAs were expected to have cis-regulatory influence over the genes they target. Furthermore, it was shown that 80 distinct lncRNAs interacted with 136 distinct miRNAs as endogenous target mimics (eTMs), indicating their role in the regulatory network for the response to salt stress. Functional analysis identified the roles of these lncRNAs in the regulation of genes associated with salt stress, including aquaporins (e.g., TIP1-2 and PIP2-5), potassium transporters, transporter family genes, serine/threonine-protein kinases, and different transcription factors (e.g., WRKY, AP2, bZIP, ERF, NAC and MYB). Additionally, about 614 lncRNA-derived simple sequence repeats (SSRs) were shown to be unique molecular markers for chickpeas that had greater efficacy and specificity (Table 2, Fig. 2) [88].

A research investigation was done to identify chickpea miRNAs linked to biotic and abiotic stresses, focusing on increasing soil salinization and *Fusarium oxysporum* f.sp. *ciceris* caused wilt disease. Three libraries were created from chickpea seedlings, one treated with salt, one untreated, and one infected with fungus, in order to study miRNA responses. In addition to 59 new miRNAs and their star sequences, the study found 122 conserved miRNAs from 25 distinct families. Four miRNAs specific to legumes were found in all libraries: miR2118, miR5232, miR5213, and miR2111 (Table 2). Notably, miR530 was significantly upregulated in response to fungal infection and targeted genes encoding zinc finger and microtubule-associated proteins (Fig. 2) [89].

Drought stress is a serious challenge to the sustainable growth and productivity of legumes such as chickpea. A study comparing the drought-tolerant cultivar Pusa 362 with the drought-sensitive SBD377 revealed that root volume was similar between the two genotypes, suggesting that drought tolerance in Pusa 362 may involve mechanisms beyond root traits. The study identified 16 validated miRNAs, among which miR171, miR167and miR168 were significantly upregulated in the roots of Pusa 362. These miRNAs regulate key components of drought stress responses, including scarecrow-like transcription factors, WD-repeat proteins, and auxin response factors. In the shoots of Pusa 362, miR390 and miR2118 were also increased. The newly identified miRNAs with the highest expression levels in Pusa 362 were nov_miR8 in the roots and nov_miR2 in the shoots. Surprisingly, nov_miR8 targets a gene encoding laccase, whilst nov_miR2 targets GMP synthase (Table 2, Fig. 2) [90].

Soyabean (*Glycine max* L.)

An early study in soybean inoculation with *Bradyrhizobium japonicum* for nodulation identified 20 conserved, and 35 novel miRNA families in its roots [91]. A subsequent study used Solexa technology to sequence eight small RNA libraries from various soybean types, including drought-sensitive and drought-tolerant seedlings and rust-susceptible and rust-resistant varieties, under both stressed and unstressed conditions. This sequencing revealed 256 miRNAs, comprising 24 novel families, six conserved families, and 22 known soybean families. miR397ab, miR166-5p, miR1513c, miR169f-3p, and miR-Seq13 were upregulated in drought-sensitive plants and downregulated in drought-tolerant ones. Under pathogen stress, miR-Seq13, miR397ab, miR166a-5p, miR169-3p, and miR166f were downregulated in susceptible plants but stable in resistant ones. miR-Seq07 was downregulated in both genotypes during rust infection, miR-Seq11 decreased in susceptible plants post-inoculation, and miR-Seq15ab was downregulated in susceptible plants but upregulated in resistant ones under stress [92] (Table 2, Fig. 2).

Common bean (*Phaseolus vulgaris*)

Legumes like common beans are crucial globally due to their high protein content and caloric value. Common bean yields are severely threatened by drought stress. However, research into microRNAs in *Phaseolus vulgaris* has been relatively limited. The first identification of miRNAs in common beans used an in silico approach in 2008 [93]. Four small RNA libraries were made from common bean cultivars that are both drought-tolerant and drought-sensitive under both drought and control circumstances in order to investigate this. Sequencing yielded 120 recognised miRNAs and 49 new miRNAs. Under drought stress, nine known miRNAs were downregulated, and seven were upregulated. Among the novel miRNAs, five were upregulated, and three were downregulated. Sixteen miRNAs that may be related to common beans' response to drought stress were identified by RT-qPCR validation [94] (Table 2, Fig. 2).

Functionally characterized non-coding RNAs in field crops: unravelling their role in stress responses

Non-coding RNAs (ncRNAs) have been functionally characterized in various cereal crops in response to different abiotic stresses, such as drought, salinity, and cold. For example, OsmiR535 negatively regulates drought, salinity, and dehydration tolerance in rice, where its knockout lines enhance resistance to these stresses [95]. Cold stress tolerance in rice has been associated with Osa-miR319b, which targets transcription factors involved in cold stress responses [96]. Osa-MIR319a and Osa-MIR319b are involved in leaf morphogenesis and cold stress tolerance. Both miRNAs are downregulated under cold stress (4 °C) but induce expression of target genes OsPCF5 and OsPCF8 [97], and miR1320, which negatively regulates cold tolerance by targeting PHD17, a protein involved in the cold signalling pathway [98]. OsmiR156 enhances cold stress tolerance by targeting OsSPL3, which regulates OsWRKY71 in rice [99] (Table 3, fig. 2). The combination of tae-miR398 and lncRNAs (LncR9A, LncR117, LncR616) regulates cold tolerance in wheat. miR398 targets CSD1 (Copper/zinc superoxide dismutase), while these lncRNAs act as competing endogenous RNAs (ceRNAs) to indirectly regulate CSD1 expression. This regulatory mechanism enhances cold stress resistance in wheat, showcasing the potential of lncRNA-miRNA interactions in stress adaptation [100].

miR408 plays a crucial role in drought tolerance in rice by targeting genes involved in blue copper proteins and other species-specific targets. Overexpression of miR408 in the drought-sensitive PB1 cultivar in rice results in improved vegetative growth, electron transport rate, photosynthetic efficiency, and dehydration stress tolerance [101]. Additionally, 83 target genes with antagonistic expression under drought stress were identified, highlighting miR408 as a positive regulator of growth and drought tolerance in rice (Table 3, Fig. 2). In Glycine max, miR166 regulates genes involved in the abscisic acid (ABA) signaling pathway, with *ATHB14-LIKE* directly activating these genes. This forms a feedback loop between miR166 and *ATHB14-LIKE*, contributing to drought resistance in soybean. Drought stress represses miR166 accumulation, leading to upregulation of its target gene *ATHB14-LIKE*. The *GmSTTM166* transgenic line, which has optimal miR166 knockdown, exhibited enhanced drought tolerance without compromising yield [102]. Another vital miRNA, Gma-miR398c, negatively regulates drought tolerance in soybean by targeting peroxisome-related genes involved in reactive oxygen species (ROS) scavenging. Overexpression of gma-miR398c in *Arabidopsis thaliana* results in reduced germination, increased leaf water loss, and decreased survival under drought conditions. In soybean, overexpression of gma-miR398c causes impaired ROS scavenging, higher electrolyte leakage, and increased stomatal opening compared to miR398c knockout and wild-type plants under drought stress [103]. In barley, drought stress accelerates flowering through two miR172b isoforms. hvu-miR172b-3p promotes flowering by cleaving AP2 genes, while hvu-miR172b-5p increases trehalose-6-phosphate synthase, boosting trehalose content for osmoprotection. After rewatering, trehalose levels decline, highlighting its role in stress mitigation and flowering induction. These modules work together to coordinate osmoprotection and flowering under drought stress [104] (Table 3, Fig. 2).

Table 3 Functionally characterized non-coding RNAs in field crops under stress conditions

Crop	Stress type	Targeted ncRNAs	Characterized ncRNAs through overexpression or loss of function	References
Oryza sativa	Drought, Salinity, Dehydration, PEG, ABA	**OsmiR535**	• OsmiR535 negatively regulates responses to drought, salinity, and dehydration stress • Knockout and inhibition of OsmiR535 enhance tolerance to NaCl, ABA, dehydration, and PEG stresses • Overexpression reduces seedling survival rates under PEG and dehydration stress recovery • CRISPR/Cas9 generated a 5 bp deletion in OsmiR535, confirming its role in stress regulation • OsmiR535 is a potential target for genetic editing to improve drought and salinity tolerance in rice	[95]
	Disease Resistance	**ALEX1 (lncRNA)**	• ALEX1 is induced by *Xanthomonas oryzae* pv. oryzae (Xoo) infection • ALEX1 activates the jasmonate (JA) signaling pathway, increasing levels of endogenous JA and JA-Ile • Overexpression of ALEX1 enhances resistance to bacterial blight	[113]
	Cold	**Osa-miR319b (miRNA)**	• Osa-miR319b is down-regulated under cold stress, but overexpression enhances cold tolerance, leading to higher survival rates and increased proline content • Osa-miR319b targets OsPCF6 and OsTCP21, transcription factors involved in cold stress response • Overexpression upregulates cold stress-responsive genes (DREB1A/B/C, DREB2A, TPP1/2) and enhances ROS scavenging, improving cold tolerance	[96]
	Cold	**Osa-MIR319a, Osa-MIR319b (miRNAs)**	• Osa-MIR319a and Osa-MIR319b are involved in leaf morphogenesis and cold stress tolerance • Overexpression of either miRNA results in wider leaf blades and increased number of longitudinal veins • Both miRNAs are downregulated under cold stress (4 °C) but induce expression of target genes OsPCF5 and OsPCF8 • RNA interference of OsPCF5 and OsPCF8 in transgenic plants enhances cold tolerance after chilling acclimation • There is a regulatory role of Osa-MIR319a and Osa-MIR319b in cold stress and leaf development	[97]
	Cold	**miR1320 (miRNA)**	• miR1320 negatively regulates cold tolerance by targeting and cleaving PHD17, a Plant Homeo Domain (PHD) protein • PHD17 affects cold tolerance by influencing ROS homeostasis, flavonoid accumulation, jasmonic acid biosynthesis and signaling • miR1320 overexpression downregulates PHD17, while knockdown upregulates it	[98]

Table 3 (continued)

Crop	Stress type	Targeted ncRNAs	Characterized ncRNAs through overexpression or loss of function	References
	Cold	**miR156 (miRNA)**	• OsmiR156 enhances cold stress tolerance by targeting OsSPL3, which regulates OsWRKY71 • OsWRKY71 negatively regulates OsMYB2 and OsMYB3R-2, which control cold stress-responsive genes like OsLEA3, OsRab16A, and OsDREB2A • Overexpression of OsmiR156 enhances cold tolerance by modulating these gene networks	[99]
	Drought	**miR408 (miRNA)**	• miR408 regulates drought tolerance by targeting genes involved in blue copper proteins and other species-specific targets • Overexpression of miR408 in the drought-sensitive PB1 cultivar improved vegetative growth, electron transport rate (ETR), photosynthetic efficiency, dehydration stress tolerance • miR408 is a positive regulator of growth and drought stress tolerance	[101]
	Salinity	**miR396b (miRNA) and GRF6 (target gene)**	• miR396b/GRF6 module enhances salt tolerance in rice, and is a suitable target for developing salt-tolerant rice with enhanced yields • MIM396 (miR396b mimic) and OE-GRF6 (GRF6 overexpression) lines showed 48.0% and 74.4% higher survival rates under salt stress compared to wild type • MYB3R is a downstream target of miR396b/GRF6 • Transgenic plants exhibited reduced H_2O_2 accumulation, increased ROS-scavenging enzymes (CAT, SOD, POD) activity	[106]

Table 3 (continued)

Crop	Stress type	Targeted ncRNAs	Characterized ncRNAs through overexpression or loss of function	References
Triticum aestivum	Alkaline (Saline-alkali soil)	**LncRNAs (L0760, L6247, L0208, L2098, L3065)**	• LncRNA–miRNA–target-mRNA networks were constructed to study roles in stress tolerance, and 19,000 novel lncRNAs were identified in wheat cultivars SR4 and JN177 • Knockdown of L0760 and L2098 increased sensitivity to alkaline stress • Knockdown of L6247, L0208, and L3065 increased stress tolerance	[108]
	Phosphate (Pi) Starvation, Salt	**TaemiR408 (miRNA)**	• TaemiR408 regulates Pi uptake under starvation, ABA signaling during salt stress • Overexpression in *Nicotiana tabacum* enhances stress tolerance, biomass, and photosynthesis; Increases NtPT2 expression for better Pi acquisition; Upregulates NtPYL2 and NtSAPK3 in ABA signaling and osmolyte synthesis • Knockdown impairs stress tolerance, confirming Tae-miR408's role in stress adaptation	[110]
	Salinity, Copper and Stripe Rust	**tae-miR408 (miRNA)**	• tae-miR408 targets TaCLP1, a chemocyanin-like protein involved in abiotic and biotic stress responses • TaCLP1 and tae-miR408 show contrasting expression patterns under Pst infection and high copper ion stress • Overexpression of TaCLP1 in *Schizosaccharomyces pombe* enhances growth under salinity and copper stress • Silencing TaCLP1 in wheat decreases resistance to stripe rust	[109]
	Nitrogen Starvation	**TaMIR444a** (miRNA)	• miR444a regulates plant tolerance to N-starvation stress by modulating NRT genes and antioxidant enzymes (CAT, POD) • Overexpression of TaMIR444a in tobacco improved growth, biomass, photosynthesis, and ROS detoxification • miR444a is a key regulator of N-starvation tolerance through N acquisition and ROS homeostasis pathways	[112]
	Cold	**tae-miR398, lncR9A, lncR117, lncR616**	• miR398 regulates cold tolerance by targeting CSD1 (Copper/zinc superoxide dismutase) • LncRNAs (LncR9A, LncR117, LncR616) act as competing endogenous RNAs (ceRNAs) to indirectly regulate CSD1 expression	[100]

Table 3 (continued)

Crop	Stress type	Targeted ncRNAs	Characterized ncRNAs through overexpression or loss of function	References
Glycine max	Drought	gma-miR398c	• gma-miR398c negatively regulates drought tolerance by targeting peroxisome-related genes involved in ROS scavenging • Overexpression of gma-miR398c in Arabidopsis thaliana resulted in reduced germination, increased leaf water loss, decreased survival under drought conditions • In soybean, overexpression of gma-miR398c caused impaired ROS scavenging, higher electrolyte leakage, increased stomatal opening compared to miR398c knockout and wild-type plants under drought	[103]
	Salinity	Gm-miR396a (miRNA)	• Gm-miR396a is crucial for soybean development and salinity tolerance • miR396a-gene-edited lines (using CRISPR/Cas9) showed enhanced salinity tolerance, increased branching, higher grain yields compared to control plants • Pre-miR396a-overexpression lines exhibited developmental defects like dwarfism, abnormal inflorescences, smaller seeds, larger stomata, downregulated photosynthesis	[107]
	Drought	**miR166** (miRNA)	• Drought stress represses miR166 accumulation, leading to upregulation of its target gene ATHB14-LIKE • The GmSTTM166 transgenic line (with optimal miR166 knockdown) exhibited enhanced drought tolerance without compromise in yield • miR166 regulates genes involved in the ABA signaling pathway, with ATHB14-LIKE directly activating these genes • ATHB14-LIKE inhibits miR166 expression, creating a feedback loop between the two, contributing to drought resistance	[102]

Table 3 (continued)

Crop	Stress type	Targeted ncRNAs	Characterized ncRNAs through overexpression or loss of function	References
Zea mays	Phosphorus (Pi) Deficiency	**miR399 (miRNA)**, **PILNCR2 (lncRNA)**	• miR399 targets ZmPHT1 to regulate Pi transport under deficiency conditions • PILNCR2, a long non-coding RNA transcribed from the opposing strand of ZmPHT1, modulates miR399's effect by preventing its cleavage of ZmPHT1 mRNAs • Overexpression of PILNCR2 increases low-Pi tolerance, while its knockdown or knockout reduces low-Pi tolerance, highlighting its role in maintaining Pi homeostasis via regulation of miR399	[111]
	Abiotic Stress (General)	**miR166** (miRNA)	• miR166 knockdown in STTM166 transgenic plants resulted in rolled leaves, enhanced stress resistance, altered vascular structure, and inferior yield-related traits • Knockdown of miR166 increased ABA levels and reduced IAA, suggesting interactions between miR166, ABA, and IAA in abiotic stress responses	[114]
Hordeum vulgare	Drought	**hvu-miR172b-3p** and **hvu-miR172b-5p** (miRNAs)	• Drought stress accelerated flowering in barley is coordinated through action of two miR172b isoforms: hvu-miR172b-3p is upregulated during drought, promoting flowering by cleaving AP2 genes, while hvu-miR172b-5p is downregulated, leading to increased levels of trehalose-6-phosphate synthase and higher trehalose content • The hvu-miR172b-5p/ trehalose-6-phosphate synthase and hvu-miR172b-3p/AP2-like modules coordinate osmoprotection and accelerated flowering under drought stress	[104]
Cicer arietinum	Drought	**miR166** (miRNA)	• miR166 overexpression in chickpea enhanced drought tolerance and improved plant survival. It improved physiological parameters and enhanced biochemical markers • miR166 positively interacted with PGPR (*Pseudomonas putida* RA) to further enhance drought resistance • ATHB15 was validated as a target via RLM-RACE	[105]

In *Cicer arietinum* (chickpea), overexpression of miR166 enhanced drought tolerance, improving photosynthesis, transpiration, and water-use efficiency. It increased proline content, SOD, peroxidase, and catalase activity, while reducing H_2O_2 levels. miR166 also interacted with *Pseudomonas putida* RA (PGPR) to boost drought resistance, and *ATHB15* was identified as its target via RLM-RACE. [105] (Table 3, Fig. 2).

The miR396b/GRF6 module enhances salt tolerance in rice. Transgenic rice plants with miR396b mimic (MIM396) and GRF6 overexpression (OE-GRF6) exhibited 48.0% and 74.4% higher survival rates under salt stress compared to wild-type plants. These lines showed reduced H_2O_2 accumulation and increased activity of ROS-scavenging enzymes (CAT, SOD, POD). ZNF9 was identified as a negative regulator of salt tolerance, while MYB3R, a downstream target of miR396b/GRF6, further enhanced salt tolerance, suggesting that the miR396b/GRF6 network could be targeted to develop salt-tolerant rice with enhanced yields [106]. Gm-miR396a is crucial for soybean development and salinity tolerance. Gene-edited miR396a-GE lines, created using CRISPR/Cas9, showed enhanced salinity tolerance, increased branching, higher grain yields, and improved growth compared to control plants. However, overexpression of pre-miR396a (Pre-miR396a-OE lines) resulted in developmental defects such as dwarfism, abnormal inflorescences, smaller seeds, larger stomata, and downregulation of photosynthesis-related genes [107].

In wheat, the identification of 19,000 novel lncRNAs in cultivars SR4 and JN177 revealed differential expression under alkaline (saline-alkali) stress. SR4 showed the differential expression of 5691 lncRNAs, while JN177 expressed 5932. Knockdown of specific lncRNAs (L0760 and L2098) increased sensitivity to alkaline stress, while knockdown of L6247, L0208, and L3065 enhanced stress tolerance. The study also constructed lncRNA–miRNA–target-mRNA networks, highlighting that some lncRNAs promote tolerance while others increase sensitivity to stress [108] (Table 3, Fig. 2).

TaemiR408 plays a key role in regulating phosphate uptake under starvation and ABA signaling during salt stress in wheat. Overexpression of TaemiR408 in *Nicotiana tabacum* enhances stress tolerance, biomass, and photosynthesis, while also improving Pi acquisition by increasing NtPT2 expression [109]. It also upregulates NtPYL2 and NtSAPK3 involved in ABA signaling and osmolyte synthesis. Knockdown of these targets impaired stress tolerance, confirming TaemiR408's role in stress adaptation [110]. Tae-miR408 targets TaCLP1, a chemocyanin-like protein involved in both abiotic and biotic stress responses. Overexpression of TaCLP1 in *Schizosaccharomyces pombe* enhanced growth under salinity and copper stress, while silencing TaCLP1 in wheat reduced resistance to stripe rust. The contrasting expression patterns of TaCLP1 and tae-miR408 under Pst infection and high copper stress suggest that tae-miR408 is a key regulator of wheat stress tolerance [109]. In maize, phosphorus (Pi) deficiency causes miR399 upregulation, which targets *ZmPHT1;1*, *ZmPHT1;3*, and *ZmPHT1;13* to regulate Pi transport. *PILNCR2*, a long non-coding RNA transcribed from the opposing strand of *ZmPHT1;1*, modulates the effect of miR399 by preventing its cleavage of *ZmPHT1* mRNAs. Overexpression of *PILNCR2* increases low-Pi tolerance, while knockdown or knockout of *PILNCR2* reduces tolerance to low phosphorus, highlighting its role in maintaining Pi homeostasis via regulation of miR399 [111].

TaMIR444a regulates wheat's tolerance to nitrogen starvation by modulating NRT genes (e.g., NtNRT1.1-s, NtNRT2.1) and antioxidant enzymes (e.g., NtCAT1;1, NtPOD1;3). Overexpression of TaMIR444a in tobacco improves growth, biomass, nitrogen content, photosynthesis, and ROS detoxification. Transcriptome analysis identified genes involved in signal transduction, metabolism, and phytohormone response, highlighting miR444a as a key regulator of nitrogen-starvation tolerance [112]. ALEX1, a lncRNA, regulates disease resistance in rice by activating the jasmonate pathway [113].

These studies underscore the critical role of ncRNAs in modulating stress responses and improving tolerance to environmental stresses in key field crops.

Conclusion

The emerging field of plant non-coding RNAs (ncRNAs) underscores their pivotal role in regulating various biological processes, yet their full potential in crop research remains underutilized. Despite the growing body of work, most of these ncRNAs have not yet been functionally evaluated in the context of stress. The increasing availability of reference genomes and advances in transcriptome sequencing and computational tools offer valuable opportunities for comparative analyses, revealing ncRNA sequence similarities and functional conservation across species. There is a need to construct a 'comprehensive genome-wide atlas' of ncRNAs for crop plants, and identify 'biomarkers for stresses' like drought, salinity, cold, metal stress, nutrient stress, herbicide stress, waterlogging and pathogen infections. The lack of specific mutant lines for ncRNAs in modal plants like Arabidopsis presents a challenge in their functional validation under stress. Future research should focus on developing mutant lines using genome-editing technologies for functionally validating novel ncRNAs and identifying 'candidate ncRNAs'. Such advancements could facilitate the targeted development of crops with enhanced resilience to abiotic and biotic stresses, advancing agricultural productivity and sustainability.

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Introduction of saffron in non-traditional areas of Jammu & Kashmir in India: farmers' perspective for policy makers

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SHORT COMMUNICATIONS

Introduction of saffron in non-traditional areas of Jammu & Kashmir in India: farmers' perspective for policy makers

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Abstract

Saffron occupies a vital place in the agricultural scenario of Jammu and Kashmir, India. India ranks only second to Iran in saffron production, yet it ranks twelfth among global saffron exporters. Therefore, we need to explore ways to promote both its production and export. Traditional saffron areas of Jammu and Kashmir are not able to meet the local demand within India, and therefore India imports saffron of around 88 tons per annum, causing an expenditure of US$ 38.2 million to the exchequer. This trend can be reversed by introducing saffron in non-traditional areas of J&K. Herein, we discuss the issue from the perspective of farmers of the non-traditional areas involved in saffron on-farm trials in 16 districts of J&K. While the environmental suitability of an area with respect to its climate is the prime requisite for growing saffron, however, the 'know-how' of farmers regarding the management practices involved in the cultivation-module of this peculiar crop (saffron) is vital for its successful introduction in the non-traditional areas. Saffron cultivation involves several unique practices, and our study revealed a low level of knowledge among the farmers of new areas about these practices. However, the most encouraging observation was that the respondents expressed a willingness to grow saffron in new areas, provided some government-sponsored schemes are set up to support them.

Keywords Saffron · Crocus sativus · Climate change · Survey · Farmers

Background and introduction

The Union Territory of Jammu and Kashmir in western Himalaya is blessed with the cultivation of saffron (*Crocus sativus* var. cashmerianus Royle), which dates back to 5th century BC. It grows on upland karewas located at an altitude of 1585 to 1677 m above the mean sea level, which makes it the highest altitude grown saffron in the whole world. Although India ranks second to Iran in saffron production, it is twelfth among global saffron exporters. Unlike Spain, France and Italy, which import Iranian saffron, add value to it, and resell it at a higher price. India imports around 88 tons of saffron annually to meet domestic demand, which represents 15.7% of the total global saffron imports. Saffron worth US$ 38.2 million was imported to India in 2024 to satisfy its demand, making it the world's second-largest importer. Unfortunately, saffron cultivation in the traditional saffron-growing regions of Kashmir has faced a severe crisis for over two decades, as evident from its dwindling share in global production (Husaini et al. 2013).

Climate change is emerging as a big challenge to saffron cultivation (Husaini 2014). The maximum area recorded under saffron crop was 5707 hectares (in 1997), with an estimated production of 16 tons at that time (Husaini et al. 2010). Several factors have led to the declining trend in its area and production (Husaini et al. 2013), amongst which climate change is the most challenging. Although the phenology of saffron is well defined for Kashmir, climate change has led to erratic weather conditions, causing variations in the onset of these developmental stages. A significant limitation to saffron cultivation in traditional areas is that saffron fields are almost entirely rain-fed, with minimal irrigation facilities. The total rainfall during the saffron growing period is usually sufficient, but its distribution is

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Table 1 Major crops grown at non-traditional sites and corresponding income generation potential

Site	Major crops grown	Annual Income generation
Malangpora (Pulwama)	Rice, Apple, Almond, Saffron	High
Pombay (Kulgam)	Rice, Apple	Medium
Balpora (Shopian)	Apple, Walnut	High
Dooru (Anantnag)	Oats, Rice, Mustard	Low
Kulangam (Kupwara)	Walnut	Low
Udina (Bandipora)	Wheat, Maize, Millets	Low
Mirgund (Baramulla)	Mulberry, Rice	Low
Harran (Budgam)	Vegetables	Low
Shuhama (Ganderbal)	Rice, Vegetables	Medium
Shiva (Doda)	Maize, Vegetables	Low
Meer (Udhampur)	Flowers, Maize, Millets	Low
Mahore (Reasi)	Maize, Oats	Low
Gandhari (Ramban)	Maize, Beans	Low
Budhal (Rajouri)	Fodder maize	Low
Mandi (Poonch)	Maize, Vegetables	Low
Benhama (Ganderbal)	Forest trees, Walnut	Low
Shalimar (Srinagar)	Vegetables, Rice	High
FOH Shalimar	Forest trees	Medium

irregular, and it has now become common for saffron to face water stress.

The huge gap between supply and the domestic demand for saffron can be met by extending its cultivation to new sites. There have been several attempts to introduce the crop in non-traditional areas of northern India through ecological modelling, some of which have shown promising results (Kumar et al. 2022). Our earlier study demonstrated the technical feasibility of cultivating saffron in the most neglected and marginal areas of the trans-Himalayan region, specifically in the Jehlum and Chenab valleys of Kashmir (Sheikh et al. 2023). Furthermore, we experimented with the introduction of organic saffron in kitchen gardens to meet the household requirements of saffron, and the results were encouraging (Husaini and Wani 2020). The areas receiving 100–150 cm of well-distributed rainfall with snow in winter are suitable for saffron cultivation, and rains in September are essential for meeting the water requirement of corms for good flower yields. Several recent studies show that the production of saffron through organic means is a way forward, and incorporating biofertilizers in its production technology can add further economic value to it (Magotra et al. 2021; Naik et al. 2024).

According to our estimate, increasing the area from the current (3785 ha) to a projected (12404 ha) will generate employment opportunities, and will increase saffron production from 16.5 tons to 78 tons, which will fetch an additional exchequer of Rs 9 billion (Rs 900 crore). Hence, there is a need to popularise saffron outside the traditional belt and share its production technology with common people in semi-arid regions and areas not suitable for irrigated crops. The results presented are based on a study that was carried out in Jammu & Kashmir, in ten districts of Kashmir and six districts of the Jammu region. Herein, we discuss the saffron diversification in non-traditional areas of J&K from the farmers' perspective.

Table 2 Variables considered for the evaluation of knowledge about saffron and their assigned coding

S. No	Variables	Answer	Coding
1.	Have you ever heard about saffron?	Yes	1
		No	0
2.	Is saffron grown in your area?	Yes	1
		No	0
3.	Do you know how saffron is grown?	Yes	1
		No	0
4.	Do you know which part of saffron is marketable?	Yes	1
		No	0
5.	Do you know how saffron is harvested?	Yes	1
		No	0
6.	Do you consume saffron?	Yes	1
		No	0
7.	Are you interested in growing saffron?	Yes	1
		No	0
8.	Do you have suitable land for growing saffron?	Yes	1
		No	0
9.	Do you feel the need of govt. sponsored scheme for growing saffron?	Yes	1
		No	0
10.	Are you satisfied with the on-field demonstration provided by NMHS sponsored project?	Yes	1
		No	0

Table 3 Responses of the farmers to the questionnaire applied

Variables	Answers		Total
	Yes	No	
Have you ever heard about saffron?	78	12	90
Is saffron grown in your area?	9	81	90
Do you know how saffron is grown?	5	85	90
Do you know which part of saffron is marketable?	32	58	90
Do you know how saffron is harvested?	5	85	90
Do you consume saffron?	38	52	90
Are you interested in growing saffron?	74	16	90
Do you have suitable land for growing saffron?	62	28	90
Do you feel the need for a government-sponsored scheme to grow saffron?	88	2	90
Are you satisfied with the on-field demonstration provided by the NMHS-sponsored project?	84	6	90

Interactive survey with the farmers

Eighteen (18) sites, across ten districts of Kashmir (Pulwama, Kulgam, Anantnag, Shopian, Budgam, Kupwara, Bandipora, Ganderbal, Baramulla, Srinagar) and six districts of the Jammu region (Poonch, Rajouri, Udhampur, Doda, Reasi and Ramban) were selected for On-Farm Trials of saffron cultivation, and ninety farmers associated with these trials were randomly selected. A survey was conducted to catalogue traditional crops grown in these areas, the knowledge of farmers about saffron and their socio-economic status (Babu and Glendenning 2019). The farmers were categorised into three income groups, viz., low income (<20,000–75,000/annum), medium income (75,000–1,50,000/annum), and high income (>1,50,000/annum). Many temperate fruits are grown in the J&K orchards, including almonds, apples,

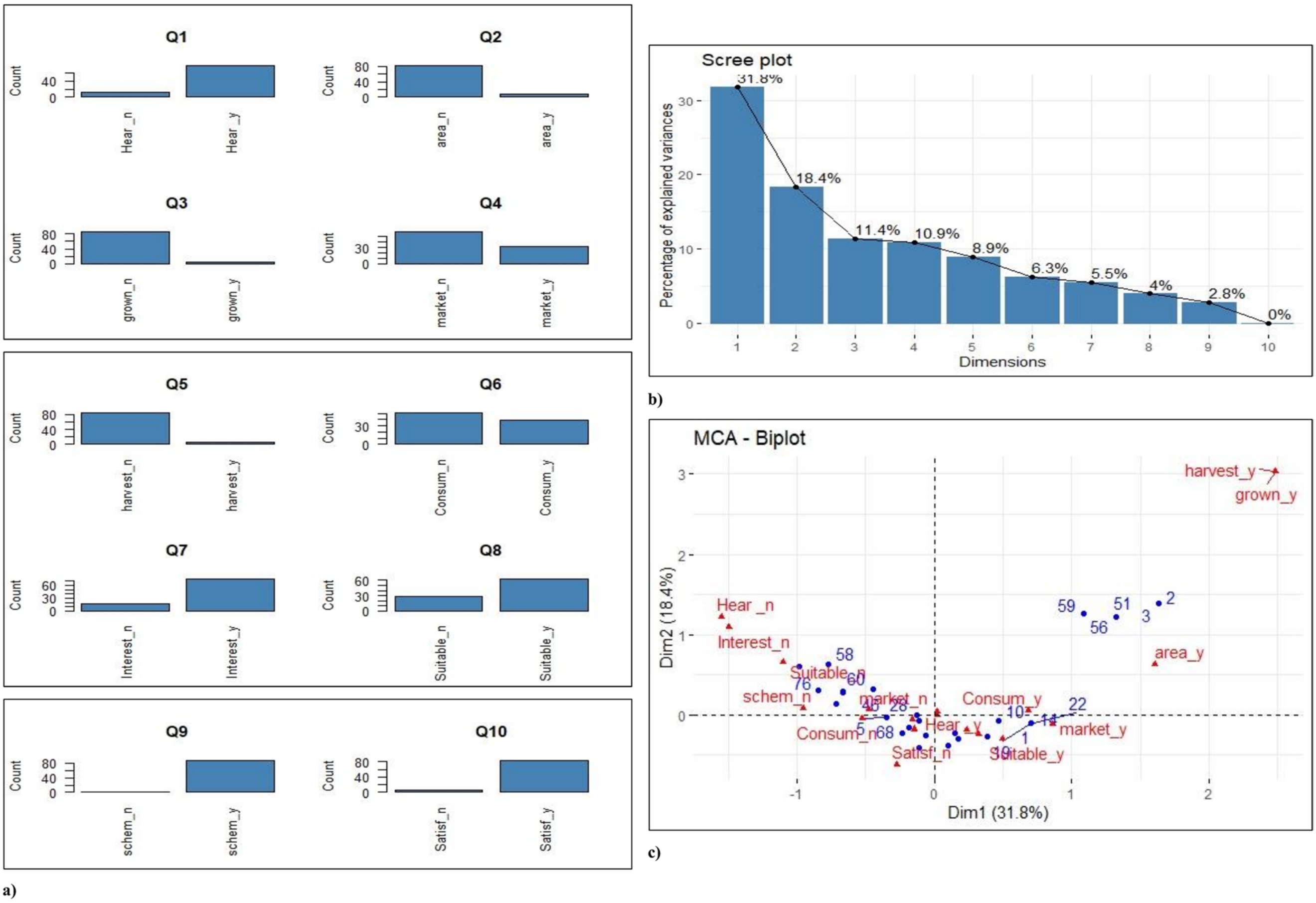


Fig. 1 Multiple Correspondence Analysis (MCA): **(a)** Pot of the frequency of all the variable categories; **(b)** Variance explained among different dimensions (axes) of the MCA; **(c)** The location of the answers within the response for each dependent (response) variable within the ordination space of the MCA

cherries, pears, peaches, walnuts, and the major cereals grown are rice, maize and wheat. The prevailing farming system, based on Maize/Wheat/Fodder farming systems, yields a profit of Rs 27,000 to 32,000/ha, compared to Rs 3,22,000/ha achieved under the saffron farming system. The areas where saffron diversification has potential have been tabulated for income in Table 1. Saffron yield in the non-traditional areas was low compared to Kashmir's traditional areas. However, if saffron cultivation in these areas is scaled up, it may lead to almost doubling the income for the concerned farmers.

A questionnaire based on ten questions was prepared (Table 2), and the farmers were asked to give their perspective on these (Pennings et al. 2002). All of the responses were given instantly in an unbiased manner. These responses were recorded and are summarised in Table 3. The approach to communication aimed at respondent farmers was mainly focused on awareness about saffron. According to the data collected in this survey, 87% of the respondents were fairly familiar with saffron, while 13% hadn't even heard about it. Moreover, 10% of the respondents were aware that saffron was grown in their respective areas, while 90% were not. About 6% of the respondent farmers knew how saffron is grown, while the rest were unsure about the process. About 36% of the respondent farmers knew which part of saffron is marketable, and 64% were unaware of which part of the saffron flower is marketable. About 6% of the respondent farmers knew how saffron is harvested, while the rest didn't. About 42% of the respondent farmers had consumed saffron, while 58% of the respondent farmers had never consumed saffron in their lives. About 82% of farmers were interested in growing saffron, and about 69% had suitable land for growing saffron. More than 97% of farmers believe that a government-sponsored scheme is necessary to support saffron cultivation. Around 93% of farmers were satisfied with the on-field demonstration provided under the project. The data used to measure the subjects' perception of saffron are presented in Figs. 1 and 2.

A multiple correspondence analysis (MCA) was performed using the collected data (Abdi and Valentin 2007). The plot of the frequency of all the variables is explained (Fig. 1a). Most of the variance was explained in the MCA's first two axes, with axis-1 having 31.8% and axis-2 with 18.3% of the variance explained (total of 50.1%) (Fig. 1b). The dependent variables: "Have you ever heard about saffron?", "Is saffron grown in your area?", "Do you know how saffron is grown?", "Do you know which part of saffron is marketable?", "Do you know how saffron is harvested?", "Do you consume saffron?", "Are you interested in growing saffron?", "Do you have suitable land for growing saffron?", "Do you feel the need for the government-sponsored scheme for growing saffron?", and "Are you satisfied with the on-field demonstration provided by the NMHS-sponsored project?" had different positions in the ordination space. Of these variables, the information corresponding to Have you ever heard about saffron and "Do you know how saffron is harvested?" did not contribute to the variance explained in axes 1 and 2 (Fig. 1b). The location of the answers within the response for each dependent (response) variable within the ordination space of the MCA is shown in Fig. 1c.

Fig. 2 Correlation among different variables

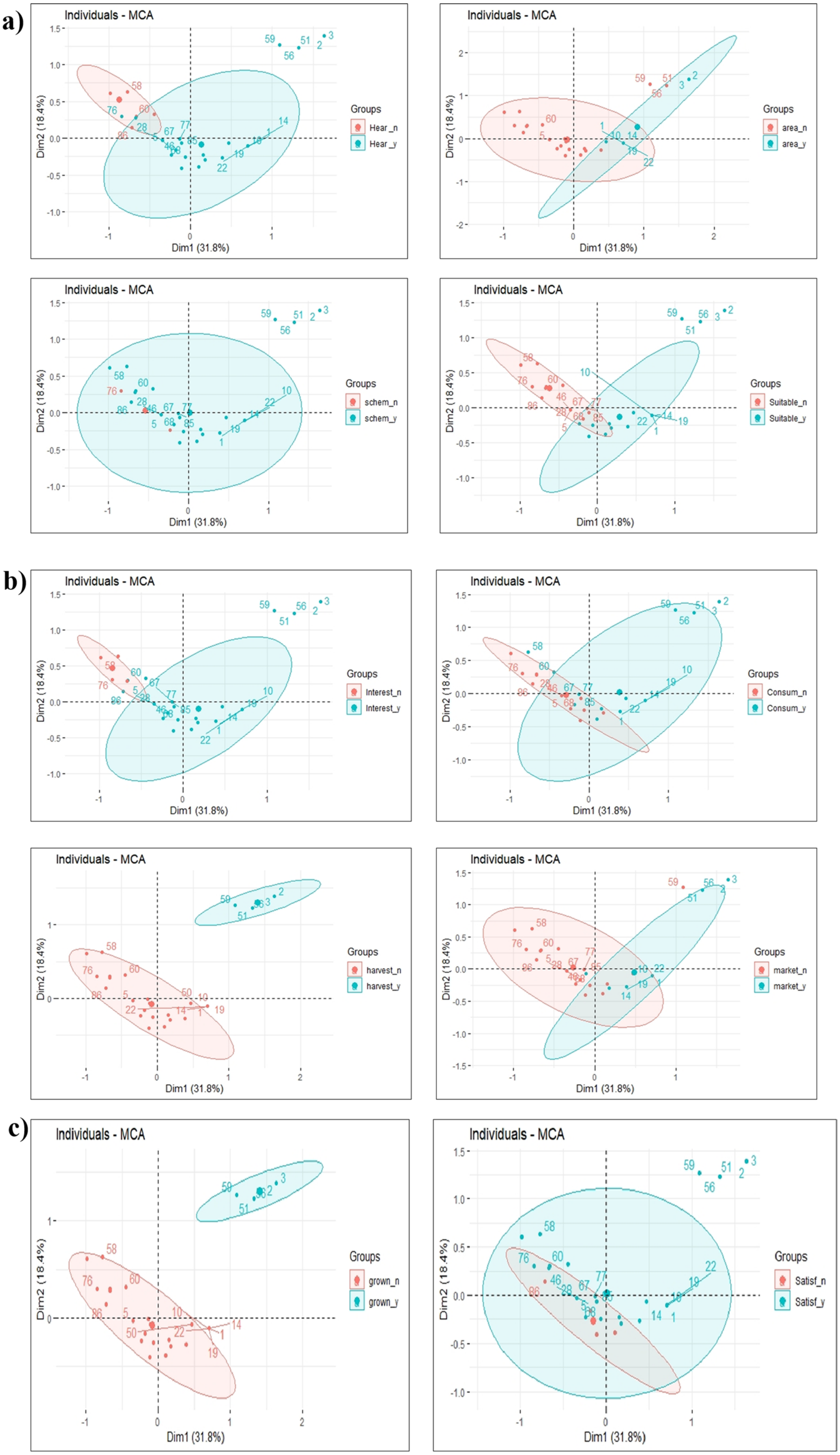
It is also noteworthy that there is a high correlation among variables indicating whether one has heard about saffron, whether saffron is grown in their area, whether they have suitable land for saffron cultivation, and the need for any government-sponsored scheme (Fig. 2a). This is also seen with the variable of "Are you interested in growing saffron?", "Do you know how saffron is harvested?", "Do you consume saffron?" and Do you know which part of saffron is marketable cognates (Fig. 2b). Also variable, "Do you know how saffron is grown?" and "Are you satisfied with the on-field demonstration provided by the NMHS-sponsored project?" (Fig. 2c). This indicates that only one variable can be selected in the future, predicting other viewpoints (i.e., Are you satisfied with the on-field demonstration provided by the NMHS-sponsored project and the need for any government-sponsored scheme?).

Most of the variance was explained in the MCA's first two axes, with axis-1 having 31.8% and axis-2 with 18.3% of the variance explained (total of 50.1%).

Conclusion

Growing saffron in areas which are eco-physiographically similar to traditional saffron growing areas of Kashmir seems promising and could enhance saffron production in Jammu and Kashmir, India. Saffron On-Farm Trials were set up in all ten districts of the Kashmir division and 6 districts of the Jammu division, which had never been used for saffron cultivation. The project was financially supported under a Government of India-sponsored National Mission on Himalayan Studies initiative. One of the important observations was that, while the feasibility of a new area for saffron cultivation depends primarily on eco-physiology, the saffron know-how of farmers regarding the management practices involved in its cultivation is pivotal for the overall success of the crop in the non-traditional areas.

Furthermore, the survey focused on questions related to the saffron area, including cultivation technology, harvesting, marketing, and local consumption. The study revealed a low level of knowledge about saffron cultivation and marketing. However, respondents expressed a willingness to


a)
Individuals - MCA
Dim1 (31.8%)
Dim2 (18.4%)
Groups
Hear_n
Hear_y
area_n
area_y
schem_n
schem_y
Suitable_n
Suitable_y
b)
Interest_n
Interest_y
Consum_n
Consum_y
harvest_n
harvest_y
market_n
market_y
c)
grown_n
grown_y
Satisf_n
Satisf_y

grow saffron under some government-sponsored scheme(s), as saffron is a peculiar crop with many distinctive characteristics that are unique to it. Saffron introduction into new areas should be encouraged as it is a unique crop in terms of its potential and is recognised as ‘red gold’. This can increase the saffron area from 3785 ha to 12,404 ha, generating employment opportunities for youth. Furthermore, saffron production will increase from 16.5 tons to 78 tons, meeting 90% of the domestic demand in India, saving on foreign exchange and fetching an additional exchequer of Rs 9 billion (900 crore) for J&K.

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Data availability It will be made available on request.

Code availability Not applicable.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

Competing Interests The authors have no conflict of interest and nothing to disclose.

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First report of *Fusarium acuminatum* from traditional-saffron region of India: Evidence of Corm Rot pathogenicity in *Crocus sativus* L.

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ABSTRACT

Saffron (*Crocus sativus* L.) is a triploid sterile plant and autumnal flowering geophyte with corms. As a subterranean organ, the corm is susceptible to soil-borne diseases and corm rot is the most destructive disease causing severe yield losses. In the present study, diseased corms showing the symptoms of rot were collected from different saffron fields in Pulwama district, Kashmir (India), to isolate the causal pathogen. Isolations from diseased corms exhibiting typical symptoms of corm rot consistently were categorized into two distinct fungal culture types. Based on pathological and morpho-cultural characteristics, one isolate type was identified as *Fusarium oxysporum*. However, in contrast to the documented morphological characteristics of *F. oxysporum*, the second isolate type displayed notable differences, suggesting a distinct fungal identity. To confirm its identity at the molecular level, a multigene-based characterization approach employing internal transcribed spacer *ITS* (*ITS1*/*ITS4*), *translation elongation factor 1-alpha* (*tef1-α*), and *beta-tubulin* (*β-tub2*) was used. The phylogenetic analysis using a polyphasic sequencing approach, identified the causal pathogen as *Fusarium acuminatum*. To our knowledge, this is the first report of *F. acuminatum* from the saffron-growing region of India and the first step towards managing saffron corm rot in India.

1. Introduction

Crocus sativus (*Iridaceae*) is a bulbous perennial herb widely cultivated globally in warm temperate regions. The vibrant crimson stigmas and styles of *Crocus sativus* flowers are utilized as a valued condiment, dye, and aroma, and they possess antioxidant and immense medicinal properties [1,2]. This species is characterized by its triploid chromosome number and male sterility, rendering it incapable of producing viable seeds for reproduction. Propagation of saffron is achieved solely through vegetative reproduction via its corms. India is one of the few countries globally engaged in the commercial cultivation of *Crocus sativus* L. Kashmirianus, with an area spanning 3674 ha and a productivity of 2.61 kg per hectare [3,4]. Jammu and Kashmir (J&K) is the only region in India where saffron is cultivated on a commercial scale. The cultivation is primarily concentrated in the districts of Pulwama, which account for 76 % of the total saffron-growing area [5].

The area dedicated to saffron cultivation worldwide has significantly decreased due to a range of biotic and abiotic factors [6,7]. Corm rot, in particular, has been identified as a critical biotic constraint that hinders the successful cultivation of saffron in both established and new growing areas [8,9]. The disease is classified as a corm rot complex due to the involvement of multiple pathogens and secondary saprophytes associated with the rotting of the corm. The intensive cultivation and monoculture of saffron in the Kashmir Valley and the persistent use of diseased planting material have led to frequent outbreaks of corm rot diseases [5,10]. Corm rot primarily affects saffron crops during the flowering period (October–November) and the grubbing period (May–July). Surveys have shown that corm rot infestations are widespread, with nearly every saffron field in Kashmir affected [5], and incidence levels ranging between 70 % and 80 %. Additionally, reports indicate a 100 per cent disease incidence with severity ranging from 6 to 46 per cent, leading to a drastic reduction in saffron yield in India [11].

Corm rot infections typically occur through injuries to the corms. Infected corms exhibit dark-brown, sunken, and irregular patches

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beneath the scales, primarily near the root and bud regions. In severe cases, the entire corm may decompose into a black, powdery mass. Additionally, the foliage of affected plants often displays symptoms of die-back [5]. Corm rot is attributed to various pathogens, including *Rhizoctonia crocorum*, *Phoma crocophila* [5] *Macrophomina phaseolina*, *Fusarium moniliforme* var. *intermedium*, *F. equiseti, Fusarium oxysporum, F. pallidoroseum, F. solani*, *Mucor* sp., *Penicillium* sp. and *Sclerotium rolfsii* [5, 12–14].

Fusarium spp. are predominant pathogens causing corm rot in saffron, and recently, *Fusarium nirenbergiae*, *Fusarium commune*, and *Fusarium annulatum* were identified as agents of corm rot in China [15, 16]. This identification was achieved through a combination of morphological and cultural characteristics, along with multilocus sequence analysis (MLSA) using concatenated partial sequences of *rpb2* (the largest subunit of *DNA-directed RNA polymerase II*), *tef1* (*translation elongation factor* 1-α), and the β-*tubulin* gene (*tub2*). No such molecular studies have been conducted on saffron corm rot in India [17].

In India, existing research reports the *F. oxysporum* as the causal organism of saffron corm rot and the identity of the pathogen was based on morphological characteristics and *ITS* sequencing [11,13], and not on multigene sequence analysis. The present study aimed to address this gap by characterizing *Fusarium* species associated with corm rot of *Crocus sativus* L. Kashmirianus in India. This was achieved through pathogenicity tests, morphological assessments, and molecular techniques involving DNA barcoding based on *including internal transcribed spacer* (*ITS*), *translation elongation factor 1-alpha* (*tef1*-α), and *beta-tubulin* (*tub2*).

2. Materials and methods

2.1. Field survey and sample collection

A survey was conducted in traditional saffron growing areas of Pulwama district Kashmir (74°58′0″E, 34°1′30″N, 5173 m above sea level), in northern Himalayan region of India during October 2023–2024 to assess the status of saffron corm rot. The survey areas included the traditional saffron growing region Pampore and its adjoining areas (Fig. 1,Table 1). Three fields were chosen randomly from each village to collect the diseased samples.

2.2. Isolation and purification of corm rot pathogen

Infected plants exhibiting typical symptoms of corm rot were collected and the fungus associated with the diseased corms was isolated by tissue bit technique, purified by single spore method and maintained on PDA slants at 25 ± 1 °C [11,13].

2.3. Identification of the isolated fungus

2.3.1. Morpho-cultural characterization

The mono-conidial isolates were characterized for their morpho-cultural characteristics and compared with authentic descriptions [18, 19]. The colony features with respect to colour, shape and size were visually assessed. For the morphology of the fungus, the wet mounts in lactophenol and cotton blue of 15 days old culture were examined under a microscope and the observations concerning different morphological characters such as shape, colour, septation of mycelium, conidiophore and conidia were recorded.

2.4. Pathogenicity studies

2.4.1. In vitro pathogenicity test

The *in vitro* pathogenicity of the isolated fungus was established on healthy saffron corms following the protocol adopted by Bayona et al. [20]. The outer skin (tunic) of the corms was peeled off, and the peeled corms were disinfected with 5 % sodium hypochlorite for 10 min, followed by 70 % ethanol for 1 min. The sterilized corms were then rinsed three times with sterile distilled water and air-dried. Using a sterile glass

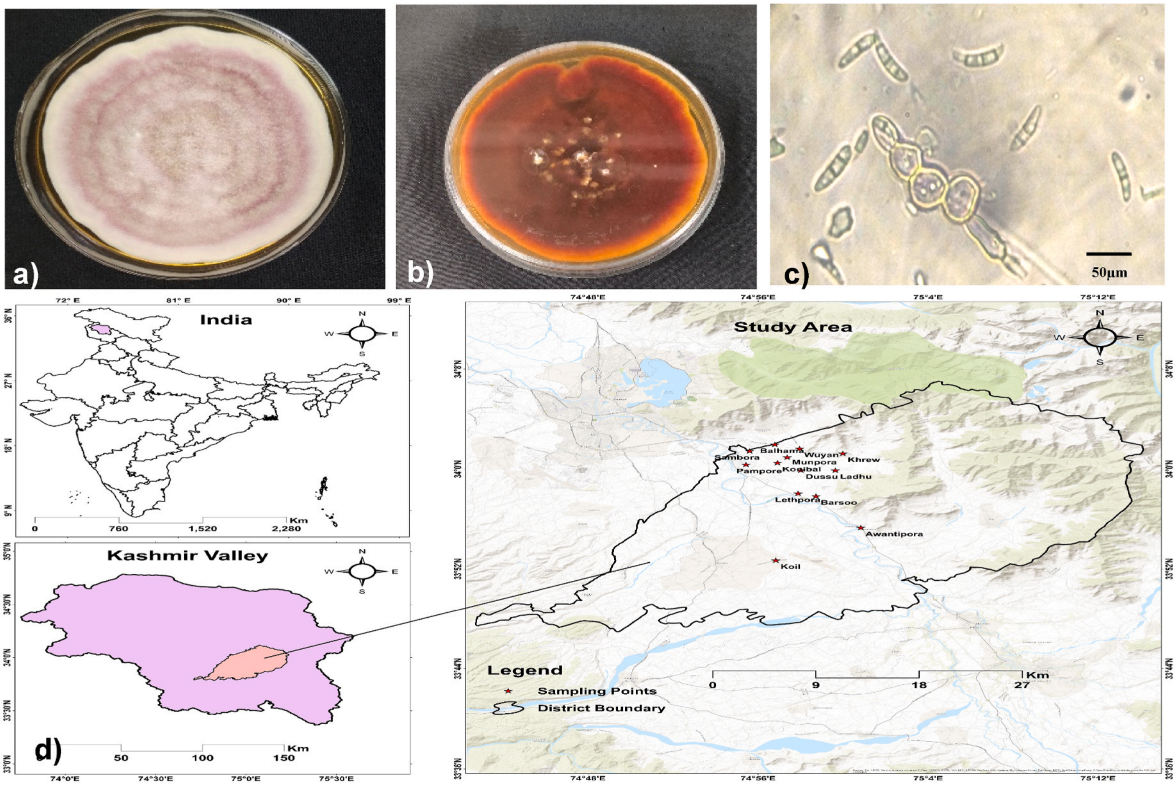

Fig. 1. Isolated fungal pathogens responsible for causing corm rot in saffron (*Crocus sativus* L. Kashmirianus) and its morpho-cultural characteristics: a) Front view, b) Back view, c) Macroconidia and Chlamydospores of *F. acuminatum* (d) Sampling area in traditional saffron growing region of India.

Table 1
Details of the thirteen sampling sites and corm rot symptoms recorded at each location.

S. No.	Location	Latitude	longitude	Symptoms observed
1)	Pampore	34.006° N	74.9238° E	Leaves turning yellow and drooping.
2)	Khrew	34.0209° N	74.9998° E	Foliage withering and dying.
3)	Ladhu	33.9984° N	74.9939° E	Foliage drooping; Small spots on corms surrounded by yellowish halos, soft rot and tissue decay with foul smell
4)	Dussu	33.9985° N	74.9669° E	Dieback of shoots; Corm disintegrating into a dark, powdery mass emitting foul smell.
5)	Munpora	34.015793° N	74.956146° E	Foliage withering; Irregular, sunken dark brown lesions beneath the corm.
6)	Sambora	34.0241° N	74.9267° E	Foliage withering; Deep, sunken brown patches forming under the corm.
7)	Balhama	34.0329566° N	74.9464764° E	Shoots wilting and collapsing, with vascular discoloration.
8)	Lethpora	33.9675° N	74.9647° E	Complete drying and death of foliage; Corms turning into dark powdery mass.
9)	Awantipora	33.9218° N	75.0139° E	Foliage withering; Corm softening and decaying.
10)	Koil	33.8782° N	74.9472° E	Leaves turning yellow and start drooping.
11)	Wayun	34.027382° N	74.965861° E	Leaves yellow; Tiny specks on corms with surrounding chlorotic halos.
12)	Barsoo	33.963921° N	74.978742° E	Wilting of the shoots.
13)	Konibal	34.0084° N	74.9485° E	Shoots gradually wilting and drying out; Corms sunken and damp.

rod, two diametrically opposite wounds, 5 mm in diameter and 5 mm deep, were made on each test corm. A spore suspension (1×10^7 spores/ml) was prepared from the seven-day-old culture of test fungus grown on potato dextrose broth at 25 °C with shaking at 150 rpm. The spore suspension so prepared was used to inoculate the detached corms. The inoculation was made by immersing the corms in a 250 ml spore suspension for 24 h, while corms immersed in sterile distilled water served as control. Following immersion, the corms were transferred in sterile plastic beakers with moist absorbent cotton covering the bottom of the beakers, and then incubated at 25 °C in the dark for 25 days [20]. Three replicates were maintained for both inoculated and control corms. After incubation, the corms were cut across the two inoculation points, and pictures were taken. The fungus was re-isolated from the artificially infected corms and compared with the initially isolated and inoculated fungus to satisfy the Koch's postulates.

2.4.2. *In vivo pathogenicity test*

The pathogenicity of the isolated fungus was also confirmed on potted saffron plants using the rhizosphere inoculation technique, as adopted by Najar et al. [21]. The soil was autoclaved to sterilize it, and pots were prepared for corm planting. The pots were inoculated with 10 per cent (w/w) test fungal cultures grown on sand maize meal medium (90 g of sand and 10 g of maize meal), and kept for 7 days inside a walk-in-plant growth chamber at 25 ± 2 °C. Before sowing, the test corms were disinfected with 5 % sodium hypochlorite for 15 min and then rinsed three times with sterile water. The corms transplanted in uninoculated sterilized soil served as control. Three replicates were maintained for both inoculated and control corms. The pots were kept for 5 weeks under controlled conditions in the walk-in-plant growth chamber, with a light/dark cycle of 18/6 h and temperatures of 25 °C during the day and 21 °C at night [21,22].

2.5. *Molecular characterization*

The DNA was isolated from seven-day-old mycelium harvested from potato dextrose agar (PDA), frozen in liquid nitrogen, ground, and homogenized with 2 mL pre-warmed cetyl trimethyl ammonium bromide (CTAB) buffer (65 °C, 10 min). The homogenate was incubated at 65 °C for 1 h, followed by extraction with chloroform:isoamyl alcohol (24:1). After centrifugation (13,000 rpm, 20 min), the aqueous phase was collected, and DNA was precipitated with chilled isopropanol and stored overnight. The DNA pellet was washed with 70 % ethanol, air-dried, and dissolved in TE buffer. RNA was removed using DNase-free RNase A. The genomic DNA bands were visualized on a 0.8 % agarose gel. The primers used for amplification were EF-1/EF-2 (F- CATCGAGAAGTTCGAGAAGG: R-TACTTGAAGGAACCCTTACC), targeting the nuclear *translation elongation factor 1-alpha* (*tef1*) gene; Tub2F/Tub2R (F-GGTAACCAAATCGGTGCTGCTTTC: R- AACCTCAGTGTAGTGACCCTTGGC), targeting the *β-tubulin* gene; and ITS1/ITS4 (F-CTTGGTCATTTAGAGGAAGTAA: R-TCCTCCGCTTATTGATATGC), targeting the *internal transcribed spacer ITS-region* [23–26](Table 3). Polymerase chain reaction (PCR) was performed using 5 μL PCR buffer (10X) 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 1 μL of dNTPs (10 mM), 2 μL of genomic DNA (2245.51 ng/μL), Taq polymerase 0.5 μL and 39.5 μL of DNase-free water. The PCR protocol included an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C, 52 °C and 55 °C respectively for 30 s, and extension at 72 °C for 2 min. A final extension was carried out at 72 °C for 10 min [25,26].

2.6. *Sequencing and phylogenetic analysis*

PCR products amplified using *EF-1/EF-2*, *Tub2F/Tub2R* and *ITS1/ITS4* were outsourced for sequencing to Barcode Biosciences, Bangalore, India. Sequences were assembled to generate the consensus sequence for phylogeny and sequence identity matrix using BioEdit Sequence Alignment Editor Version 7.0.9.0. The nucleotide sequences were confirmed through basic local alignment search tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/). The searches were performed against the Nucleotide collection (nr/nt) and restricted to sequences from type material. The dendrogram was constructed using the MEGA 11 (Molecular Evolutionary Genomics Analysis Version 7) software [27]. The sequences were submitted to GenBank. Pairwise sequence identity matrices were generated using the Sequence Demarcation Tool (SDT) v1.2 (http://web.cbio.uct.ac.za/) to assess sequence similarity.

3. Results

3.1. *Morpho-cultural characterization*

Isolations from diseased corms exhibiting typical symptoms of corm rot consistently categorized into two distinct fungal culture types. The cultural and morphological characteristics of the isolates were studied on PDA medium in pure culture. One culture type displayed a cottony or floccose colony, varying in color from white to pale violet. Based on its morphological characteristics, particularly its coloration, this isolate was identified as *F. oxysporum* which has been previously reported as a causal agent of saffron corm rot (Table 1S) [5,12–14].

The second fungal culture type exhibited a distinct pink to salmon pigmentation with yellow edges. Concentric ring formations were observed on both the upper and lower surfaces, characteristic of its growth pattern. In contrast to the documented morphological characteristics of *F. oxysporum*, this isolate displayed notable differences, suggesting a distinct fungal identity. The colony demonstrated rapid growth, reaching 90 mm in diameter after 14 days of incubation at 25 ± 1 °C. Initially, the purified culture formed abundant whitish cottony colonies with extensive aerial hyphae. As the culture matured, the colony surface developed a distinct pink to salmon pigmentation, while the edges remained yellow.

Microscopic examination revealed smooth, branched, and septate mycelium. Mycelium was smooth, branched, septate, slendrical conidiophores were short, simple, and measured 32.5 μm–53.5 μm in length and 3.5 to 5.1 width in size. The macroconidia were falcate, slender, and distinctly curved at the base, with 3–5 septa and measured 23.4–52.1 μm x 4.5–6.8 μm in length and width, respectively. The microconidia were rare. Chlamydospores were intercalary, produced singly or in chains, nearly spherical, hyaline and measured 10.7–14.2 μm in diameter (Fig. 1,Table 2). Based on these characteristics and their comparison with the authentic descriptions, the fungus was identified as the *F. acuminatum* [19,28]. Further studies were conducted to confirm its identity through molecular studies and to evaluate its pathogenic potential.

3.2. Pathogenicity test

Pathogenicity of the isolated fungus was established on detached corms under *in vitro* conditions and in vivo on potted saffron plants grown under greenhouse conditions. Symptoms were observed on inoculated corms and potted plants, whereas no symptom development was observed in control plants in both the cases (Fig. 2). The inoculated corms rotted and subsequently turned the white surface of corms to yellow and ultimately to black, resulting in the rotting and death of the corms after 25 days of inoculation. The inoculated potted saffron plants produced symptoms like chlorosis, drooping, and rolling of the leaves resulting in the death of foliage and subsequently rotting of the corms and death of the whole plant after 35 days post-inoculation. The fungus on re-isolation from the artificially infected tissue resembled the initially isolated and inoculated pathogen, satisfying Koch's postulates (1884).

3.3. Molecular characterization

The PCR amplification of genes *tef1-α, tub2* and *ITS* (*ITS1/ITS4*) from *Fusarium* generated amplicon sizes ~300bp, ~400bp and ~600bp, respectively (Fig. 3,Table 3). The PCR amplicons were sequenced and BLASTn analysis revealed the sequence similarity of 100 % with *F. accuminatum* type strain JX397865 [29] at the *tef1* locus, 100 % at *ITS1/ITS4* locus (OL832226) [30] and 99.40 % at the *tub2* locus (ON960209) [31]. BLASTn of *ITS*, *tef1-α,* and *tub2* loci sequences indicated similarity with the type strain of *F. acuminatum,* which supported our preliminary morphological identification of this isolate. The Sequences obtained were deposited in GenBank, with accession numbers PQ319729 (ITS), PQ492270 (tef1-α), PQ492271 (*tub2*) (Table 3).

Table 2
Morpho-cultural characteristics of *Fusarium acuminatum* causing saffron corm rot.

Fungal propagule	Shape	Color	Size	Septation
Colony	Smooth, branched	Distinct pink to salmon pigmentation, while the edges were yellow	90 mm diameter in 14 days	Septate
Hyphae	Aerial/ Smooth, branched	Hyaline	3.20–4.20 μm	Septate
Macroconidia	Falcate, slender, and distinctly curved at the base	Hyaline	23.4–52.1 μm × 4.5–6.8 μm	3-5 septa
Conidiophores	Cylindrical, short and simple	Hyaline	32.5–53.5 μm × 3.5–5.1 μm	Septate
Chlamydospores	Singly or in chains, nearly spherical	Hyaline	10.7–14.2 μm in diameter	–

3.4. Phylogenetic analysis

An optimal dendrogram was constructed using MEGA 11 software v 5.05, and different taxa were clustered together in a bootstrap test 1000 replicates using sequences of *tef1*, *tub2* and *ITS* regions that were compared with their respective hits retrieved from the NCBI database and compared with the already available *F. acuminatum* sequences (Fig. 4,Table 4). The phylogenetic analysis of the query sequences PQ492270, PQ492271, and PQ319729 (all fungal origin) was conducted to evaluate their evolutionary relationships within the *Fusarium* genus. The phylogenetic clustering revealed five distinct clusters within the *Fusarium* genus. The query sequence

Corm rot in *C. sativus* has increasingly restricted crop production in India over recent years. Observations of disease distribution suggest that the pathogen has spread extensively across saffron-producing regions. It indicates that infested corms are likely a primary source of inoculum [14]. The present study was initiated with the aim of isolating the pathogen responsible for corm rot disease in *C. sativus* L. Kashmirianus. Previous studies have reported that corm rot in saffron is caused by different formae specialis of *Fusarium oxysporum*, including *F. oxysporum* f. sp. *gladioli*, *iridiacearum*, and *saffrani* [40]. In the saffron-growing regions of the Kashmir Valley, corm rot caused by *F. oxysporum* and *F. solani* is considered highly destructive [5,7]. Traditionally, the identification of *Fusarium* species has relied on morphological characteristics. However, distinguishing between different Fusarium species can be challenging [13,34,41]. *Fusarium* species share similar characteristics, and their morphological traits can be affected by factors such as culture media, temperature, and light. Additionally, these methods do not account for intraspecific variability. Consequently, a polyphasic approach is required for accurate species identification. This approach integrates morphological, biological, and genetic characteristics to provide a comprehensive analysis [42–44]. Previous studies have suggested that combining single-locus datasets to construct multilocus phylogenetic trees offers greater overall support compared to single-gene phylogenies [45,46].

Recent advances in molecular biology and morphological surveys have led to the discovery of numerous cryptic species within *F. oxysporum* [15,41,47]. Consequently, the taxonomy of the genus has undergone significant revisions [48]. Several genomic sequences have been used to analyse intraspecific variability in *Fusarium*, the *β-tubulin gene* (*β-tub2*), regions of the *ITS* rDNA region including ITS1, 5.8 S and ITS4 and the *translation elongation factor* gene *tef*-1*α* to discriminate FOSC population diversity [16,35,49,50]. The *tef1* locus is frequently chosen for taxonomic studies of *Fusarium* due to its single-copy nature and significant sequence polymorphism among closely related species [51,52]. *β-Tubulin* proteins are fundamental to cellular function, and their genes are highly conserved across different species, similar to other 'housekeeping' genes. The *β-tubulin* genes display at least 60 % amino acid similarity between the most distantly related lineages, reflecting their essential role and conservation [53,54]. *ITS* rDNA is most frequently studied because of species specificity of this region and they are known to provide better resolution at the sub-species level and thus sequence analysis is a superior choice for phylogenetic studies in the *F. oxysporum* species complex [55–57]. There are no such studies on saffron corm rot in Kashmir, India.

In the present study, morpho-cultural identification, pathogenicity testing and multigene phylogenies identified *F. acuminatum* as the causal agent of corm rot in saffron fields in Pampore. Pathogenicity tests conducted in vivo and *in vitro* demonstrated that the isolate was virulent on

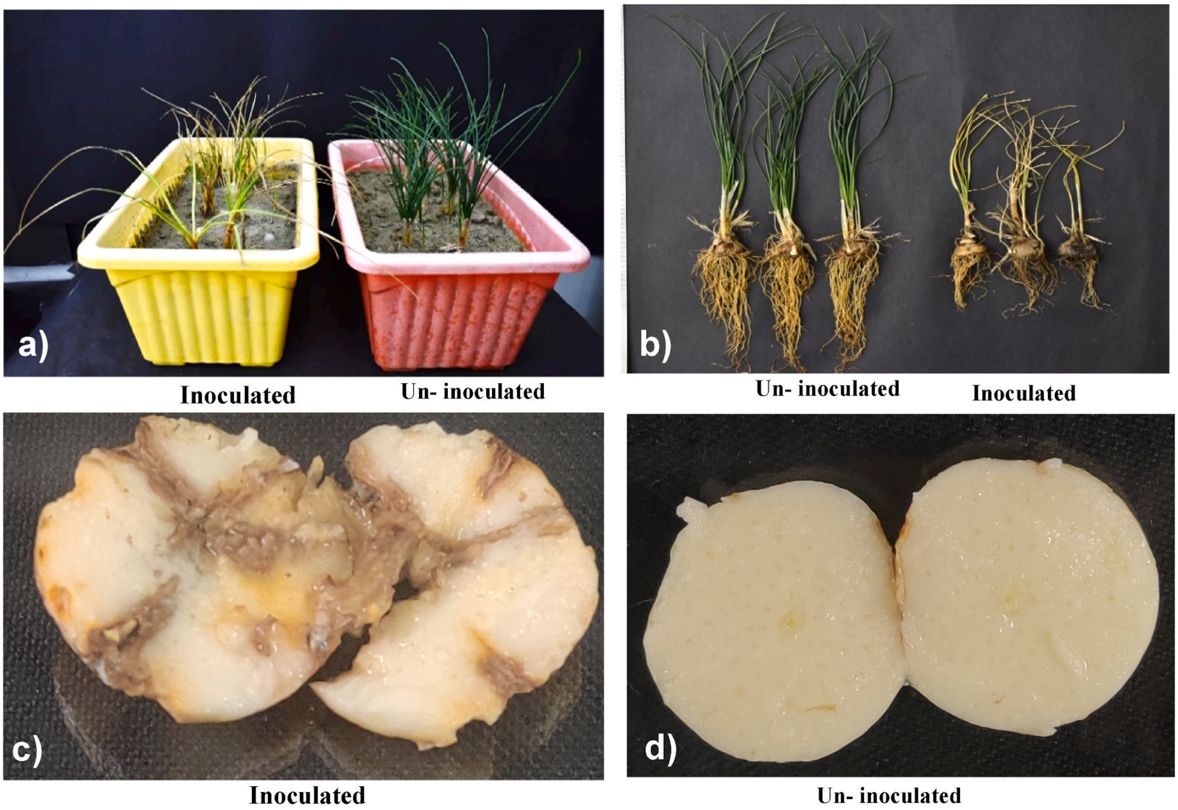

Fig. 2. Pathogenicity test on *Crocus sativus* L. Kashmirianus plants and detached corms with fungal isolate *F. acuminatum*. Typical external symptoms of saffron corm rot were seen in plants inoculated with 35dpi a) in pots, b) roots and shoots, c, d) in corm sections inoculated with 25dpi and uninoculated controls.

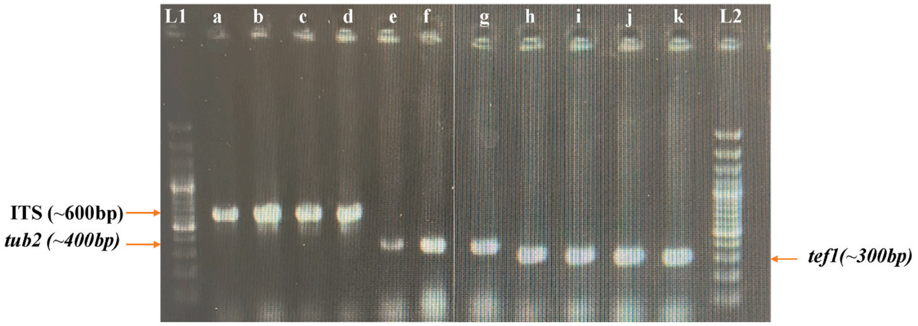

Fig. 3. Molecular detection of *Fusarium acuminatum* by PCR using the ITS (ITS1/ ITS-4), *tef1* and *tub2* primer pair sets. The purified gDNA from the Fusarium isolate was used as template. Lanes: L1: 100bp ladder, Lanes- a to d: gDNA amplified using ITS: Lanes e to g :gDNA amplified using tub2; Lanes h to k:gDNA amplified using tef1 primer pair sets and L2 : 100bp ladder.

Table 3
Details of PCR and DNA sequencing.

Primer name	Primer sequence	Size of amplicon (bp)	Tm (°C)	Accession numbers
ITS1	CTTGGTCATTTAGAGGAAGTAA	~600	**55**	PQ319729 (*ITS1/ITS4*) https://www.ncbi.nlm.nih.gov/nuccore/PQ319729
ITS-4	TCCTCCGCTTATTGATATGC			
β-*tubulin* -F	GGTAACCAAATCGGTGCTGCTTTC	~400	**52**	PQ492271 (*tub2*) https://www.ncbi.nlm.nih.gov/nuccore/PQ492271.1?report=fasta
β-*tubulin* -R	AACCTCAGTGTAGTGACCCTTGGC			
tef-F	CATCGAGAAGTTCGAGAAGG	~300	**54**	PQ492270 (*tef1*) https://www.ncbi.nlm.nih.gov/nuccore/PQ492270
tef-R	TACTTGAAGGAACCCTTACC			

intact plants and detached corms, respectively. *In vitro* assays have been previously conducted for plant pathogenicity tests (potato, hyacinth) [20,58], and a similar assay was used in the present study. After 20–25 days of inoculation with fungal culture, the *F. acuminatum* inoculated corm was completely covered with fungal mass, in contrast to the uninoculated control, which showed no signs of fungal infection. The results support the earlier reports, which recount almost the same incubation period in different species of *Fusarium* infecting corm [11,35]. Further, the rhizosphere inoculation of the potted saffron plants produced characteristic symptoms like chlorosis, drooping, and rolling of

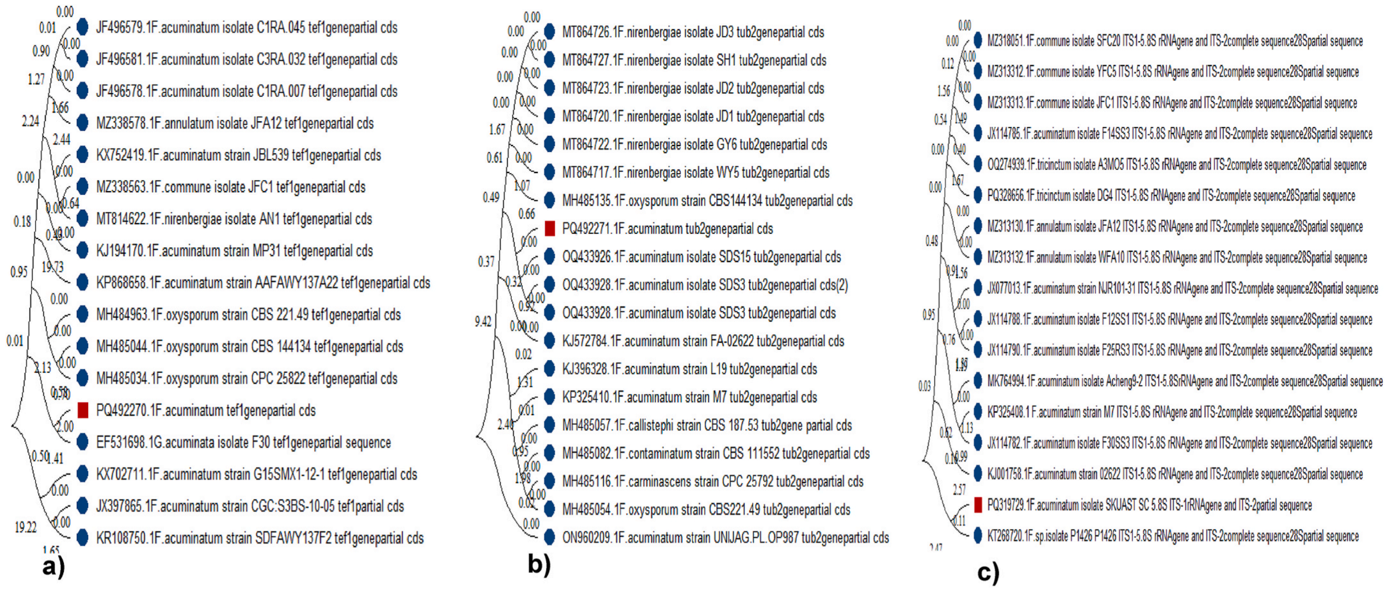

Fig. 4. Phylogenetic relationships of *Fusarium acuminatum* causing corm rot in *Crocus sativus* L. Kashmirianus from India, based on a) *tef1*, b) *tub2*, and c) ITS1/4 sequences. The isolate obtained in this study is marked by red triangle. The Neighbor-Joining method was used to construct the phylogenetic trees with branch lengths of 66.258 (tef1), 37.000 (Tub2), and 21.045 (ITS1/ITS4). Analyses included 857 (17 taxa), 1311 (19 taxa), and 603 (17 taxa) nucleotide sites, respectively. Bootstrap consensus trees were derived from 1000 replicates, with branches supported by bootstrap values ≥50% retained. Evolutionary distances were calculated using the p-distance method, expressed as base differences per site. Codon positions included 1st, 2nd, 3rd, and noncoding regions. Ambiguous sites and positions with <95% site coverage were excluded (partial deletion option). Analyses were performed in MEGA 11.

the leaves, resulting in the death of foliage, subsequently rotting of corms and death of the whole plant after 3–5 weeks of inoculation [5].

The pathogen was re-isolated from sym s PQ492270, PQ492271, and PQ319729 showed close evolutionary relationships, particularly with *F. acuminatum*. PQ492270 clustered distinctly within the fungal clade, showing evolutionary proximity to F. acuminatum strains (e.g., JF496579.1, JF496581.1), with slight genetic divergence indicated by moderate branch lengths. Similarly, PQ492271 grouped closely with *F. acuminatum* isolates (e.g., KJ572784.1, OQ433926.1), reflecting evolutionary similarity while maintaining unique traits. PQ319729 formed a subgroup within the *F. acuminatum* clade (e.g., KP325408.1, JX114782.1, MK764994.1), indicating close genetic ties with minor variability. Other Fusarium species, such as *F. nirenbergiae* (e.g., MT864726.1, MT864727.1), *F. oxysporum* (e.g., MH485135.1, MH485054.1), *F. commune*, and *F. annulatum*, contributed to the remaining distinct clusters, highlighting genetic diversity and evolutionary separation from the query sequences. Overall, the placement of PQ492270, PQ492271, and PQ319729 within or near the *F. acuminatum* group underscores their evolutionary relatedness to this species. The pairwise identity matrices for *tef1-α*, *tub2*, and *ITS* gene sequences were generated to evaluate sequence similarity among isolates. The percentage identity ranged from 70 to 100 % for *tef1-α,* 82–100 % for *tub2*, and 92–100 % for *ITS* sequences (Fig. 5). The matrices, visualized using the Sequence Demarcation Tool (SDT) v1.2 (http://web.cbio.uct.ac.za/), depicted the degree of similarity between sequences, with each colored cell representing a pairwise comparison.

4. Discussion

ptomatic tissues, fulfilling Koch's postulates and confirming that *F. acuminatum* is pathogenic to saffron. The fungal isolate obtained from saffron corms in this study was initially presumed to be *F. oxysporum*, given its global prevalence and documented association with saffron corm rot in India and other parts of the world [5,40]. Previous studies have identified *F. oxysporum* as the causal organism of saffron corm rot, and these studies are based solely on morphological characteristics and *ITS* sequencing [11]. This initial assumption was further supported by the pinkish pigmentation observed during the preliminary stages of growth. However, the pigmentation of the isolate remained consistently pink to salmon, whereas authentic literature describes *F. oxysporum* as producing pink to purple pigmentation on culture media. The macroconidia of the fungus were observed to be slightly curved with pointed ends, a characteristic feature of *F. acuminatum*, while *F. oxysporum* typically produces straight or slightly curved macroconidia with blunt ends [18,19,28]. Additionally, the retention of pink to salmon pigmentation further aligned with the characteristics of *F. acuminatum*. Moreover, a detailed examination employing multigene sequence analysis, as discussed below, distinguished them as *F. acuminatum*. Earlier, *F. acuminatum* has been identified as the causal agent of saffron corm rot in Khorramabad, Iran [59].

The validity of the morphological identification was confirmed through phylogenetic analysis using molecular data. Amplification of the *Internal Transcribed Spacer* (*ITS*) region with genus- and species-specific primers, along with the *Transcription Elongation Factor* (*tef1-α)* gene and *β-tubulin* (*tub2*) primers, was carried out for the accurate identification of the pathogen. The PCR products were sequenced, and the pathogen was accurately identified based on sequencing of the *ITS1-ITS4*, *tef1-α*, and *tub2* regions. These genetic markers are widely recognized for their utility in the taxonomic and phylogenetic identification of fungi [6,41]. Sequence alignment using CLUSTALW revealed that sequence similarity was consistent, irrespective of the geographic origin of the isolates, confirming the reliability of these regions in distinguishing fungal species [24,60].

In the phylogenetic study, *F. acuminatum* sequences of *ITS, tef1-α* and *tub2* genes were compared with their respective hits retrieved from NCBI database and were compared with the already available F. acuminatum sequences. The phylogenetic analysis of the fungal query sequences PQ492270, PQ492271, and PQ319729 revealed close evolutionary relationships with *F. acuminatum.* All three sequences clustered within the *F. acuminatum* clade, with PQ492270 and PQ492271 showing moderate genetic divergence from other strains of *F. acuminatum* (Fig. 4). PQ319729 formed a subgroup within this clade, indicating minimal genetic variability. Other *Fusarium* species, such as *F. nirenbergiae*, *F. oxysporum*, *F. commune*, and *F. annulatum*, formed distinct clusters,

Table 4
List of fungal species used for phylogenetic analysis.

Taxon	GenBank Acc. No.	DNA locus	Specimen code	Isolation source	Country	References
Fusarium commune	MZ338563.1	*tef1-α*	JFC1	*Crocus sativus*	China	[15]
Fusarium annulatum	MZ338578.1	*tef1-α*	JFA12	*Crocus sativus*	China	[15]
Fusarium nirenbergiae	MT814622.1	*tef1-α*	AN1	*Crocus sativus*	China	[16]
Fusarium oxysporum	MH485044.1	*tef1-α*	CBS 144134	*Solanum tuberosum*	Germany	[32]
	MH484963.1	*tef1-α*	CBS 221.49	*Camellia sinensis*	Netherlands	[32]
	MH485034.1	*tef1-α*	CPC 25822	Protea sp.	South Africa	[32]
Fusarium acuminatum	KX702711.1	*tef1-α*	G15SMX1-12-1	Wheat	China	[33]
	JX397865.1	*tef1-α*	CGC:S3BS-10-05	Wheat and barley	Canada	[29]
	KR108750.1	*tef1-α*	SDFAWY137F2	*Saposhnikovia divaricata*	China	Direct Submission
	PQ492270.1	*tef1-α*		*Crocus sativus*	India	Present study
	JF496578.1	*tef1-α*	C1RA.007	Barley	Spain	[34]
	JF496579.1	*tef1-α*	C1RA.045	Barley	Spain	[34]
	JF496581.1	*tef1-α*	C3RA.032	Barley	Spain	[34]
	KX752419.1	*tef1-α*	JBL539	–	Serbia	Unpublished
	KP868658.1	*tef1-α*	AAFAWY137A22	*Artemisia argyi*	China	Direct Submission
	KJ194170.1	*tef1-α*	MP31	*Alfa-alfa*	China	Unpublished
Gibberella acuminata	EF531698.1	*tef1-α*	F30	*Astragalus racemosus*	USA	Unpublished
Fusarium nirenbergiae	MT864720.1	*β-Tubulin*	JD1	*Crocus sativus*	China	[16]
	MT864723.1	*β-Tubulin*	JD2	*Crocus sativus*	China	[16]
	MT864726.1	*β-Tubulin*	JD3	*Crocus sativus*	China	[16]
	MT864727.1	*β-Tubulin*	SH1	*Crocus sativus*	China	[16]
	MT864717.1	*β-Tubulin*	WY5	*Crocus sativus*	China	[16]
	MT864722.1	*β-Tubulin*	GY6	*Crocus sativus*	China	[16]
Fusarium callistephi	MH485057.1	*β-Tubulin*	CBS 187.53	*Callistephus chinensis*	Netherlands	Direct Submission
Fusarium carminascens	MH485116.1	*β-Tubulin*	CPC 25792	*Zea mays*	South Africa	[32]
Fusarium contaminatum	MH485082.1	*β-Tubulin*	CBS 111552	Pasteurized fruit juice	Netherlands	[32]
Fusarium acuminatum	KJ572784.1	*β-Tubulin*	FA-02622	Ginseng	China	[35]
	KP325410.	*β-Tubulin*	M7	*Actinidia arguta*	China	[36]
	KJ396328.1	*β-Tubulin*	L19		China	Unpublished
	PQ492271.1	*β-Tubulin*	–	*Crocus sativus*	India	This study
	ON960209.1	*β-Tubulin*	UNIJAG.PL.OP987	*Arabidopsis arenosa*	Poland	[31]
	OQ433926.1	*β-Tubulin*	SDS15	*Divaricate Saposhniovia* Root	China	Direct Submission
	OQ433928.1	*β-Tubulin*	SDS3	*Divaricate Saposhniovia* Root	China	Direct Submission
Fusarium oxysporum	MH485054.1	*β-Tubulin*	CBS 221.49	*Camellia sinensis*	Netherlands	[32]
	MH485135.1	*β-Tubulin*	CBS 144134	*Solanum tuberosum*	Germany	[32]
Fusarium sp	KT268720.1	ITS	P1426	*Microthlaspi perfoliatum*	Spain	[37]
Fusarium tricinctum	PQ328656.1	ITS	DG4	*Angelica sinensis*	China	Unpublished
	OQ274939.1	ITS	A3MO5	–	Iran	Unpublished
Fusarium commune	MZ313313.1	ITS	JFC1	*Crocus sativus*	China	[15]
	MZ318051.1	ITS	SFC20	*Crocus sativus*	China	[15]
	MZ313312.1	ITS	YFC5	*Crocus sativus*	China	[15]
Fusarium annulatum	MZ313130.1	ITS	JFA12	*Crocus sativus*	China	[15]
	MZ313132.1	ITS	WFA10	*Crocus sativus*	China	[15]
Fusarium acuminatum	KP325408.1	ITS	M7	*Actinidia arguta*	China	[36]
	MK764994.1	ITS	Acheng9-2	Alfalfa	China	Unpublished
	KJ001758	ITS	02622	Ginseng	China	[35]
	JX077013.1	ITS	NJR101-31	Wetland sediment	China	[38]
	JX114788.1	ITS	F12SS1	Crown of Aleppo pine seedling	Algeria	[39]
	JX114785.1	ITS	F14SS3	Crown of Aleppo pine seedling	Algeria	[39]
	JX114782.1	ITS	F30SS3	Crown of Aleppo pine seedling	Algeria	[39]
	JX114790.1	ITS	F25RS3	Crown of Aleppo pine seedling	Algeria	[39]
	PQ319729.1	ITS	SKUAST_SC	*Crocus sativus*	India	Present study

highlighting the genetic separation from the query sequences. Similar species were grouped in a single clade irrespective of their geographic origin. The pairwise identity analyses of *tef1-α*, *tub2*, and *ITS* gene sequences provided valuable insights into the genetic diversity among the isolates. The percentage identity ranged from 70 to 100 % for *tef1-α*, 82–100 % for *tub2*, and 92–100 % for *ITS* sequences, highlighting varying levels of conservation across these loci (Fig. 5). A new fungal species *F. acuminatum* was found associated with causing saffron corm rot in Kashmir valley, and this is the first report of this pathogen from India.

5. Conclusion

The current study provides comprehensive insights into the etiology of corm rot disease in saffron (*C. sativus* L. Kashmirianus) cultivated in the Kashmir valley, identifying *Fusarium acuminatum* as the causal agent for the first time in India. Through a polyphasic approach involving morpho-cultural characterization, pathogenicity testing, and multigene sequence analysis, *F. acuminatum* was confirmed as the pathogen responsible for this destructive disease. Pathogenicity assays validated its virulence on saffron plants, fulfilling Koch's postulates and establishing its role in disease progression. The study also highlights the genetic distinctiveness of *F. acuminatum* compared to other *Fusarium* species. This research provides a foundation for effective disease management strategies to mitigate the impact of *F. acuminatum* induced saffron corm rot in India.

CRediT authorship contribution statement

Tanzeel Bashir: Writing – original draft, Investigation, Formal analysis. **Syed Anam ul Haq:** Writing – original draft, Investigation. **Zahoor A. Bhat:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization. **Sajad Un Nabi:** Resources, Methodology, Formal analysis. **Shahid A. Padder:** Software, Data curation. **Amjad M. Husaini:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

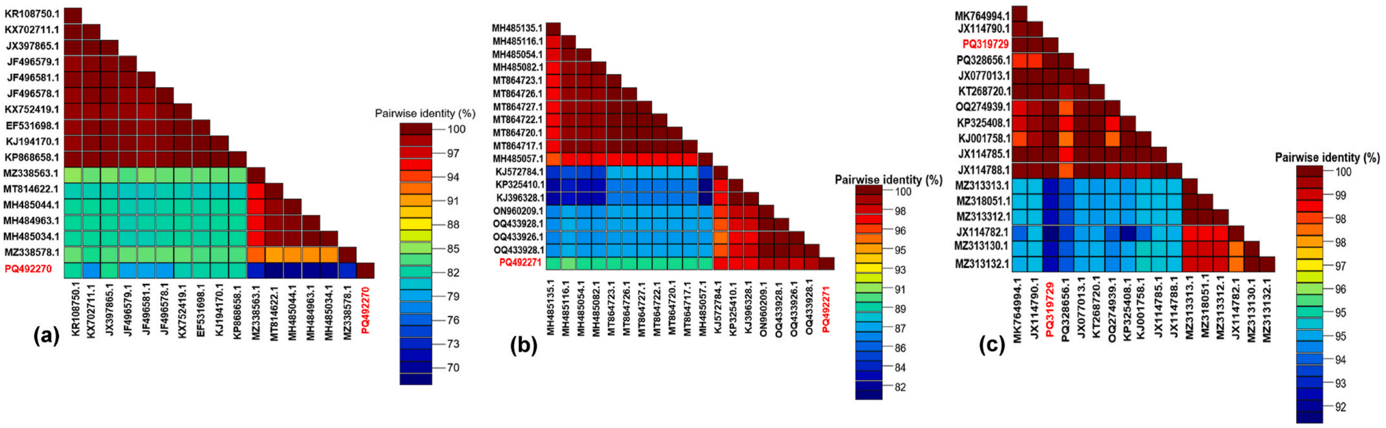

Fig. 5. Pairwise identity matrices for (a) *tef1*, (b) *tub2*, and (c) ITS gene sequences. Each colored cell represents a pairwise comparison, with percentage identity ranging from 70 to 100 %, 82–100 %, and 92–100 %, respectively. Analyses were performed using SDT v1.2 (http://web.cbio.uct.ac.za/).

Informed consent

Not applicable.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

It will be made available on request.

Code availability

Not applicable.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2025.102702.

Data availability

Data will be made available on request.

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Role of non-coding RNAs in quality improvement of horticultural crops: computational tools, databases, and algorithms for identification and analysis

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REVIEW

Role of non-coding RNAs in quality improvement of horticultural crops: computational tools, databases, and algorithms for identification and analysis

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Abstract

Horticultural crops, including fruits, vegetables, flowers, and herbs, are essential for food security and economic sustainability. Advances in biotechnology, including genetic modification and omics approaches, have significantly improved these crops'traits. While initial transgenic efforts focused on protein-coding genes, recent research highlights the crucial roles of non-coding RNAs (ncRNAs) in plant growth, development, and gene regulation. ncRNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), influence key biological processes through transcriptional and post-transcriptional regulation. This review explores the classification, functions, and regulatory mechanisms of ncRNAs, emphasizing their potential in enhancing horticultural crop quality. This growing understanding offers promising avenues for enhancing crop performance and developing new horticultural varieties with improved traits. Additionally, we elucidate the role of ncRNA databases and predictive bioinformatics tools into modern horticultural crop improvement strategies.

Keywords Horticulture · Non-coding RNAs · MicroRNAs · Gene regulation · Bioinformatics · Crop improvement

Introduction

Horticultural plants, including fruits, ornamental trees, vegetables, flowers, herbs, and tea plants, have been developed to address human needs for food and aesthetic value through techniques such as hybridization, mutation breeding, and genetic modification (Bashir et al. 2023; Xiong et al. 2015). Early transgenic breeding efforts primarily focused on protein-coding genes associated with specific agricultural traits(Husaini and Xu 2016a, b, Husaini et al. 2010a, b, Husaini 2010, Nerkar et al. 2022). Different approaches were developed for addressing the concerns raised against transgenic technologies (Brookes and Barfoot 2018; Husaini et al. 2011; Husaini and Tuteja 2013).However, overexpression of different protein coding genes remained as a method of choice for improving horticultural crops(Husaini and Xu 2016a, b; Husaini 2010; Husaini and Abdin 2008). Later, the challenges of climate change emerged as a major threat causing multiple biotic and abiotic stresses, and crops tolerant against these were developed using modern biotechnological and omics- approaches (Campbell et al. 2018; Gil et al. 2019; Husaini and Xu 2016a, b; Husaini et al. 2012; Husaini and Khurshid 2021). Genetic modification or modulation of pleiotropic genes is increasingly being explored as a strategy to develop climate-resilient, nutrient-dense crops suitable for high-value farms (Husaini 2022; Husaini and Rafiqi 2012). Apart from the coding regions of the plant genome, the other prominent targets for the development of better horticultural crops are non-coding RNAs(ncRNAs).

Non-coding RNAs are acknowledged for their essential roles in plant growth, development, and environmental stress responses, operating through both transcriptional and post-transcriptional mechanisms(Haq et al. 2022; Yang et al. 2023). Though up to 90% of the eukaryotic genome is transcribed into RNA, only about 2% of these RNA molecules encode proteins. Most of these transcripts are non-coding RNAs (ncRNAs) derived from regions once thought to be inactive or irrelevant, including intergenic areas, repetitive sequences, transposons, and pseudogenes(Zhang et al. 2019).Initially, transcripts originating from these regions were thought to be mere

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transcriptional noise because they either lacked significant protein-coding potential or had poorly conserved sequences.

While experimental research has provided much insight into the roles of non-coding RNAs (ncRNAs) in different biological processes, next-generation sequencing (NGS) and computational biology plays a crucial role in advancing this knowledge by predicting possible new interactions between ncRNAs and other molecules (Rincón-Riveros et al. 2021). Computational predictions provide a crucial alternative for discovering new insights, creating a feedback loop where experimental findings enhance computational models. These models, in turn, suggest potential interactions that can be tested and validated experimentally.

Non-coding RNAs (ncRNAs) regulate gene expression and key physiological processes in plants. Advances in sequencing and bioinformatics have expanded ncRNA databases and predictive tools, yet their applications in horticultural crop improvement remain underexplored. This review classifies ncRNAs, outlines their regulatory roles, and summarizes key databases and tools for functional analysis. It further highlights the potential of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in enhancing horticultural crop traits. By integrating bioinformatics with functional insights, this study underscores ncRNAs' role in advancing horticultural research.

Classification of non-coding RNAs

Since their discovery, non-coding RNAs (ncRNAs) have been divided into different categories. The major categories of ncRNA transcripts are housekeeping ncRNAs and regulatory ncRNAs(Fig. 1)(Yang et al. 2016). Housekeeping ncRNAs play a crucial role in fundamental cellular and ribosomal processes. This category includes ribosomal RNAs (rRNAs), transfer RNAs (tRNAs, ranging from 50–200 nucleotides), small nuclear RNAs or spliceosomal RNAs (snRNAs, typically 50–200 nucleotides), and small nucleolar RNAs (snoRNAs, around 50–200 nucleotides). On the other hand, regulatory ncRNAs modulate gene expression by interacting with mRNAs or other molecules. They play key roles in controlling developmental processes, cellular responses, and gene silencing. They encompass small RNAs such as microRNAs (miRNAs, approximately 20–24 nucleotides), which are the most abundant class of small non-coding RNAs, small interfering RNAs (siRNAs, 20–24 nucleotides), piwi-interacting RNAs (piRNAs, 24–32 nucleotides), and long non-coding RNAs (lncRNAs, longer than 200 nucleotides) (Zhao et al. 2022). Regulatory ncRNAs or ribo-regulators serve as crucial regulatory RNA molecules transcribed from DNA but not translated into proteins. Additionally, circular RNAs (circRNAs), a distinct class of endogenous ncRNAs characterized by covalently closed structures without 5'or 3'ends, are produced through

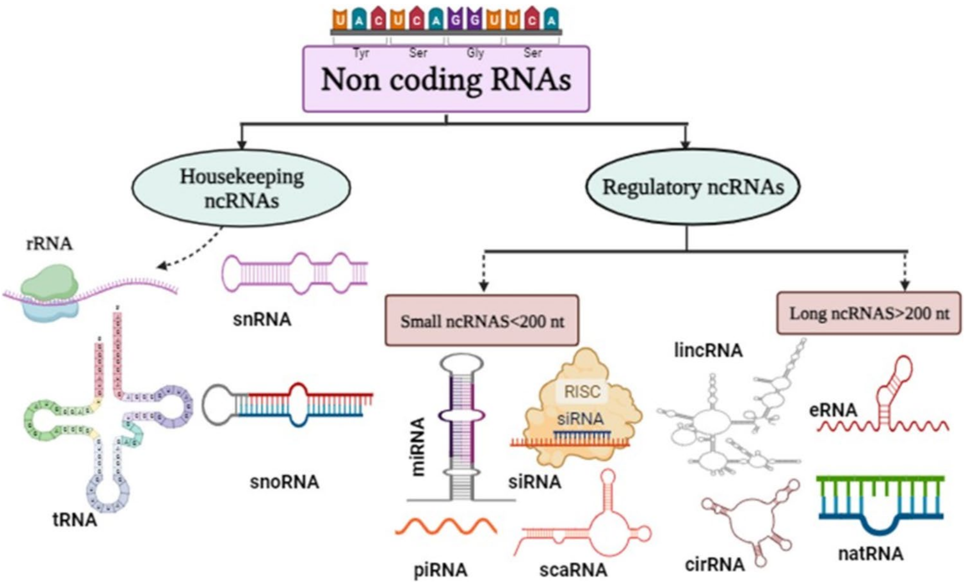

Fig. 1 The major categories of ncRNAs and their sub-categories: Housekeeping ncRNAs play a crucial role in fundamental cellular and ribosomal processes, and include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs, ranging from 50–200 nucleotides), small nuclear RNAs or spliceosomal RNAs (snRNAs, typically 50–200 nucleotides), and small nucleolar RNAs (snoRNAs, around 50–200 nucleotides). The regulatory ncRNAs modulate gene expression by interacting with mRNAs or other molecules, and encompass microRNAs (miRNAs, approximately 20–24 nucleotides), small interfering RNAs (siRNAs, 20–24 nucleotides), piwi-interacting RNAs (piRNAs, 24–32 nucleotides), and long non-coding RNAs (lncRNAs, longer than 200 nucleotides)

non-sequential back-end splicing from precursor mRNAs by RNA polymerase II (Bhogireddy et al. 2021). They are categorized into exonic, intronic, intergenic, and exon-intronic circRNAs based on their genomic origin, and play a role in gene expression regulation by acting as miRNA sponges.

Exploring non-coding RNAs and their regulatory functions in plants

miRNAs and siRNAs regulate gene expression through various mechanisms. These include mRNA cleavage, inhibition of translation, and suppression of transcription (Wang et al. 2021). Likewise, lncRNAs regulate gene expression through interactions with proteins and miRNAs. They impact mRNA stability and translation and can also modify chromatin structure (Waititu et al. 2020).

Target transcript cleavage by miRNAs and siRNAs

Sequence-specific gene silencing is achieved when miRNAs or siRNAs bind to complementary regions of target mRNA molecules, leading to their cleavage (Lam et al. 2015). Regarding miRNAs, the processing of MIR genes involves DCL1, HYL1, and the SE complex (Li and Yu 2021). This processing produces mature miRNA-miRNA duplexes. These duplexes are then transported to the cytoplasm by the HASTY protein, where they associate with the RNA-Induced Silencing Complex (RISC), which includes AGO1. The RISC-AGO1 complex binds to complementary sites within the target RNA transcript's sense sequence, leading to its degradation. Importantly, the antisense strand of miRNAs remains associated within the RISC complex (Lelandais-Brière et al. 2010). For siRNAs, one strand is incorporated into the RISC-AGO1 or AGO7 complex, guiding the cleavage of target gene transcripts approximately 10–11 nucleotides upstream of the 5'end of the antisense strand (Liu et al. 2017). Subsequently, the enzyme EXONUCLEASE 4 (XRN4) participates in the degradation of both the 3'and 5'cleaved fragments (Ren et al. 2014). Recent research has identified the RNA binding and target cleavage functions of AGO2, AGO4, and AGO10 in plants. These discoveries highlight the intricate nature of small RNA-mediated gene silencing processes (Zhu et al. 2011).

Translational suppression by miRNAs and siRNAs

AGO1 and AGO10 facilitate translational inhibition through imperfect pairing of small RNAs (sRNAs) with target mRNA in plants (Lee et al. 2018). However, the effectiveness of this inhibition process depends considerably on the quantity of miRNA binding sites (Cuellar and McManus 2005). By binding to the target gene's open reading frame (ORF) or 5'untranslated region (UTR), the RISC-AGO1 complex regulates translation. This binding restricts the recruitment or mobility of ribosomes. More factors like VARICOSE, GW-repeat proteins, the microtubule enzyme KATANIN, and ALTERED MERISTEM PROGRAM 1 influence this translation inhibition mechanism (Li et al. 2013a, b, c). The mechanisms behind sRNA-mediated translation repression and how the repressed target mRNAs escape endonucleolytic cleavage need further investigation.

DNA methylation directed by miRNAs and siRNAs

The Arabidopsis DCL family consists of multiple copies that are essential for generating small RNAs (sRNAs) of various lengths. DCL1 specifically converts partially paired double-stranded RNA (dsRNA) precursors into mature 21-nucleotide (nt) miRNAs (Pikaard and Scheid 2014). On the contrary, 20 to 22 nucleotide siRNAs are produced from entirely complementary dsRNA precursors via DCL2 and DCL4. 24 nucleotide siRNAs, often referred to as hc-siRNAs, are produced by DCL3 and frequently contribute to gene silencing through the RdDM pathway (Creasey et al. 2014). These hc-siRNAs are produced during the transcription process from heterochromatic domains, where they lead to cytosine methylation in the CG, CHG, and CHH sequence contexts in cis. DCL3-dependent miRNAs bind to AGO4 and form a complex that methylates histones and cytosines to restrict gene expression (Ye et al. 2012). In contrast, the de novo hc-siRNA-induced RdDM process needs the cooperation of Pol IV and V, DCL, AGO, and RNA-Dependent RNA Polymerase (RDR). These elements promote methylation at particular target sites and transcribe double-stranded precursors. Systemic silencing is thus caused by methylation of DNA and lysine at histone H3's ninth site (H3 K9). Transcriptional gene silencing is achieved through the hc-siRNA/AGO4 RNA-Induced Silencing Complex (RISC), which targets DNA and H3 K9 methyltransferases to the target sequence.

Gene expression modulation by long non-coding RNAs

Long non-coding RNAs (lncRNAs) function as intermediaries between RNA molecules and proteins. They play a crucial role in regulating gene expression. Depending on their interactions, lncRNAs can act as transcriptional activators, enhancing gene expression, or as repressors, inhibiting it (Dey et al. 2022). Nevertheless, the exact molecular mechanisms through which they exert these functions in plants remain partially understood. Plant lncRNAs function in both cis and trans contexts (Wu et al. 2020). Cis-acting lncRNAs function near their sites of synthesis. They act directly on local nucleotide sequences or chromosomal regions associated with one or more adjacent genes. Conversely, trans-acting lncRNAs migrate from their point of synthesis and can impact multiple genes, even across considerable distances, including those located on different chromosomes. Furthermore, lncRNAs can also serve as precursors for small RNAs (sRNAs) (Lambert et al. 2019). Certain lncRNAs can form double-stranded RNA duplexes with Natural Antisense Transcripts (NAT), generating sRNAs that perform regulatory functions. A Natural Antisense Transcript (NAT) pair was formed between the complementary regions of the Rab2-like gene and a pentatricopeptide repeat gene. This process was facilitated by an endogenous siRNA in *Arabidopsis* (Liu et al. 2015). Additionally, lncRNAs can serve as miRNA decoys, binding to miRNAs and preventing them from interacting with their target mRNAs. This interference reduces miRNA activity and relieves the repression of the target gene. For instance, in Arabidopsis experiencing phosphate deficiency, the lncRNA"Induced by Phosphate Starvation 1 (IPS1)"was discovered to mimic miRNA399 (Yuan et al. 2016). Moreover, lncRNAs often act through various mechanisms such as protein–protein interactions or post-translational modifications or subcellular localization, and via epigenetic regulatory mechanisms, including methylation of DNA, histone modification as well as chromatin remodelling (Matzke and Mosher 2014).

Computational tools for identifying and analyzing ncRNAs

Over the past twenty years, extensive research has been devoted to identifying non-coding RNAs (ncRNAs) and exploring their roles in various cellular processes (Garikipati and Uchida 2021). The introduction of next-generation sequencing (NGS) has revolutionized our ability to analyze ncRNA transcriptomes under different conditions. This advancement has opened new avenues for discovering novel ncRNAs and detecting changes in their expression levels (Mubarak and Zahir 2022). Recent advancements in transcriptomics methodologies and computational resources are significantly improving our ability to identify, classify, annotate, and analyze non-coding RNAs (ncRNAs). These developments are vital for the scientific community as they facilitate the discovery, annotation, archiving, prediction, and interpretation of ncRNA data (Dindhoria et al. 2022; Thind et al. 2022). However, details about these sequences are frequently hidden in supplementary materials associated with publications or are only indicated by the chromosomal location of the genes that encode them. This can pose difficulties for biologists and bioinformaticians in efficiently accessing and extracting the relevant data (Burley et al. 2022). To address this issue, specialized databases have been developed for various types of non-coding RNAs (ncRNAs) to extract, abstract, and present this information in a consolidated manner online. In 2010, a meeting of RNA researchers at Hinxton emphasized the swift growth in ncRNA sequence data and functional insights (Bateman et al. 2011). They advocated for the creation of a unified ncRNA sequence database that would harness the expertise of the RNA research community by integrating information from various existing ncRNA databases. With the continuous release of genomic information from diverse plant species, a strong foundation is being established for the discovery of novel ncRNAs. Additionally, growing knowledge about the function, structure, and conservation of ncRNAs is enhancing our ability to distinguish different ncRNA types. Since their discovery, numerous bioinformatics tools, including databases and software, have been developed to study ncRNAs. A significant proportion of these resources have been introduced in recent years (Tables 1 and 2).

ncRNA databases

ncRNA databases serve as comprehensive repositories that consolidate diverse types of ncRNA-related data, including sequence information, regulatory interactions, and expression profiles. Many of these databases integrate experimentally validated datasets to enhance reliability. For example, miRbase (Kozomara et al. 2019) systematically curates published mature miRNA sequences alongside their precursor hairpin structures. Likewise, resources such as miRTarBase (Huang et al. 2020) and NPInter (Teng et al. 2020) document interactions between ncRNAs and their molecular targets, supplementing these records with supporting experimental evidence. Currently, miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) are catalogued across multiple specialized online platforms (Jin et al. 2021) (Table 1). The rapid advancements in high-throughput sequencing technologies, combined with the continuous expansion of genomic datasets, have significantly enriched these repositories, enabling large-scale comparative analyses of ncRNA evolution. These developments facilitate the establishment of conserved models for predicting and functionally characterizing novel ncRNAs. Despite these advancements, several challenges persist in utilizing these databases for cross-species investigations. One of the primary constraints is the difficulty in distinguishing conserved ncRNAs from species-specific counterparts. Furthermore, achieving high-confidence functional annotation of novel ncRNAs remains a considerable challenge, necessitating the development of more sophisticated computational frameworks for accurate prediction and validation.

ncRNA prediction tools

The computational prediction of ncRNAs encompasses both their identification and functional annotation. While

Table 1 Bioinformatics databases for ncRNA analysis

ncRNA Type	Database	Description	Web Address	Reference
lncRNA	PLNlncRbase	• Curated database of experimentally identified plant long non-coding RNAs (lncRNAs) mir828 • Provides sequences, classifications, expression profiles, and detection methods • Includes predicted functions and target genes for studying regulatory roles	http://bioinformatics.ahau.edu.cn/PLNlncRbase	(Xuan et al. 2015)
	JustRNA	• Offers expression profiles and network interactions of plant lncRNAs • Enables exploration of regulatory roles and functional associations in various plant species	http://justrna.itps.ncku.edu.tw/	(Tseng et al. 2023)
	GreeNC	• A wiki-based database of plant non-coding RNAs (ncRNAs) • Provides curated information on lncRNAs and other ncRNAs across plant species	http://greenc.sciencedesigners.com/	(Paytuví Gallart et al. 2016)
	CANTATAdb 2.0	• Covers lncRNAs from 39 plant species and algae • Provides annotations, expression data, and functional predictions	http://cantata.amu.edu.pl/ http://yeti.amu.edu.pl/CANTATA/	(Szcześniak et al. 2019)
	AlnC	• Focuses on lncRNAs in angiosperms • Offers annotations, expression patterns, and functional insights	http://www.nipgr.ac.in/AlnC	(Singh et al. 2021)
	lncRNAdb	• A database for regulation mRNA and lncRNAs that have or associate with biological functions in eukaryotes	http://www.lncrnadb.org/	(Amaral et al. 2011)
	NONCODE	• A database of expression and biological functions of lncRNAs	http://www.noncode.org/	(Liu et al. 2005)
	CPC	• Calculate protein-coding potential of lncRNAs and other RNAs	http://cpc2.cbi.pku.edu.cn/	(Kong et al. 2007)
circRNA	AtCircDB	• Specialized for circular RNAs (circRNAs) in *Arabidopsis thaliana* • Provides annotations, expression profiles, and functional predictions	http://genome.sdau.edu.cn/circRNA	(Ye et al. 2019)
	CropCircDB	• Dedicated to circRNAs in crop plants • Focuses on their expression patterns and regulatory roles under abiotic stress	http://genome.sdau.edu.cn/crop/ http://deepbiology.cn/crop/	(Wang et al. 2019)
	PlantcircBase	• Database for plant circular RNAs (circRNAs) • Integrates high-throughput sequencing data for annotation • Provides expression profiling and functional analysis • Links circRNAs to miRNA and RNA-binding protein (RBP) interactions • Supports evolutionary studies of plant circRNAs	http://ibi.zju.edu.cn/plantcircbase/index.php	(Chu et al. 2017)

Table 1 (continued)

ncRNA Type	Database	Description	Web Address	Reference
miRNA	miRbase	• Comprehensive database of known microRNA (miRNA) sequences and annotations • Includes miRNA identification, classification, genomic locations, and target interactions	http://www.mirbase.org/	(Kozomara and Griffiths-Jones 2014)
	PNRD	• Comprehensive database for plant non-coding RNAs (ncRNAs) • Contains 25,739 entries covering different ncRNAs • Includes 11 types of non-coding RNAs • Covers 150 plant species • Provides a user-friendly interface for easy navigation	http://structuralbiology.cau.edu.cn/PNRD/index.php	(Yi et al. 2015)
	miRIAD	• Focuses on intragenic miRNAs and their host genes • Provides insights into genomic organization, regulatory relationships, and functional interactions	http://www.miriad-database.org/	(Hinske et al. 2014)
	MetaMirClust	• Database for miRNA clusters and their conservation across species • Offers insights into co-transcription, evolutionary relationships, and regulatory functions	http://fgfr.ibms.sinic.aedu.tw/MetaMirClust	(Chan and Lin 2016)
	miRTarBase	• Collection of experimentally validated miRNA-target interactions • Provides curated evidence for functional studies on miRNA-mediated gene regulation	http://mirtarbase.mbc.nctu.edu.tw/	(Hsu et al. 2010)
	PmiREN	• Comprehensive plant miRNA database • Offers curated annotations, expression profiles, target predictions, and evolutionary insights	http://www.pmiren.com/	(Guo et al. 2020)
	Rfam	• A comprehensive database of non-coding RNA (ncRNA) families, including lncRNAs, miRNAs, riboswitches, and other structured RNA elements • Provides multiple sequence alignments, consensus secondary structures, and covariance models for RNA families • Facilitates the identification and annotation of ncRNAs across different species using computational tools • Supports comparative genomics and evolutionary studies of structured RNAs • Widely used for RNA classification and functional predictions in diverse organisms	http://rfam.xfam.org/	(Kalvari et al. 2018)

Table 1 (continued)

ncRNA Type	Database	Description	Web Address	Reference
	mirPub	• A curated database providing literature-based information on microRNAs (miRNAs) • Collects and organizes experimentally validated miRNA-related publications • Offers insights into miRNA functions, regulations, and disease associations • Facilitates miRNA research by linking publications to relevant miRNA annotations and interactions • Supports researchers in identifying key references for miRNA functional studies	http://www.microrna.gr/mirpub/	(Vergoulis et al. 2015)
	PeTMbase	• A specialized database for plant-encoded transfer-messenger RNAs (tmRNAs) • Provides curated information on tmRNA sequences, structures, and functional roles in plants • Supports research on the role of tmRNAs in ribosome rescue and stress responses • Includes comparative analysis across different plant species	http://petmbase.org/	(Karakülah et al. 2016)
	AtmiRNET	• A database for Arabidopsis thaliana microRNA (miRNA) regulatory networks • Provides curated miRNA-target interactions, co-expression data, and functional annotations • Supports network-based analysis of miRNA-mediated gene regulation in Arabidopsis • Helps researchers explore miRNA roles in plant growth, development, and stress responses	http://AtmiRNET.itps.ncku.edu.tw/	(Chien et al. 2015)
	PmiRExAt	• A plant microRNA expression atlas providing expression profiles of miRNAs across various tissues, developmental stages, and stress conditions • Supports comparative analysis of miRNA expression patterns in different plant species • Facilitates functional studies by linking miRNA expression data with biological processes and environmental responses • A valuable resource for understanding the regulatory roles of miRNAs in plant development and stress adaptation	http://pmirexat.nabi.res.in/	(Gurjar et al. 2016)

Table 1 (continued)

ncRNA Type	Database	Description	Web Address	Reference
	PMTED	• A comprehensive database integrating plant microRNAs (miRNAs) and their target gene expression profiles • Provides experimentally validated miRNA-target interactions in plants • Supports comparative analysis of miRNA-target expression across different conditions and tissues • Helps researchers study miRNA-mediated gene regulation in various plant species	http://pmted.agrinome.org/	(Sun et al. 2013a, b)
	PASmiR	• A database focusing on plant miRNAs and associated small RNAs, providing comprehensive annotation and interaction data • Includes experimentally validated and predicted miRNA-target interactions across various plant species • Offers functional analysis tools for studying miRNA regulatory networks in plants • Supports comparative expression profiling of miRNAs and small RNAs under different conditions	http://pcsb.ahau.edu.cn:8080/PASmiR/	(Zhang et al. 2013)
	tasiRNAdb	• A database dedicated to trans-acting small interfering RNAs (tasiRNAs) in plants • Provides information on tasiRNA sequences, biogenesis pathways, and regulatory functions • Includes target predictions and expression profiles across different plant species • Supports research on tasiRNA-mediated gene silencing and its role in plant development and stress responses	http://bioinfo.jit.edu.cn/tasiRNADatabase/	(Zhang et al. 2014)
	starBase	• A comprehensive database integrating CLIP-seq, degradome-seq, and RNA-seq data • Provides information on miRNA-target interactions, ceRNA (competing endogenous RNA) networks, and RNA-binding protein (RBP) interactions • Supports multiple species, including plants and animals • Enables visualization and functional analysis of miRNA-mediated gene regulation	http://starbase.sysu.edu.cn/	(Yang et al. 2011)

Table 1 (continued)

ncRNA Type	Database	Description	Web Address	Reference
	RNAcentral	• Comprehensive Database for non-coding RNAs • Integrates data from multiple expert databases (e.g., miRBase, Rfam, Ensembl, RefSeq) • Contains millions of RNA sequences from various species • Covers different types of ncRNAs, including miRNAs, rRNAs, lncRNAs, tRNAs, and snoRNAs • Provides functional annotations and sequence alignments	https://rnacentral.org/	(Bateman et al. 2011)
	miRNEST	• A comprehensive database of plant and animal microRNAs (miRNAs) • Includes both known and predicted miRNAs along with their genomic locations and secondary structures • Provides expression data, target predictions, and comparative evolutionary analysis • Supports miRNA identification across multiple species	http://mirnest.amu.edu.pl/	(Szcześniak et al. 2012)
	siRNAdb	• A specialized database for small interfering RNAs (siRNAs) in plants • Provides curated annotations of siRNAs, including their sequences, origins, and regulatory roles • Supports functional analysis of siRNA-mediated gene silencing mechanisms • Helps researchers explore the involvement of siRNAs in plant defense, stress responses, and epigenetic regulation	http://siRNA.cgb.ki.se	(Chalk et al. 2005)

Table 1 (continued)

ncRNA Type	Database	Description	Web Address	Reference
	PmiRKB	• A specialized plant miRNA database with four major functional modules: • SNP module provides insights into single nucleotide polymorphisms (SNPs) within miRNA-related regions, helping researchers study genetic variations affecting miRNA function • Pri-miR module focuses on primary miRNA (pri-miR) structures, offering valuable information for understanding miRNA biogenesis and regulation in plants • MiR–Tar module contains data on miRNA-target interactions, aiding in the functional annotation of plant miRNAs and their regulatory roles • Self-reg module analyzes self-regulatory feedback loops involving plant miRNAs, contributing to the understanding of complex gene regulatory networks	http://bis.zju.edu.cn/pmirkb/	(Meng et al. 2010)
	scaRNAbase	• Dedicated to scaRNAs, a class of small non-coding RNAs • Provides sequence data and annotations for scaRNAs • Includes functional information related to snRNA modifications • Supports research on RNA processing and ribonucleoprotein biogenesis	http://gene.fudan.edu.cn/snoRNAbase.nsf	(Xie et al. 2007)
	NPInter	• Provides interaction information between non-coding RNAs (ncRNAs) and other biomolecules (proteins, DNAs, and RNAs) • Integrates experimentally validated ncRNA interactions from published literature • Covers various ncRNA types, including miRNAs, lncRNAs, circRNAs, and snoRNAs • Supports cross-species interaction analysis to explore conserved regulatory mechanisms • Helps researchers study the functional roles of ncRNAs in gene regulation and disease pathways	http://bigdata.ibp.ac.cn/npinter4/	(Teng et al. 2020)

numerous algorithms have been developed for ncRNA discovery, relatively few specialize in functional prediction. These tools can be categorized based on their specific applications (Table 2). A major challenge in the field lies in accurately identifying novel ncRNAs and their molecular targets. Recent advancements in predictive methodologies primarily focus on three key areas: miRNA sequence and precursor identification (Fei et al. 2021), detection of ncRNA binding sites on target molecules (Brousse et al. 2014), and modeling or visualizing secondary and tertiary RNA structures (Biesiada et al. 2016). Sequence alignment remains the cornerstone of ncRNA prediction; however, sequence divergence presents a significant hurdle in achieving high accuracy. Evolutionarily conserved ncRNAs, particularly those governing fundamental developmental pathways, exhibit strong conservation across

Table 2 *In silico* tools for the identification, prediction, annotation and analysis of important non-coding RNAs

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
lncRNA	PLncPRO	Random Forest	• Predicts lncRNAs using transcriptome data in plants • Uses a machine learning strategy (random forest algorithm) • Employs 71 distinct features to differentiate coding and non-coding transcripts	http://ccbb.jnu.ac.in/plncpro/	(Singh et al. 2017)
	ICAnnoLncRNA	Machine Learning-based Classification	• Automated workflow for detecting, classifying, and annotating plant lncRNAs • Processes transcriptomic sequences from RNA-seq data • Streamlines lncRNA characterization	https://github.com/artempronozin95/ICAnnoLncRNA-identification-classification-and-annotation-of-LncRNA	(Pronozin and Afonnikov 2023)
	PLIT	L1 Regularization + Iterative Random Forests	• Detects plant lncRNAs from RNA-seq datasets • Utilizes L1 regularization and iterative Random Forests for feature selection • Focuses on sequence and codon-bias features to classify transcripts	https://github.com/deshpan4/PLIT	(Deshpande et al. 2019)
	lncRNADetector	Alignment-based or Machine Learning Methods	• Tailored for discovering lncRNAs in medicinal and aromatic plants • Identifies lncRNAs from NGS data • Integrated with MAPslnc database	https://lncrnapipe.cimap.res.in/	(Shukla et al. 2021)
	UClncR	Transcript Assembly + Machine Learning Annotation	• Performs transcript assembly and predicts novel lncRNAs • Quantifies and annotates known and newly discovered lncRNAs • Generates user-friendly reports for downstream analysis	http://bioinformaticstools.mayo.edu/research/UClncR	(Sun et al. 2017)
	LncMachine	Machine Learning	• Identifies and annotates plant lncRNAs • Uses machine learning algorithms for classification • Supports large-scale transcriptome analysis	https://github.com/hbusra/lncMachine	(Cagirici et al. 2021)
	ASLncR	Machine Learning	• Predicts abiotic stress-responsive lncRNAs in plants • Uses machine learning and sequence-derived features • Complements experimental methods for stress-related lncRNA identification	https://iasri-sg.icar.gov.in/aslncr/	(Pradhan et al. 2023)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	Prelnc2	Ensemble Learning	• Predicts lncRNAs using multi-dimensional RNA features • Integrates sequence characteristics, secondary structure, and evolutionary conservation • Uses an ensemble learning model for improved classification	https://github.com/spu112005/prelnc2	(Gao et al. 2023)
	COME	Not specified	• A tool for identification and characterization of coding potential	https://github.com/lulab/COME	(Hu et al. 2017)
	CNCI	SVM (Triplet Nucleotide Patterns)	• distinguishing coding and non-coding RNAs • Works independently of reference genomes, making it useful for non-model organisms • Effectively differentiates lncRNAs from protein-coding transcripts • Accepts transcript sequences in FASTA format for analysis • Does not rely on evolutionary conservation or homology-based methods • Widely used in transcriptome studies for lncRNA identification and functional annotation	https://github.com/www-bioinfo-org/CNCI	(Sun et al. 2013a, b)
	CPAT	Logistic Regression	• Annotates lncRNAs • Predicts coding vs. non-coding RNAs • Uses logistic regression model based on ORF length, Fickett score, and hexamer usage bias • Requires species-specific training data for optimal accuracy • Works efficiently with large-scale transcriptome datasets • Outputs coding probability scores, helping in lncRNA identification	http://rna-cpat.sourceforge.net/	(Wang et al. 2013)
circRNA	CircPCBL	Deep Learning	• Uses deep learning to differentiate circRNAs from lncRNAs • Analyzes raw sequence data	https://github.com/Peg-Wu/CircPCBL	(Wu et al. 2023)
	CircPrime	Primer Design Algorithms (Thermodynamic Modeling)	• Web-based tool for designing DNA primers for circRNA detection • Optimizes thermocycling parameters for PCR-based identification	http://circprime.elgene.net/	(Sharko et al. 2023)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	CIRIquant	Advanced Statistical Models	• Facilitates quantification and differential expression analysis of circRNAs • Constructs pseudo-circular references for accurate analysis • Uses advanced statistical models	https://sourceforge.net/projects/ciri	(Zhang et al. 2020a, b, c)
	DCC	Read Alignment + Back-Splice Junction Detection	• Identifies and quantifies circRNAs from RNA-seq data • Detects back-spliced junctions • Provides differential expression analysis	https://github.com/dieterich-lab/	(Cheng et al. 2016)
	nf-core/circrna	Read Alignment-based Quantification Methods	• Comprehensive workflow for circRNA analysis • Includes quantification, differential expression, and miRNA target prediction	https://nf-co.re/circrna https://github.com/nf-core/circrna	(Digby et al. 2023)
	CircPlant	Sequence Alignment + Motif Discovery	• Identifies plant-specific circRNAs • Highlights their roles as competing endogenous RNAs (ceRNAs)	http://bis.zju.edu.cn/circplant	(Zhang et al. 2020a, b, c)
	StackCirRNAPred	Stacking Ensemble Learning	• Classifies circRNAs using stacking ensemble learning • Integrates multiple machine learning models for improved accuracy • Utilizes sequence-derived features, secondary structure, and conservation data	https://github.com/xwang1427/StackCirRNAPred	(Wang et al. 2022)
	CirComPara2	Multiple circRNA Detection Pipelines (CIRI, DCC, find_circ)	• Detects and quantifies both linear and circular RNAs from RNA-seq data • Uses multiple approaches for circRNA detection	https://github.com/egaffo/CirComPara2	(Gaffo et al. 2022)
	PcircRNA_finder	Back-Splice Junction Detection + Machine Learning	• Predicts plant circRNAs with high sensitivity and accuracy • Outperforms traditional detection methods	http://ibi.zju.edu.cn/bioinplant/tools/manual.htm	(Chen et al. 2016)
	CircPro	Junction Read-based Detection + Coding Potential Analysis	• Detects circRNAs from high-throughput sequencing data • Assesses coding potential and junction reads from Ribo-Seq data	http://bis.zju.edu.cn/CircPro	(Meng et al. 2017)
miRNA	miRDeep-P2	Not specified	• Optimized for plant miRNA analysis using small RNA sequencing data • Enhanced accuracy over previous versions	https://sourceforge.net/projects/mirdp2/	(Kuang et al. 2019)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	miRCat2	Entropy-Based Approach	• Identifies miRNA loci using an entropy-based approach • Provides graphical representations of hairpin structures and sequence alignments	http://srna-workbench.cmp.uea.ac.uk/	(Paicu et al. 2017)
	iwa-miRNA	Homology-Based Annotation + Machine Learning	• Supports miRNA annotation in plants • Integrates computational analysis with manual curation	http://iwa-mirna.omicstudio.cloud/	(Zhang et al. 2022)
	miRkwood	Quality Scoring Algorithm	• Identifies plant miRNAs from small RNA sequencing data • Includes quality scoring to rank predictions and reduce false positives	http://bioinfo.cristal.univ-lille.fr/mirkwood	(Guigon et al. 2019)
	miR-Island	Pseudo-Genome Approach	• Efficiently annotates and quantifies miRNAs in plant genomes • Uses pseudo-genome approaches	https://github.com/janeyurigao/miR-Island	(Gao et al. 2019)
	Mirador	Sequence Alignment + Probabilistic Models	• Accurately predicts miRNAs based on widely accepted standards	https://github.com/rkweku/miRador	(Hammond et al. 2023)
	miWords	Deep Learning (Transformers + CNN)	• Uses deep learning with transformers and convolutional neural networks • Detects pre-miRNA regions in plant genomes	https://scbb.ihbt.res.in/miWords/index.php	(Gupta and Shankar 2023)
	PmiRDiscVali	Machine Learning + Sequence-based Feature Selection	• Large-scale identification of plant miRNAs • Supports both reference-based and de novo prediction methods	https://github.com/unincrna/pmirdv	(Yu et al. 2019a, b)
	PlantMirP2	Machine Learning	• Predicts precursor and mature miRNAs in plants • Uses machine learning for enhanced accuracy • Optimized for large RNA-seq datasets	https://github.com/wuqiansibai/plantMiRP2/releases/tag/v1.0/	(Fan et al. 2021a, b)
	TarHunter	Sequence Complementarity Scoring Methods	• Predicts conserved miRNA targets and target mimics in plants • Identifies miRNA binding sites in coding and non-coding regions	http://www.biosequencing.cn/TarHunter/	(Ma et al. 2018)
	miRanalyzer	Alignment-Based + Machine Learning Methods	• Detects and analyzes miRNAs from RNA sequencing data • Identifies both known and novel miRNAs • Evaluates miRNA expression levels and biogenesis	http://bioinfo5.ugr.es/miRanalyzer/miRanalyzer.php	(Hackenberg et al. 2009)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	miRA	Machine Learning	• Identifies miRNAs in organisms without existing annotations • Uses machine learning for novel miRNA discovery	https://github.com/mhuttner/miRA	(Evers et al. 2015)
	PsRobot	Sequence Complementarity + Secondary Structure Analysis	• A bioinformatics tool designed for plant small RNA analysis • Predicts miRNA precursors and their corresponding mature miRNAs • Identifies potential miRNA targets in plant genomes • Facilitates functional studies on small RNA-mediated gene regulation • Useful for analyzing high-throughput sequencing data in plants • A web-based easy-to-use tool dedicated to the identification of smRNAs with stem-loop shaped precursors (such as microRNAs and short hairpin RNAs) and their target genes/transcripts	http://omicslab.genetics.ac.cn/psRobot/	(Wu et al. 2012)
	psRNATarget	Modified Smith-Waterman Algorithm	• A widely used web-based tool for predicting plant microRNA targets • Utilizes a modified Smith-Waterman algorithm for sequence complementarity analysis • Provides insights into miRNA-mediated gene regulation by identifying potential target genes • Supports multiple plant species with customizable target prediction parameters • Facilitates functional genomics and transcriptome-wide miRNA studies	http://plantgrn.noble.org/psRNATarget/	(Dai and Zhao 2011)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	CleaveLand	Scoring-Based System	• A computational tool designed for detecting cleaved microRNA targets using degradome (PARE) sequencing data • Identifies and validates miRNA-mediated target cleavage sites in plant transcriptomes • Uses a scoring system based on target site complementarity and degradome signal strength • Facilitates genome-wide discovery of miRNA-regulated genes • Supports high-throughput analysis for functional genomics studies	http://www.bio.psu.edu/people/faculty/Axtell/AxtellLab/Software.htm/	(Addo-Quaye et al. 2009)
	miRNApath	Pathway Enrichment Algorithms (KEGG, DAVID-based)	• A bioinformatics tool designed for pathway enrichment analysis of miRNA target genes • Helps identify biological pathways regulated by miRNAs • Integrates miRNA-target interactions with gene ontology and pathway databases • Supports functional annotation of miRNA-mediated regulatory networks • Useful for studying miRNA roles in plant and animal systems	http://lgmb.fmrp.usp.br/mirnapath/	(Chiromatzo et al. 2007)
	miR-PREFeR	Probabilistic Model	• A computational tool designed for the accurate prediction of novel miRNAs from deep sequencing data • Utilizes expression patterns and secondary structure features to distinguish miRNAs from other small RNAs • Implements a probabilistic model to enhance prediction accuracy • Supports large-scale miRNA identification in plants and other organisms	https://github.com/hangelwen/miRPREFeR/	(Lei and Sun 2014)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	CleaveLand4	Alignment-based prediction, PARE data integration	• Predict the binding sites of miRNAs in target • Detects miRNA-mediated cleavage sites in degradome sequencing data • Supports high-throughput analysis for multiple plant species • Provides target plots (t-plots) for visualizing miRNA-induced cleavage events • Widely used in plant miRNA research to study post-transcriptional gene regulation	https://github.com/MikeAxtell/CleaveLand4	(Addo-Quaye et al. 2009)
	TAPIR	Sequence Complementarity + Free Energy Calculations	• A bioinformatics tool designed for predicting miRNA targets in plants • Supports both fast and sensitive target prediction modes, balancing speed and accuracy • Uses sequence complementarity and free energy calculations to identify miRNA-target interactions • Helps researchers study gene regulation by miRNAs in various plant species	http://bioinformatics.psb.ugent.be/webtools/tapir/	(Bonnet et al. 2010)
	comPARE	Statistical models for miRNA-mediated cleavage events	• Computational tool for analyzing PARE (Parallel Analysis of RNA Ends) data • Designed for high-throughput identification of miRNA-target interactions • Supports cross-species comparisons to identify conserved miRNA functions • Provides visualization tools for exploring degradome sequencing results • Helps in understanding post-transcriptional gene regulation in plants	https://mpss.danforthcenter.org/tools/mirna_apps/comPARE.php	(Riffo-Campos et al. 2016)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	pssRNAMiner	Small RNA Sequencing Analysis + Degradome Data Integration	● A bioinformatics tool designed for identifying phased small RNAs (phasiRNAs) and their regulatory networks in plants ● Detects phased siRNA loci from small RNA sequencing data ● Helps in understanding the role of phasiRNAs in gene regulation and stress responses ● Supports functional annotation and target prediction for identified small RNAs	http://bioinfo3.noble.org/pssRNAMiner/	(Dai and Zhao 2008)
	comTAR	High-throughput sequencing-based detection	● Computational tool for identifying transcriptionally active regions (TARs) in genomic data ● Utilizes high-throughput sequencing data to detect active regulatory regions ● Helps in studying gene expression dynamics and regulatory elements ● Supports comparative analysis across different species or conditions ● Assists in identifying functional ncRNAs and their roles in transcriptional regulation	http://rnabiology.ibr-conicet.gov.ar/comtar/	(Liao et al. 2018)
	RNAComposer	Fragment-based assembly and computational modeling	● an automated 3D structure prediction tool for RNA molecules ● Uses sequence and secondary structure information to generate 3D models ● Supports visualization and analysis of RNA tertiary structures ● Widely used for studying RNA folding, interactions, and functional mechanisms ● Helps in understanding the structural basis of RNA function in biological processes	http://rnacomposer.ibch.poznan.pl/	(Xu et al. 2022)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	RNAshapes	Abstract shape representation and thermodynamic stability	● Tool for predicting and analyzing RNA secondary structures ● Supports efficient exploration of RNA folding space with a focus on biologically relevant structures ● Helps in understanding RNA structure–function relationships in various biological processes	https://bibiserv.cebitec.uni-bielefeld.de/rnashapes	(Liao et al. 2018)
	RNAcon	Machine learning-based identification of RNA base pair interactions	● Tool designed for RNA contact prediction based on sequence and structural features ● Helps in understanding RNA 3D folding, stability, and intermolecular interactions ● Supports applications in RNA structure modeling, drug discovery, and functional annotation ● Enhances the accuracy of RNA secondary and tertiary structure predictions	http://crdd.osdd.net/raghava/rnacon/	(Panwar et al. 2014)
	Inferna	Energy-based models with sequence alignment	● Computational tool for predicting RNA secondary structures using energy-based models ● Integrates thermodynamic parameters and sequence alignment to improve prediction accuracy ● Useful for identifying conserved RNA structural motifs across species ● Supports applications in ncRNA function annotation, RNA evolution studies, and structural analysis ● Enhances understanding of RNA folding dynamics and interactions	http://eddylab.org/infernal/	(Liao, Li et al. 2018)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	NUPACK	Thermodynamic modeling for RNA/DNA interactions	● computational tool for analyzing and designing nucleic acid secondary structures ● Supports DNA and RNA structure prediction, hybridization analysis, and reaction pathway modeling ● Useful for designing multistranded complexes and evaluating thermodynamic stability ● Applies to fields like synthetic biology, nanotechnology, and RNA-based therapeutics ● Provides a web-based interface and command-line tools for customized nucleic acid simulations	http://www.nupack.org/partition/new	(Wang et al. 2012)
	RNAz	Thermodynamic stability and evolutionary conservation	● Computational tool for detecting functional ncRNAs in multiple sequence alignments ● Uses thermodynamic stability and evolutionary conservation to identify structured RNA elements ● Predicts whether an RNA sequence is likely to form a stable secondary structure with functional significance ● Supports genome-wide screening for conserved and structured ncRNAs across species ● Useful in comparative genomics, ncRNA discovery, and regulatory RNA analysis	https://www.tbi.univie.ac.at/software/RNAz/	(Hertel and Stadler 2006)
	CentroidAlign	Probabilistic models incorporating base-pairing probabilities	● Multiple sequence alignment of RNA sequences ● Uses probabilistic models to predict structurally informed alignments ● Incorporates base-pairing probabilities to improve alignment accuracy ● Useful for analyzing ncRNAs, conserved RNA motifs, and secondary structures ● Helps in phylogenetic studies and functional RNA characterization	http://www.ncrna.org/software/centroidalign/	(Hamada et al. 2009)

Table 2 (continued)

ncRNA Type	Tool	Algorithm Used	Role	Web Address	Reference
	RNAfold	Minimum free energy (MFE) thermodynamic modeling	• Predicting RNA secondary structures based on minimum free energy (MFE) • Part of the Vienna-RNA package, widely used in RNA structure analysis • Provides base-pairing probabilities, centroid structures, and ensemble free energy calculations • Supports thermodynamic modeling to predict the most stable RNA conformation • Useful for studying ncRNAs, riboswitches, miRNAs, and RNA-RNA interactions	http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi	(Denman 1993)
	Randfold	MFE comparison with randomized sequences	• Statistical tool for assessing the significance of predicted RNA secondary structures • Compares the minimum free energy (MFE) of a given RNA sequence to a set of randomly shuffled sequences • Helps determine whether an RNA structure is biologically relevant or just a random fold • Commonly used in ncRNA identification, miRNA prediction, and functional RNA analysis • Useful for filtering false positives in RNA secondary structure predictions	https://github.com/erbon7/randfold	(Thakur et al. 2011)

species (Jarroux et al. 2017). In contrast, recently evolved ncRNAs often demonstrate species specificity. Moreover, different ncRNA classes display varying degrees of conservation, further complicating cross-species predictions. Enhancing the precision of ncRNA prediction remains an ongoing challenge, which could potentially be addressed through the development of conserved computational models tailored to specific ncRNA families, leveraging the rapidly expanding repository of ncRNA sequence and functional data.

Regulatory roles of ncRNAs in enhancing quality traits of horticultural crops

Horticulture, one of the oldest and most globally significant agricultural practices, encompasses a diverse range of crops, including fruits, vegetables, ornamental plants, herbs, and tea trees. Over time, various breeding strategies-such as hybridization, mutation breeding, and transgenic approaches have been employed to enhance desirable traits in these crops. Traditionally, transgenic breeding primarily targeted protein-coding genes associated with key agronomic traits. However, recent discoveries have revealed that non-coding RNAs (ncRNAs) play pivotal roles in regulating plant growth, development, and responses to environmental stimuli at both transcriptional and post-transcriptional levels. As a result, ncRNAs are emerging as promising molecular targets for accelerating the domestication and genetic improvement of horticultural crops.

Although ncRNA discovery and functional characterization have been ongoing for over half a century, their widespread presence and diverse regulatory roles were not fully appreciated until the post-genomic era. A surprising revelation from genome annotations is that protein-coding sequences occupy only a small fraction (2–25%) of the genomic landscape. Functional insights into ncRNAs have been gained through molecular genetic approaches, including gain-of-function and loss-of-function analyses. By examining recent advancements in ncRNA research in horticultural crops, this review aims to provide a foundation for further investigations and practical applications in the field. It explores critical biological processes such as pigment biosynthesis, organ size determination, flavor and texture modulation, secondary metabolite production, reproductive tissue differentiation, and the intricacies of fruit ripening across a range of horticultural crops. These processes, intricately regulated by ncRNAs, are central to enhancing the qualitative attributes and overall performance of horticultural crops, offering valuable insights into their molecular control.

Fruits

The biosynthesis and accumulation of pigments are fundamental processes that drive the maturation and ripening of horticultural crops, contributing significantly to their characteristic color transitions. These pigments not only define the visual appeal of fruits and vegetables but also serve as bioactive compounds, enhancing the nutritional and health-promoting properties of horticultural produce. Pigment accumulation is largely governed by the progression of ripening stages, making it an essential biochemical marker for development of horticultural crops, with direct implications for post-harvest storage and quality management (Kapoor et al. 2022). Anthocyanins, a prominent class of pigments, play a key role in determining the coloration of crops and are primarily regulated by MYB transcription factors. In tomato (*Solanum lycopersicum*), silencing of miR858 significantly upregulates MYB7-like, thereby promoting anthocyanin accumulation (Jia et al. 2015). Likewise, miR828 negatively regulates anthocyanin biosynthesis under phosphate-deficient conditions (Xiao-yun et al. 2015). Additionally, long non-coding natural antisense transcript (lncNAT-ACoS-AS1) mediates trans-splicing of the PSY1 gene, encoding phytoene synthase, a key enzyme in carotenoid biosynthesis, resulting in yellow pigmentation in tomatoes (Xiao et al. 2020) (Table 3, Fig. 2). Beyond their role in color formation, anthocyanins serve as secondary metabolites, modulating photosynthesis, filtering UV rays, and enhancing antioxidant capacity in fruits and vegetables. In *Hippophae rhamnoides* (sea buckthorn), LNC1 and LNC2 act as endogenous target mimics (eTMs) for miR156a and miR828a, respectively, modulating the expression of transcription factors SPL9 and MYB114, which regulate anthocyanin content (Zhang et al. 2018) (Table 3, Fig. 2). Similarly, carotenoids, which determine the color of leaves, flowers, and fruits, also attract pollinators and seed dispersers while offering protection against photodamage. In sea buckthorn, 61 differentially expressed lncRNAs were identified, with 23 specifically expressed in red fruit and 22 in yellow fruit, regulating carotenoid biosynthesis via cis- and trans-regulatory mechanisms (Zhang et al. 2017).

In *Morus spp.* (mulberry), lncNAT ABCB19 AS, derived from the ABCB19 gene, modulates miR477-mediated cleavage and enhances anthocyanin accumulation (Dong et al. 2021) (Table 3, Fig. 2). The regulation of anthocyanin biosynthesis in *Malus × domestica*, an economically significant fruit, involves a network of miRNAs, lncRNAs, and transcription factors. Notably, miR828 and TAS4, along with MYB1, form a feedback loop that modulates anthocyanin biosynthesis, with miR828 expression increasing during fruit pigmentation (Zhang et al. 2020a, b, c). A WRKY1-LNC499-ERF109 cascade also plays a role, with WRKY1 activating LNC499 expression and subsequently enhancing

Table 3 Target Identification and Computational Approaches used for functional validation of ncRNAs in Horticultural crops

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
Fruits	*Malus domestica*	miR828	TAS4	_	_	• Overexpression of mdm-miR828 suppressed anthocyanin production	(Zhang et al. 2020a)
		miR172	AP2	_	RNA sequencing (RNA-seq)	• Overexpressing mdm-miR172 resulted in smaller fruit size and diminished red coloration with a notable increase in russeting on the fruit skin and a reduction in flavonoid levels	(Ding et al. 2022)
		miR7125	CCR	_	_	• Overexpression of miR7125 increased anthocyanin production and decreased lignin synthesis when exposed to light. In contrast, inhibiting miR7125 produced the reverse effects	(Hu et al. 2021)
		miR172	AP2	_	_	• Overexpressing miR172 led to smaller fruit or complete fruit absence and disrupted the development of floral organs	(Yao et al. 2015)
		miR156,MLNC3.2, MLNC4.6	(SPL2-like, SPL33)	_	RNA Sequencing (RNA-seq)	• Act as endogenous target mimics (eTMs) of miR156, preventing its cleavage of SPL2-like and SPL33 transcripts, thereby participating in photoinduced anthocyanin biosynthesis	(Yang et al. 2019a)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
		LNC499	ERF109	_	Hi-C sequencing	• WRKY1 binds to the LNC499 promoter, activating its transcription and upregulating ERF109, which enhanced anthocyanin biosynthesis during early fruit coloring, promoting pigment accumulation	(Ma et al. 2021)
	Hippophae rhamnoides	miR156a	LNC1	_	Strand-Specific RNA Sequencing (ssRNA-seq)	• LNC1 acted as an endogenous target mimic (eTM) for miR156a, influencing SPL9 expression, which regulates anthocyanin content	(Zhang et al. 2018)
		miR828a	LNC2			• LNC2 acted as an endogenous target mimic (eTM) for miR828a, influencing MYB114 expression, which regulates anthocyanin content	(Zhang et al. 2018)
	Hippophae rhamnoides	61 differentially expressed lncRNAs, 23 in red fruit, 22 in yellow fruit		CPC (Coding Potential Calculator)	RNA Sequencing (RNA-seq)	• Modulated carotenoid biosynthesis through cis- and trans-regulatory mechanisms	(Zhang et al. 2017)
	Fragaria ananassa	miR397	FRILAIR/LAC11a	_	High-throughput sequencing	• Knockdown of miR397 accelerated fruit maturation, whereas overexpression of miR397 yielded the opposite effects	(Li et al. 2016)
		miR399	PHO2			• Fruit with increased miR399a expression showed higher sugar levels	(Wang et al. 2017)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Vitis vinifera	miR828	MYB113	–	Small RNA Sequencing (sRNA-seq)	• Overexpressing vvi-miR828 led to leaves with a lighter coloration	(Chen et al. 2019)
	Morus alba	lncNAT ABCB19 AS	miR477	–	High-Throughput Small RNA Sequencing, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis	• Modulated ABCB19 expression by facilitating miR477-mediated cleavage, enhancing anthocyanin accumulation	(Dong et al. 2021)
		lncRNAs (LNC- 0132, LNC- 0521, LNC-0782)	–	NONCODE4.0 database, TAIR and PlncDB, BLASTN, Cufflinks, RefSeq, Ensembl, UCSC, and Vega	–	• 1,133 lncRNAs exhibit tissue-specific expression, with some associated with floral organ formation	(Song et al. 2016)
	Pyrus bretschneideri	miR397	LAC	psRNATarget, MEGA5, ClustalW	–	• Transient overexpression of miR397a caused a decline in lignin content and reduced the quantity of stone cells in the fruit	(Xue et al. 2019)
	Actinidia arguta	miR858	MYBC1	–	Small RNA Sequencing	• Fruit that transiently overexpressed miR858 showed no color development, and anthocyanin levels were almost undetectable	(Li et al. 2020)
	Cucumis melo	miR393	AFB2	Mireap, FastQC, CleaveLand, Target Finder, Metascape, Rfam 11 database, miRBase 21, SRA database	–	• Overexpression of miR393 postponed fruit ripening	(Bai et al. 2020)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Prunus mume	_	_	TopHat, MIRANDA, PITA and RNAHYBRID	_	• 2,572 unique lncRNAs mapped and 24,648 genes, predicting 591 novel lncRNAs • Significant differences were found, with 186 known and 89 novel lncRNAs differentially expressed between the cultivars • lncRNAs XR_514690.2 and TCONS_00032517 may play a role in the development of multiple pistils	(Wu et al. 2019a, b)
	Pyrus pyrifolia	Pp-miRn182	PP2 C1	psRNATarget, NCBI Sequence Read Archive, GDR database, psRNATarget, Bowtie2, DESeq2, OmicShare online tools	Illumina HiSeq2500	• Regulated PP2 C1 expression; • Played a crucial role in hydrogen cyanide-induced endodormancy release via the PP2 C–H_2O_2 pathway	(Li et al. 2021)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Sand pear (Pyrus spp.)	PB.6461.1, PB.13941.1	4-coumarate: CoA ligase (4 CL)	BluePippin™ Size Selection System, and Pfam database 27.0, SWISS-PROT (a manually annotated and reviewed protein sequence database), NR (NCBI non-redundant), Pfam (a database of conserved protein families or domains), BLASTX, Coding Potential Calculator (CPC), Coding–Non-coding Index (CNCI), Coding Potential Assessment Tool (CPAT),	COG (Clusters of Orthologous Groups), eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes)	• Regulated 4-coumarate: CoA ligase (4 CL), modulating enzymatic browning in sand pear flesh	(Fan et al. 2021)
	Citrus limon	11,814 ncRNAs	_	_	_	• 632 ncRNAs correlate with 5,810 mRNAs; 113 ncRNAs involved in terpenoid metabolism, 29 in flavonoid biosynthesis	(Bordoloi et al. 2022)
Vegetables	*Solanum lycopersicum*	miR828	MYB7	psRNATarget, MegAlign of DNAStar, MEGA5	_	• Seedlings overexpressing miR828 exhibited reduced anthocyanin levels under phosphate deficiency	(Xiao-yun et al. 2015)
		miR858	MYB7	psRNATarget, MegAlign of DNAStar, MEGA5	_	• Inhibition of miR858 led to increased anthocyanin accumulation	(Jia et al. 2015)
		lncRNA2155	*RIN*	_	_	• Delayed fruit ripening	(Zuo et al. 2020)
		lncRNA *ACoS-AS1*	*SlPSY1*	ORFfinder	_	• Trans-splicing and carotenoid biosynthesis	(Xiao et al. 2020)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
		miR396	GRFs	miRBase, PlantTFDB database	_	• Inhibition of miR396 resulted in enhanced fruit size and weight	(Cao et al. 2016)
		miR1917	CTR4	_	_	• The miR1917 knockdown mutants exhibited elongated terminal leaflets, larger floral organs, and increased fruit and seed size	(Yang et al. 2020)
		miR171	HAM/NSP	Tomato Sol Genomic Network database, Cuffdiff, RSeQC-2.3.2 program	Illumina HiSeq™ 2000 System	• Downregulation of miR171 led to abnormal compound leaf development, an increase in axillary branch formation, and disruptions in pollen development	(Huang et al. 2017)
		miR168	AGO1	BLAST- 2.2.28 +	_	• The expression of miR168-resistant AGO1 A/B resulted in defects that affected growth rate, flowering timing, leaf morphology, and fruit development	(Xian et al. 2014)
		miR159	GAMYB1/2	miRbase version 21, Solanaceae Genome Network Database, mFOLD	_	• Plants overexpressing miR159 exhibited early fruit initiation and displayed obligatory parthenocarpy	(da Silva et al. 2017)
		miR164	NAM2/3	RNAfold, Tomato Functional Genomics Database	_	• Loss of function in MIR164a resulted in small fruit with a pronounced red color and a matte appearance	(Gupta et al. 2021)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
		miR166	REV	Tomato Sol Genomic Network database	_	• Overexpression of an miR166-resistant variant of REV caused continuous flower production at the pedicel abscission zone and led to the formation of ectopic fruit on the receptacles	(Hu et al. 2014)
		miR160	ARF10	BLASTN	_	• Knockdown of miR160 led to pronounced elongation of the proximal regions of mutant ovaries and a reduction in placental thickness	(Damodharan et al. 2016)
		miR156	SPLs	TargetFinder v1.6, CleaveLand, FASTX Toolkit version 0.0.13, Vmatch version 2.0,	Degradome Sequencing (PARE), High Throughput Sequencing	• Overexpression of the AtMIR156b precursor resulted in fruits with additional carpels and ectopic growth	(Karlova et al. 2013)
		miR172	AP2a	mfold	_	• Overexpression of miR172 accelerated the fruit ripening process	(Chen et al. 2015)
		miR157	SPL-CNR	_	_	• Viral delivery of miR157 resulted in a delay in fruit ripening	(Chung et al. 2020)
		miR1917	CTR4	BLASTp, MEGA5 software, psRNATarget, ClustalW, TBtools	_	• Overexpression of miR1917 enhanced the ethylene response, resulting in expedited pedicel abscission and accelerated fruit ripening	(Wang et al. 2018)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Brassica oleracea	miR390	TAS3/ARFs	miRBase 22, MEGA 6	_	• Ectopic overexpression of bol-miR390a in transgenic Arabidopsis lines led to enhanced growth of lateral organs and increased overall biomass	(He et al. 2020)
		miR171	SCL6/27	TargetScan, miRDB, MirTarget, PITA (Probability of Interaction by Target Accessibility), RNA22	_	• Transgenic broccoli lines with elevated levels of bol-miR171b exhibited dark green foliage and were unable to produce sterile flowers	(Li et al. 2018)
	Brassica rapa	miR319	TCP4	_	_	• miR319a overexpression led to wavy leaf margins, changes in head shape and straightened leaf tips	(Mao et al. 2014)
		miR166	REV1/2	_	_	• Overexpression of miR166 g led to changes in leaf curvature	(Ren et al.2018)
	Beta vulgaris	GL15X1, AGL15X2, CAULIFLOWERA	_	BLAST, ClustalW, RNAfold	_	• Mediates vernalization responses	(Liang et al. 2017)
	Brassica campestris	BcMF1	_	_	_	• Required for efficient pollen germination and tube elongation; • Suppression leads to abnormal pollen development, delayed tapetal degradation, and pollen atrophy	(Song et al. 2013)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Lactuca sativa	miR408	BBP/BCP/PAA2	BLASTN, ExPaSy, MEME program, BRAD, MapInspect, iTOL, PmiREN, BLASTP, BLASTN, GRAMENE, Pfam database, HMMER, MEGA 7.0	–	• Overexpression of miR408 led to an increase in leaf size and petiole length at the seedling stage	(Zhang et al. 2021)
		miR396	GRF5			• Lines overexpressing miR396a exhibited smaller leaves	(Zhang et al. 2021)
	Solanum tuberosum	StFLORE	*StCDF1*	–	–	• Tuber development	(Ramírez Gonzales et al. 2021)
	Capsicum chinense	– –	– –	miRanda, RNAhybrid, LncTar	–	• Identified 2,525 lncRNAs, 47 miRNAs, and 71 circRNAs in the pepper hybrid • The downregulation of miR156, miR169, and miR369 suggested a potential association with enhanced growth vigor	(Shu et al. 2021)
	Capsicum annuum	2505 lncRNAs, 1066 differentially expressed during fruit development	–	RNAplex	RNA-seq	• Regulated carotenoid biosynthesis by targeting pigment-related genes (PGs) via cis or trans interactions	(Ou et al. 2017)
Ornamental plants	*Chrysanthemum indicum*	miR396	GRF1/5	psRNATarget,	KEGG Pathway Analysis, Gene Ontology (GO) Analysis	• Plants with elevated levels of miR396a exhibited extended internodes and reduced numbers of epidermal hairs	(Liu et al. 2021)
	Chrysanthemum morifolium	miR156	NF-YB8	psRNATarget, BioEdit, ClustalW, MEGA version 5, TAIR database, PopGenIE,	Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis	• Overexpression of miR156 reversed early flowering caused by NF-YB8 silencing	(Wei et al. 2017)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Sinningia speciosa	miR159	GAMYB	_	_	• Transgenic plants with elevated miR159 levels displayed a marked delay in flowering	(Li et al. 2013)
		miR172	AP2	_	_	• Transgenic plants with overexpression of miR172 exhibited early flowering, while suppression of miR172 resulted in late flowering	(Li et al. 2013b)
	Petunia hybrida	*miR156/157*	SPLs	psRNATarget, MEGA	_	• Overexpression of ph-miR157a caused delayed flowering, shorter plant height, increased branching, and smaller organ size • Overexpression of Ph-miR156f led to delayed flowering but did not influence plant height or branching • Reduced levels of ph-miR156/157 resulted in taller plants with fewer branches, elongated internodes, and earlier flowering	(Zhou et al. 2021)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Rosa hybrida	-	-	BLAST, LncTar, Coding-Non-Coding Index (CNCI), Coding Potential Calculator (CPC)	Strand-Specific RNA-Seq	• RNA-Seq analysis identified 13,957 lncRNAs, including 10,887 annotated and 3,070 novel ones. Of these, 10,075 lncRNAs were linked to 29,622 target genes involved in floral scent synthesis • Additionally, 425 lncRNAs were differentially expressed during flowering, with 19 showing differential expression across all flowering stages	(Shi et al. 2022)
		miR164	RhNAC100	_	_	• Ethylene regulated RhNAC100 via miR164, influencing petal size • Silencing NAC100 enhanced petal cell expansion, leading to larger petals, while its overexpression results in smaller petals	(Pei et al. 2013)
	Jasminum sambac	_	_	Hisat2, Cuffmerge, Pfam, and CPC2, TBtools, CANTATAdb 2.0, StringTie (v1.3.3)	CNCI	• 31,079 novel lncRNAs were identified from RNA-Seq data of flowers at different stages • Found 2,752 differentially expressed lncRNAs and 8,002 differentially expressed protein-coding genes at full bloom	(Lu et al. 2023)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Cymbidium goeringii	miR156, miR167, miR319, miR396	SPL ARF TCP4 GRF	PTFDB, BLASTX, RNA hybrid, trinity, Nr database, Swis-sProt, RepeatMasker, Rfam, UCSC, BLASTN, Mireap, TAIR10	–	• Transcriptome analyses identified these modules in reproductive organ development and cell proliferation in multi-tepal orchids	(Yang et al. 2017)
	Liriodendron chinense	–	–	Cufflinks, GOSeq miRDeep2, miREvo, R package DESeq2, KOBAS	–	• Identified critical miRNA-lncRNA-TF regulatory networks for leaf and flower development, revealing 105 miRNAs, 258 lncRNAs, 393 TFs, and 22 endogenous target mimics • lch-lnc7374-miR156h was identified as potential regulator for stamen development and lch-lnc7374-miR156j for pistil development	(Tu et al. 2022)
	Ginkgo biloba L	–	–	BLAST, LncTar, Coding Potential Calculator (CPC), Coding-Non-Coding Index (CNCI)	–	• Both leaf types revealed 2,044 lncRNAs • Identified 238 differentially expressed lncRNAs, including 32 DELs and 49 differentially expressed mRNAs • 48 cis-acting DELs regulated 72 target genes, while 31 trans-acting DELs controlled 31 different target genes	(Wu et al. 2019b)
	Ipomoea nil	–	–	LncTar, BLAST, Coding Potential Calculator (CPC), Coding-Non-Coding Index (CNCI)	–	• Identified 11,203 expressed lncRNAs, including 961 known and 10,242 novel lncRNAs	(Zhou et al. 2023)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
	Camellia oleifera	_	_	BLAST, miRDeep-P, psRobot, LncTar, CPC, CNCI,	Small RNA Sequencing, Single-Molecule Real-Time (SMRT) Sequencing, Illumina RNA Sequencing,	• Identified 18,393 transcripts, 414 lncRNAs, and 372 miRNAs • 37 lncRNAs and 30 miRNAs regulated 44 and 92 target genes, respectively • Upregulated miR396 and miR156 families correlated with downregulated growth regulating factors	(Kong et al. 2022)
Plantation crops	*Camellia sinensis*	miR156, miR164a, miR166a, miR167 d, miR396 d	SBP3, NAC TF, HD-ZIP4, ARF, GRF1, GRF4, GRF13	miRDeep-P, psRobot, Goseq	Illumina Small RNA Sequencing	• Regulated catechin accumulation by targeting key transcription factors; probably through interaction with IAA, JA, ABA, ZA, and SA	(Zhao et al. 2020)
		LTCONS_00026271, LTCONS_00020084	novel_miR44, miR169 d- 5p_1	BLAST, LncTar, Goseq, CPC, CNCI	Illumina RNA Sequencing	• Functioned as endogenous target mimics (eTMs) promoting volatile terpenoid accumulation in withered tea leaves	(Zhu et al. 2019)
		miR169	NF-YA	_	_	• Regulated caffeine and theanine synthesis by targeting NF-YA, with its expression positively correlating with these metabolites	(Zhao et al. 2020)

Table 3 (continued)

Type	Species	miRNA	Target	Tools/Databases used to Identify Target	Computational Techniques	Quality and Functional Roles	Reference
Spice Crops	*Nigella sativa*	240 conserved, 34 novel miRNAs	-	miRDeep-P, psRobot	Illumina Small RNA Sequencing	• Identified miRNAs target genes involved in terpenoid, phenylpropanoid, flavonoid, and carotenoid metabolism • qPCR validation confirmed expression patterns	(Uriostegui-Pena et al. 2024)
	Zingiber officinale	16,346 identified lncRNAs, 1,184 DELs	-	BLAST, Goseq, CPC, CNCI,	Illumina RNA Sequencing	• Regulatory network proposed for gingerol biosynthesis • Key transcription factors (MYB1, ERF100, WRKY40) and gingerol biosynthesis enzyme genes (GBEGs) identified	(Zhang et al. 2023)

ERF109, which promotes anthocyanin biosynthesis during fruit color development (Ma et al. 2021) (Table 3, Fig. 2). Moreover, MLNC3.2 and MLNC4.6, as endogenous target mimics of miR156, prevent cleavage of SPL2-like and SPL33 transcripts, thus influencing photoinduced anthocyanin biosynthesis (Yang et al. 2019a, b). Another important regulatory pathway, the miR172-AP2-MYB10 module, controls flavonoid biosynthesis, with miR172 suppressing AP2 to decrease MYB10 expression, resulting in reduced anthocyanin content and increased russeting in mature apples (Ding et al. 2022) (Table 3, Fig. 2). Additionally, light-induced pigmentation in apples is linked to lignin metabolism through miR7125, which targets cinnamoyl-CoA reductase (CCR) and is regulated by MYB16 and MYB1 transcription factors, thereby coordinating anthocyanin and lignin production (He et al. 2022). In grape (*Vitis vinifera*), overexpression of miR828 in Arabidopsis has been shown to downregulate MYB113, resulting in lighter leaf color, suggesting miR828's role as a negative regulator of anthocyanin biosynthesis (Chen et al. 2019). Similarly, in *Actinidia arguta* (red kiwifruit), miR858 inhibits anthocyanin biosynthesis by targeting MYBC1, a gene encoding an R2R3-type MYB transcription factor, with overexpression of miR858 leading to reduced anthocyanin accumulation (Li et al. 2020) (Table 3, Fig. 2).

In the context of horticultural crops, particularly fruits, the size and development of fruit are essential factors influencing marketability and consumer preference. In *Malus × domestica*, fruit development is regulated by complex genetic interactions, with the AP2 gene playing a crucial role in fruit size determination. The microRNA miR172 modulates fruit size by post-transcriptionally silencing AP2, and variations in this regulation are influenced by evolutionary adaptations. For instance, a transposon insertion in MIR172 leads to reduced miR172 accumulation, resulting in larger fruit due to elevated AP2 expression (Yao et al. 2015) (Table 3, Fig. 2). This highlights the importance of miRNA regulation in fruit size, with species-specific variations observed due to evolutionary selection pressures.

In *Solanum lycopersicum* (tomato), multiple miRNAs are implicated in fruit initiation and size determination. The AGO1 s-miR168 interaction plays a critical role in early fruit development, while miR159 overexpression induces parthenocarpy by silencing the GAMYB1/2 genes, thereby affecting fruit initiation and growth (da Silva et al. 2017). Moreover, the suppression of miR396a/b using the short tandem target mimic (STTM) strategy upregulates growth-regulating factors (GRFs), promoting fruit enlargement (Cao et al. 2016). Conversely, miR171-targeted overexpression of GRAS24 results in smaller fruit due to altered hormone balances, particularly gibberellin and auxin, which negatively affect cell division and expansion (Huang et al. 2017). Additionally, *miR164a* has been identified as a key regulator of

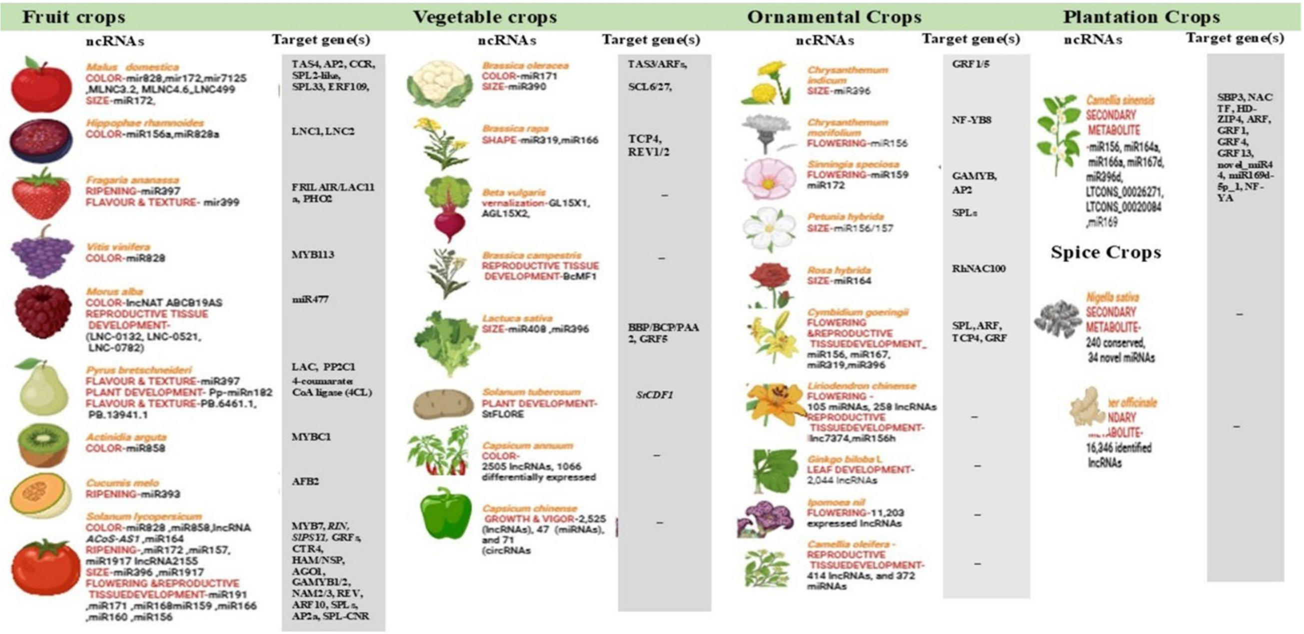

Fig. 2 The involvement of various non-coding RNAs (ncRNAs) in regulating key quality traits in different horticultural crops. The crops are categorized into fruit crops, vegetable crops, ornamental crops, plantation crops, and spice crops. Specific ncRNAs associated with traits such as color, size, ripening, flavor, texture, reproductive tissue development, plant growth, and secondary metabolite production are highlighted. Experimentally validated targets and transcription factors interacting with these ncRNAs are also indicated, showcasing their regulatory influence on plant development and quality enhancement

tomato fruit growth, as its silencing negatively affects fruit size (Gupta et al. 2021). Moreover, the interaction between AGO1 proteins and miR168 plays a crucial role in regulating fruit initiation and growth (Xian et al. 2014) (Table 3, Fig. 2).

In *Solanum lycopersicum* (tomato), miRNAs play a pivotal role in shaping fruit morphology. For example, overexpression of miR166b results in a"fruit-growing-out-of-fruit"phenotype, while expressing an miR166-resistant REV mutant (35S::REVRis) leads to the formation of ectopic carpels and fruit fusion. Interestingly, overexpressing REV alone does not affect fruit morphology, indicating that its function is largely regulated by miR166 post-transcriptionally (Hu et al. 2014). Another important miRNA, miR160, regulates fruit expansion by targeting ARF10 A from the Aux/IAA gene family. Silencing miR160 in tomato causes elevated ARF10 A expression, which results in pear-shaped fruit (Damodharan et al. 2016) (Table 3, Fig. 2).

In *Prunus mume* (Japanese apricot), lncRNAs regulate pistil number, with differential expression of known and novel lncRNAs between cultivars leading to changes in fruit morphology and interactions with miRNAs like ppe-miR172 d and ppe-miR160a/b (Wu et al. 2019a). miRNAs and lncRNAs influence the flavor and texture of fruits, key factors in edible quality. In *Fragaria ananassa* (strawberry), miR399 enhances sugar content, boosting fructose, glucose, and soluble solids, which improves fruit quality (He et al. 2022, Wang et al. 2017) (Table 3, Fig. 2). In *Pyrus bretschneideri* (Asian pear), miR397a overexpression reduces lignin and stone cell formation, improving taste (Xue et al. 2019). In *Malus domestica* (Philippot et al. 2024), miR7125 targets the CCR gene to balance lignin and anthocyanin levels, affecting fruit quality(Hu et al. 2021). These molecular mechanisms play a significant role in enhancing the edible quality of fruits.

Initial insights into miRNA involvement in ripening emerged from tomato (*Solanum lycopersicum*), where *miR156* and *miR172* negatively regulate *SPL-CNR* and *AP2a*, key transcription factors essential for ripening (Chen et al. 2015; Karlova et al. 2013). Similarly, *miR157* overexpression delays ripening by targeting *SPL-CNR* (Chung et al. 2020), whereas *miR1917* enhances ethylene response and accelerates ripening by targeting *CTR4* spliced variants (Wang et al. 2018) (Table 3, Fig. 2). In *Cucumis melo*, *miR393* overexpression delays ripening by repressing the auxin receptor gene *AFB2*(Bai et al. 2020). In *Fragaria ananassa*, *miR397* cleaves *FRILAIR* lincRNA transcripts, with *FRILAIR* overexpression accelerating ripening (Tang et al. 2021). miRNAs influence fruit flavor and texture, key determinants of edible quality. In *Fragaria ananassa*, *miR399* expression is linked to sugar content, with *miR399a* overexpression significantly increasing fructose, glucose, and soluble solids, thereby enhancing fruit quality (He et al. 2022, Wang et al. 2017) (Table 3, Fig. 2).

Long non-coding RNAs (lncRNAs) are also crucial in ripening regulation. In tomato, lncRNAs such as lncRNA2155 act as competing endogenous RNAs (ceRNAs) for miRNAs, affecting the expression of genes involved in ethylene and carotenoid pathways, which are key to ripening. The RIPENING INHIBITOR (RIN) targets lncRNA2155, and its knockout delays ripening (Yu et al. 2019a, b). Furthermore, lncRNA ACoS-AS1 regulates carotenoid biosynthesis, influencing fruit color during ripening (Xiao et al. 2020) (Table 3, Fig. 2). Together, miRNAs and lncRNAs coordinate the complex molecular networks that drive fruit ripening.

Reproductive tissue development is a key process in the life cycle of fruit-bearing plants, directly influencing their reproduction and yield. In tomatoes (*Solanum lycopersicum*), 62% of identified lncRNAs in the Heinz 1706 variety and 44% in *S. pimpinellifolium* LA1589 exhibit reproductive tissue specificity, playing important roles in floral organogenesis and reproductive tissue development (Wang et al. 2016). Additionally, a comprehensive study in tomato identified 10,919 lncRNAs across leaves, flowers, and roots, with many contributing to floral organogenesis (Yang et al. 2019a, b). In mulberry (*Morus alba*), 1,133 lncRNAs demonstrate tissue-specific expression, some of which are involved in floral organ formation (Song et al. 2016) (Table 3, Fig. 2).

Other metabolic processes, such as post-harvest disorders and secondary metabolite production, play a crucial role in fruit quality and market value. In navel oranges, granulation, a disorder affecting fruit from the stem, leads to reduced sugar and organic acid content. Transcriptomic analysis identified 486 ncRNAs involved in granulation, regulating genes related to cell wall metabolism, cellulose biosynthesis, and enzyme activity (Yao et al., 2020). In *Pyrus pyrifolia* (Asian pear), the ncRNA Pp-miRn182, derived from lncRNA PpL-T31511, regulates type 2 C protein phosphatase 1 (PP2 C1), playing a key role in hydrogen cyanide-induced endodormancy release (Li et al. 2021) (Table 3, Fig. 2).Browning, a common post-harvest issue, affects the appearance and storage of fruits. Analysis of enzymatic browning in sand pear identified 254 ncRNAs, including PB.15038 and PB.156.1, which regulate genes encoding peroxidase (POD), polyphenol oxidase, and other enzymes (Fan et al. 2021a, b).Secondary metabolites like vitamin C, citric acid, flavonoids, and stress-responsive terpenoids are essential for fruit quality. In lemon, 11,814 ncRNAs were identified, 113 of which were linked to terpenoid metabolism, while in peach, 575 lncRNAs were associated with flavonoid biosynthesis and aroma compound accumulation (Bordoloi et al. 2022) (Zhou et al. 2022) (Table 3, Fig. 2).

Vegetables

The biosynthesis and accumulation of pigments play a critical role in the maturation and ripening of vegetables, contributing to their characteristic color transitions and influencing their nutritional and health-promoting properties. Pigment accumulation is predominantly regulated by the progression of ripening stages and is crucial for post-harvest storage and quality management. Color in vegetables is primarily determined by the accumulation of anthocyanins, carotenoids, and other pigments. In *Capsicum annuum* (bell pepper), 2,505 lncRNAs have been identified, with 1,066 differentially expressed during fruit development, many of which regulate carotenoid biosynthesis by targeting pigment-related genes through cis or trans interactions (Ou et al. 2017) (Table 3, Fig. 2).

In leafy vegetables like *Lactuca sativa* (lettuce), miRNA-mediated regulation plays a crucial role in determining leaf and achene size. Overexpression of miR408 enhances both traits by downregulating copper-related target genes, while miR396a overexpression negatively impacts leaf expansion through suppression of GRF5, a critical gene for plant growth and development (Zhang et al. 2021) (Table 3, Fig. 2). Furthermore, by concentrating on GRAS family members (SCL6/27), overexpression of miR171b in *Brassica oleracea* (broccoli) enhances chlorophyll levels in the leaves (Li et al. 2018). Similarly, *broccoli* miR390a ectopic expression in *Arabidopsis* stimulates the development of lateral organs and increases biomass (He et al. 2020) These studies highlight the significant role of miRNAs and lncRNAs in controlling the size and development of key vegetable crops, influencing their growth and productivity. Studies on potato sprouting have revealed that 723 lncRNAs show significant expression changes as tubers transition from dormancy to sprouting, influencing cellular and metabolic processes in apical buds. Notably, the lncRNA *StFLORE* and *StCDF1* are involved in tuber development and drought response(Ramírez Gonzales et al. 2021). In *Capsicum chinense*, transcriptome analysis identified 2,525 lncRNAs, 47 miRNAs, and 71 circRNAs, with downregulation of miR156, miR169, and miR369 indicating their role in regulating growth vigor in hybrid peppers (Shu et al. 2021) (Table 3, Fig. 2).

In vegetables, miRNA-mediated regulation plays a crucial role in determining morphological traits. In *Brassica rapa* (Chinese cabbage), overexpression of MIR319a alters leaf architecture by suppressing TCP4, resulting in wavy leaf margins and altered leafy heads (Mao et al. 2014). Similarly, MIR166 g overexpression affects leaf curvature, further influencing vegetable shape (Ren et al. 2018). Additionally, miR164 targets NAC transcription factors involved in ethylene-induced leaf senescence, suggesting a potential strategy for improving leafy vegetable quality (Li et al. 2013a, b, c) (Table 3, Fig. 2).

Vernalization, the process by which prolonged cold exposure induces flowering, is a critical requirement for the flowering of several crops. In *Beta vulgaris*, three long non-coding RNAs (lncRNAs)-GL15X1, AGL15X2, and CAULIFLOWERA have been identified as key mediators of the vernalization response, regulating the timing of flowering under cold conditions (Liang et al. 2017). Additionally, lncRNAs are involved in the regulation of pollen development and fertility. In *Brassica campestris*, the pollen-specific lncRNA BcMF1 is essential for efficient pollen germination and tube elongation. Knockdown of BcMF1 leads to abnormalities in pollen development, delayed tapetal degradation, and pollen atrophy, highlighting its crucial role in reproductive success (Song et al. 2013) (Table 3, Fig. 2).

Ornamental plants

Ornamental plants, renowned for their aesthetic appeal, exhibit intricate mechanisms of pigment biosynthesis and accumulation, which are pivotal in determining their characteristic coloration. Pigments such as anthocyanins, carotenoids, and flavonoids play key roles in this process, with regulation occurring through complex networks of miRNAs, long non-coding RNAs (lncRNAs), and transcription factors. In *Ginkgo biloba*, a species noted for its golden autumn foliage, lncRNAs and transcription factors are involved in the regulation of leaf pigmentation, with pathways linked to chloroplast thylakoid membranes and photosynthesis playing a crucial role (Wu et al. 2019a, b) (Table 3, Fig. 2).

In ornamental plants, miRNAs and lncRNAs play a key role in shaping floral morphology. In *Liriodendron chinense*, a tree valued for its ornamental leaves and tulip-like flowers, miRNA-lncRNA-transcription factor networks regulate phenylpropanoid metabolism, affecting flower and leaf development. Key regulators identified include lch-lnc7374-miR156 h and lch-lnc7374-miR156j, which influence stamen and pistil development, respectively (Tu et al. 2022). In *Rosa hybrida*, ethylene regulates RhNAC100 expression via miR164, influencing petal size. Silencing NAC100 enhances petal cell expansion, leading to larger petals, while its overexpression results in smaller petals (Pei et al. 2013) (Table 3, Fig. 2).

In *Rosa hybrida* (rose), long non-coding RNAs (lncRNAs) play a pivotal role in regulating floral scent production, with differential expression of 425 lncRNAs identified during flowering stages. These lncRNAs target genes involved in scent synthesis, offering insights into the genetic control of fragrance (Shi et al. 2022). Similarly, in *Jasminum sambac* (jasmine), lncRNAs influence the production of floral scents, with differentially expressed lncRNAs associated with terpenoid and phenylpropanoid biosynthesis pathways (Lu et al. 2023) (Table 3, Fig. 2). These studies underscore the critical role of lncRNAs and miRNAs in the size, scent, and color of ornamental plants, providing valuable insights for breeding and horticultural improvements.

Ipomoea nil, known for its diverse flower colors, has been studied for its lncRNA profiles. A recent study using whole transcriptome RNA sequencing identified 11,203 lncRNAs, including 961 known and 10,242 novel lncRNAs in *I. nil*. These findings contribute to understanding the genetic regulation of flower color and development (Zhou et al. 2023). In *Cymbidium ensifolium*, a miR172-AP2-like module governs petal formation, contributing to multi-tepal flowers (Yang et al. 2015) (Table 3, Fig. 2). Transcriptome analysis of multi-tepal orchids revealed miR156-SPL and miR167-ARF modules involved in reproductive organ development, as well as the miR319-TCP4-miR396-GRF cascade regulating cell proliferation (Yang et al. 2017). In *Chrysanthemum indicum*, miR396a targets GRF1 and GRF5, affecting internode elongation and epidermal hair density (Liu et al. 2021) (Table 3, Fig. 2). *Camellia oleifera*, an evergreen shrub from the *Theaceae* family, is a key oil source used in various products. Despite prolific flowering, seed production remains low, with limited understanding of flower bud development. A study on gene expression, long non-coding RNA (lncRNA), and miRNA during anther development revealed 18,393 transcripts, 414 lncRNAs, and 372 miRNAs. Differential expression analysis identified 5,324 genes, 115 lncRNAs, and 44 miRNAs. Gene ontology showed lncRNA targets in anther development processes, while miRNA targets were linked to microspore development. Interaction networks identified key miRNA families and lncRNAs involved in pollen wall formation (Kong et al. 2022). The timing and duration of flowering are essential for plant reproduction and their commercial value, with miRNA-target modules playing critical roles in floral transition. In *Sinningia speciosa*, miR159 delays flowering by targeting GAMYB, whereas miR172 promotes flowering by suppressing AP2 (Li et al. 2013a, b, c) In *Chrysanthemum morifolium*, silencing NF-YB8 induces early flowering, which can be reversed by miR156 overexpression (Wei et al. 2017). Similarly, in *Petunia hybrida*, overexpression of miR156/157 delays flowering through the targeting of SPLs (Zhou et al. 2021) (Table 3, Fig. 2).

Plantation crops

Tea is a widely consumed non-alcoholic beverage, valued for its secondary metabolites (SMs) such as catechins, theanine, caffeine, and volatile compounds, which contribute to its flavor and health benefits (Jia et al., 2021). Several ncRNA-mediated regulatory pathways influence SM biosynthesis in tea leaves. miRNAs, including miR156, miR164a, miR166a, miR167 d, and miR396 d, regulate catechin accumulation by targeting key transcription factors like SBP3, NAC, HD-ZIP4, ARF, and GRFs (Zhao et al. 2020)These regulatory modules potentially interact with phytohormones such as IAA, JA, ABA, ZA, and SA, although direct evidence is lacking. Additionally, miR169 has been experimentally validated to regulate caffeine and theanine synthesis by targeting NF-YA, with its expression positively correlating with these metabolites (Zhao et al. 2020) (Table 3, Fig. 2). Long non-coding RNAs (lncRNAs) also play a crucial role in tea plant metabolism. Transcriptome analyses identified 32,036 lncRNAs across different developmental stages and tissue types, with some specifically linked to flavonoid and terpenoid biosynthesis. Notably, LTCONS_00026271 and LTCONS_00020084 function as endogenous target mimics (eTMs) for novel_miR44 and miR169 d- 5p_1, respectively, promoting volatile terpenoid accumulation in withered tea leaves (Zhu et al. 2019). These findings highlight the complex genetic regulation of tea quality traits, offering potential targets for breeding and metabolic engineering (Table 3, Fig. 2).

Spice crops

MicroRNAs (miRNAs) play a crucial role in regulating secondary metabolite biosynthesis, although their precise involvement remains largely unexplored. In *Nigella sativa* (black cumin), a medicinal plant with limited genomic data, next-generation sequencing (NGS) was employed to profile miRNAs and investigate their role in secondary metabolism. A total of 240 conserved and 34 novel miRNAs were identified, targeting 6,083 potential genes involved in key biosynthetic pathways, including terpenoid, phenylpropanoid, flavonoid, and carotenoid metabolism. qPCR validation confirmed the expression patterns of selected miRNAs, reinforcing their regulatory function. This study provides valuable insights into miRNA-mediated control of secondary metabolism and potential strategies for metabolite enhancement in *N. sativa* (Uriostegui-Pena et al. 2024) (Table 3, Fig. 2).

Gingerols, the primary bioactive compounds in ginger (*Zingiber officinale*), are known for their significant health benefits. A combined metabolomic and transcriptomic analysis of three major ginger cultivars in China identified 744 metabolites, including 21 gingerol derivatives, with shogaol and gingerol showing significant accumulation. Transcriptomic analysis further revealed 16,346 long non-coding RNAs (lncRNAs), with differentially expressed lncRNAs linked to secondary metabolism and hormone responses. Correlation analysis identified key gingerol biosynthesis enzyme genes (GBEGs) alongside transcription factors such as MYB1, ERF100, and WRKY40, as well as 1,184 potential regulatory lncRNAs. Additionally, protein–protein interaction analysis suggested that MYB4, MYB43, and WRKY70 interact with essential GBEGs, including *PAL1, PAL2, PAL3*, and *4 CL- 4*(Table 3, Fig. 2). This study proposed a comprehensive regulatory network involving lncRNAs, transcription factors, and GBEGs in gingerol biosynthesis, offering novel insights into the genetic and molecular mechanisms governing gingerol metabolism (Zhang et al. 2023).

Conclusion

While significant advances have been made in understanding the roles of non-coding RNAs (ncRNAs) in plants, several research opportunities remain. ncRNAs regulate essential processes in plants, including ripening, growth, pigmentation, secondary metabolite biosynthesis, and reproductive development in crops such as fruits, vegetables, and plantation crops. Advances in transcriptomic and metabolomic integration have deepened our understanding of ncRNA-mediated regulatory networks. However, cross-tissue and developmental stage analyses should be prioritized to better understand how these RNAs coordinate gene expression across different plant organs. In metabolic engineering, ncRNAs offer potential for enhancing the nutritional value, flavor, and health properties of crops.

The conservation of ncRNAs across species highlights the need for extensive species-specific data collection and improved prediction models. Further research is needed in plantation crops, such as tea, to explore the regulation of secondary metabolites like catechins and theanine, and in vegetable ripening to improve post-harvest quality. ncRNAs may also be harnessed for ornamental plant breeding, regulating traits like color and scent. Further improvement in the computational tools, including RNA sequencing, bioinformatics pipelines, and machine learning algorithms, can be pivotal in uncovering the functional roles of miRNAs and lncRNAs. Furthermore, developing user-friendly tools for both Linux and Windows systems will enhance research accessibility. RNA interference technologies and genome editing tools like CRISPR-Cas provide promising avenues for utilizing ncRNAs to improve crop yield and quality.

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Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

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ORIGINAL ARTICLE

Upregulated expression of MYB4, DREB1 and AP37 transcription factors modulates cold stress response in high-altitude Himalayan rice via time-dependent ROS regulation

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Abstract

Background Cold stress is an upcoming challenge for rice (*Oryza sativa* L.) cultivation, especially at the seedling establishment stage. It causes serious constraints in its production and productivity as it is a thermophilic cereal crop. North-western Himalayan region has a rich repository of temperate rice genotypes, and there is a need to identify cold-tolerant rice varieties from these available genetic resources.

Methods and results The present study screened 90 rice accessions (indica and japonica) grown in the high-altitude regions at 2200 m amsl for cold tolerance (5 °C) at the seedling stage, and found 14 highly cold-tolerant accessions. Almost eighty per cent of the indica types clustered into cold-sensitive class. One cold-tolerant japonica (GS-74) accession and one cold-susceptible (SR-4) accession were used to compare their biochemical and gene expression response during cold stress and after recovery. A wide range of differences was noticed at different time points in the accumulation of ROS scavengers, osmoprotectants and antioxidant enzymes, with significant differences between the contrasting genotypes. Similarly, gene expression of five transcription factors OsMYB4, OsAP37, OsDREB1A, OsDREB1B and OsDREB1D revealed their role in cold responsiveness at the seedling stage, critically modulating the cold-induced osmoprotectant-mediated tolerance mechanism.

Conclusion This is the first study that explored the high-altitude Himalayan rice germplasm for cold tolerance at the critical S3 seedling stage under controlled conditions. It demonstrated that the upregulation of OsDREB1A, OsDREB1B, OsMYB4 and OsAP37 transcription factors modulates cold stress response in rice via a complex mechanism involving ROS scavengers and osmoprotectants.

Keywords *Oryza sativa* L. · Cold stress · Himalayas · Antioxidants · Gene expression

Introduction

Rice (*Oryza sativa* L.) is the second most-consumed cereal in the world, with over 3.6 billion people around the globe (50% population) taking it as an essential meal in their diets. Over 400 million people worldwide are associated with rice production, and Asia produces more than 90% of the world's rice [1]. Since rice is a thermophilic crop and a sensitive agro-ecosystem, cold stress affects it adversely [2]. Due to cold stress, about 15 million hectares of land worldwide is unsuitable for rice cultivation [3, 4]. The optimum seed germination temperature for rice is 25 °C, while temperatures below 15 °C cause a severe decrease in germination, vigour and seedling emergence, which further results in delayed initial growth and seedling establishment, yellowing of the leaves, overall growth inhibition, late and limited tillering as well as high seedling mortality [5]. With a drop in temperature, grain loss of approximately 26% ranging from 0.5 to 2.5 t/ha has been recorded [6, 7].

Plants respond to abiotic stresses at the molecular level through signal perception, transduction, gene expression alterations, and metabolic changes [8]. The common effects caused by stress are increased production of intracellular Reactive-Oxygen Species (ROS), including superoxide anion (O_2•−), hydroxyl radical (•OH), as well as non-radical

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molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) [9, 10]. ROS are major signalling molecules that maintain the plant's normal growth and responses to stress. If left unchecked, ROS levels in cells rise, causing irreversible damage to membranes (lipid peroxidation), DNA, RNA, and proteins, altering tissue and organ development, stomatal activity, and eventually programmed cell death (PCD) [11]. A network of transcription factors and regulatory genes that modulate defence enzymes, proteins, and pathways is essential for plants' tolerance to abiotic stresses [12–16]. Elucidating these molecular mechanisms of specific gene activation/repression through transcription factors is essential for crop improvement [17, 18].

Based on the available literature, we chose five stress-responsive transcription factors OsMYB4, AP37, DREB1A, DREB1B, and DREB1D for the present study. Except AP37, the rest of the transcription factors are known for their role in drought and salt stress [19, 20]. Anther-specific aspartic protease (AP37), though involved in tapetal programmed cell death (PCD), has also been implicated in drought tolerance [21]. While there is a limited understanding about the involvement of these transcription factors in cold tolerance in rice, we aimed to investigate their role in the metabolic adjustments that aid rice plants in responding to cold stress. Furthermore, the Himalayan rice germplasm accessions are assumed to possess cold tolerance, but this claim needed validation under controlled conditions [2, 22, 23]. The present study repudiated the assumption and fished out 14 cold-tolerant rice genotypes, which would help breed cold-tolerant rice varieties. One cold-tolerant japonica accession (GS-74) and one cold-susceptible indica accession (SR-4) were compared under cold stress and post-stress recovery phase for gene expression of transcription factors *Oryza sativa* myeloblastosis transcription factor 4 (OsMYB4), Aspartyl protease 37 (AP37), Dehydration-responsive element-binding protein 1 (DREB1A, DREB1B, DREB1D), and biochemical profiles of important metabolites as well as phenotypic characters to advance our understanding into their role in cold-stress.

Materials and methods

Planting material

Over one hundred rice accessions were collected from high-altitude areas of the Western Himalayas (30.25–35.20 N latitude and 74–75.25 E longitude), primarily landraces cultivated at 2200 m above mean sea level (amsl) or higher. These were purified for five years at the Mountain Research Centre for Field Crops, Khudwani, India, and genetic stability and diversity were determined using Distinctness, Uniformity, and Stability (DUS) descriptors of the International Rice Research Institute [22, 23]. At a temperature of 30 °C and relative humidity of 60–80%, ninety different rice accessions from the indica and japonica ecotypes were germinated in petri dishes within a seed germinator. Seeds were then sown in triplicates in plastic pots filled with clay soil (0.5 kg each) and kept in a walk-in plant growth chamber under controlled conditions (16 °C, relative humidity 60–80%, photoperiod 16/8 h day/night, and light intensity 3000 lm/m^2). Two-week-old seedlings were transplanted into pots (one in each pot) containing a 2:1:1 mixture of clay, sand, and FYM (5 pots for each accession) and grown in a walk-in plant growth chamber at a temperature of 25 ± 2 °C and relative humidity of 60%.

Screening for cold tolerance

Twenty-day-old seedlings were subjected to cold stress at 5 °C for seven days (Fig. 1) and were scored on LD Scale of 0–9 as per the Standard Evaluation System (SES) for rice [24–26]. The screening of the entire germplasm set was repeated twice, and the highest score (9) was assigned to accessions that resulted in the death of the largest number of seedlings (highly susceptible) and the lowest score (0) to those that exhibited no damage to the leaves/plants (strongly resistant) (Table 1S).

Gene expression analysis

Sample collection

The selected resistant (R) and susceptible (S) rice accessions were grown in a plant growth chamber at 25 °C and relative humidity of 60–80% till the seedling (S1) stage. Cold stress (5 °C) was given to twenty-day-old rice seedlings for 24 h, and the leaf samples (200 mg) were collected at five different time points as per the following details: 0 h before cold stress (T0), 2 h (T1), 6 h (T2), 24 h during cold stress (T3), and 24 h after stress recovery at 25 °C (T4).

The samples were immediately immersed in RNA later (Sigma Aldrich), and stored in 2 ml microfuge tubes (Tarson) at − 80 °C for expression analysis.

RNA extraction:

RNA was extracted from leaf samples according to the manufacturer's instructions using Trizol reagent (Genetix Biotech.). The quality and quantity of RNA were determined using agarose-formaldehyde gel electrophoresis and spectrophotometry. Thermo Scientific US's Revert Aid first-strand cDNA synthesis kit was used to synthesize first-strand cDNA. The real-time PCR reactions were performed with a CFX96 Real-Time PCR detection system (Bio-Rad) in 96 well plates in triplicates using SYBR Green master mix (Bio-Rad Laboratories) with actin gene

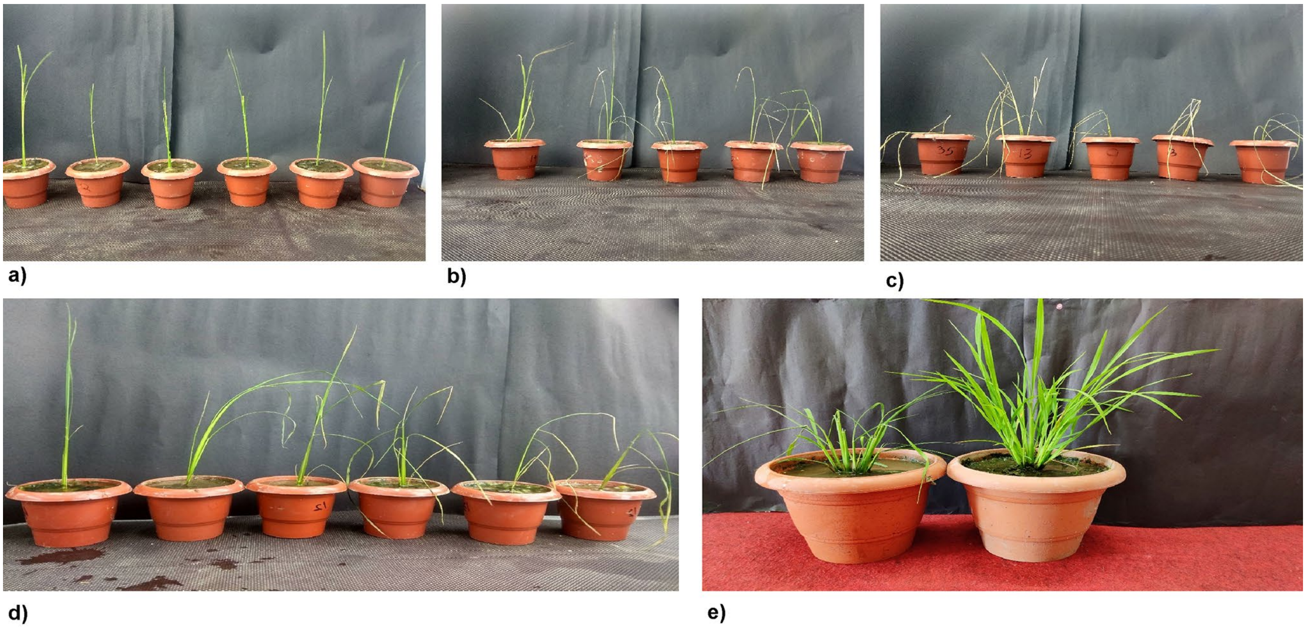


Fig. 1 Twenty day old seedlings of rice genotypes subjected to cold stress treatment at 5 °C for 7 days: **a** Resistant genotypes (GS-74, GS-51, GS-34, GS-37, GS-49, GS-88) showing no symptoms of stress; **b** Moderately resistant genotypes (Jehlum, Mushk budji, GS-61, Kamad, K-78) with almost half seedlings dry and having turned pale yellow; **c** Susceptible genotypes (IR-42, PS-3, SR-4, SR-3, SR-2) with maximum seedlings dead and dehydrated; **d** Genotypes after cold stress- resistant (left two), moderately tolerant (central two) and susceptible (left two); **e** Cold susceptible SR-4 (Lt) and cold tolerant GS-74 (Rt) genotypes selected for molecular and biochemical studies

as endogenous control [27]. The amplification was carried at 95 °C for 2 min incubation and 39 cycles of 95 °C for 30 s, 95 °C for 5 s, varied amplification temperatures for different primers (58 °C for OSMYB4, 57.5 °C for AP37, 57 °C for DREB1A, 55.5 °C for DREB1B and 59 °C for DREB1D), followed by final extension of 72 °C for 30 s. The supporting table (Table 2S) shows the primer details. The relative expression of each sample was calculated using the ΔΔCT method [28].

Biochemical analysis

As detailed above, leaf samples collected at five different time points were dipped in liquid N_2 for 30 s and immediately stored in a deep freezer at − 80 °C. The samples were analyzed for osmoprotectants, viz. free proline [29], glycine betaine [30], sucrose [31], glucose [32]. Antioxidant enzyme activities were estimated for SOD [33, 34], CAT [35], APOD [36], Glutathione reductase [37]. The total antioxidant activity was determined by the Phosphomolybdenum method [38]. Ascorbate and Glutathione contents were estimated as described by [39, 40], respectively.

Statistical analysis

The screening of tolerant and susceptible accessions was done using a completely randomized design (CRD), and analyzed by ANOVA using Graphpad Prism and Opstat softwares [41, 42]. For biochemical analyses, the experiment was laid in CRD with five levels of treatment. The data were interpreted according to two-way ANOVA using CPCS1 statistical software, Dunn-Sidak and Tukey tests for pairwise comparisons using Graphpad Prism [43, 44]. Each treatment had at least three replicates for estimating biochemical parameters and ten for estimating phenotypic characters. The data are presented as mean ± SE in figures, and the number of biological replicates is mentioned under each figure.

Results and discussion

Rice is an important cereal crop and is consumed by over half of the world's population. The current study screened ninety Himalayan rice accessions for cold tolerance (Fig. 1S), and classified them into different categories of cold stress tolerance (Fig. 2S). The present study highlights several critical aspects of subcellular activities that occur in rice seedlings at S3 stage under severe cold stress of 5 °C.

Rice seedlings responded to cold stress by accumulating osmolytes like proline, glycine betaine, and soluble sugars, which are known to activate defence-related genes, lower the cellular water potential, maintain turgor high pressure sufficient for growth, and protect cellular membranes from desiccation and cold damage. Cold stress caused ROS elevation in the rice seedlings. Since superoxide radicals are toxic to cells, antioxidant machinery was activated to reduce H_2O_2 levels and protect the plants from cold-induced ROS production [45–47].

Proline and glycine-betaine (GB) content

In the present study, proline (proteinogenic amino acid) content of the resistant genotype increased significantly for 24 h of chilling cycle compared to the susceptible genotype and decreased significantly post-24 h of chilling (Table 3S; Fig. 2a). Similar proline content variations were observed in earlier studies on rice plants [48, 49]. Once plants are exposed to stress, it is well known that they accumulate high levels of proline in the cytoplasm and chloroplast [50, 51]. The role of proline in singlet oxygen quenching, ROS scavenging, and sub-cellular structure stabilization makes it important for cold tolerance [52, 53].

Plants increase their cell osmolality under abiotic stress, and Glycine-betaine (GB), a quaternary amine, plays an important role in the process. In the present study, glycine betaine content followed a similar trend as proline. GB content increased sharply due to cold stress in the susceptible genotype (SR-4), reaching a maximum value at 24 h (Table 1; Fig. 2b) [54, 55], but it was significantly lower than in the tolerant genotype (GS-74). Both genotypes showed a decline in its concentration during the post-chilling recovery phase. In a similar study, glycine betaine content in rice genotypes increased five-fold under cold stress [56]. The interactions based on Sidak's multiple comparisons test between treatments and genotypes imply that proline and glycine-betaine levels vary between the contrasting genotypes and play a significant role in tolerating cold stress (Table 3S).

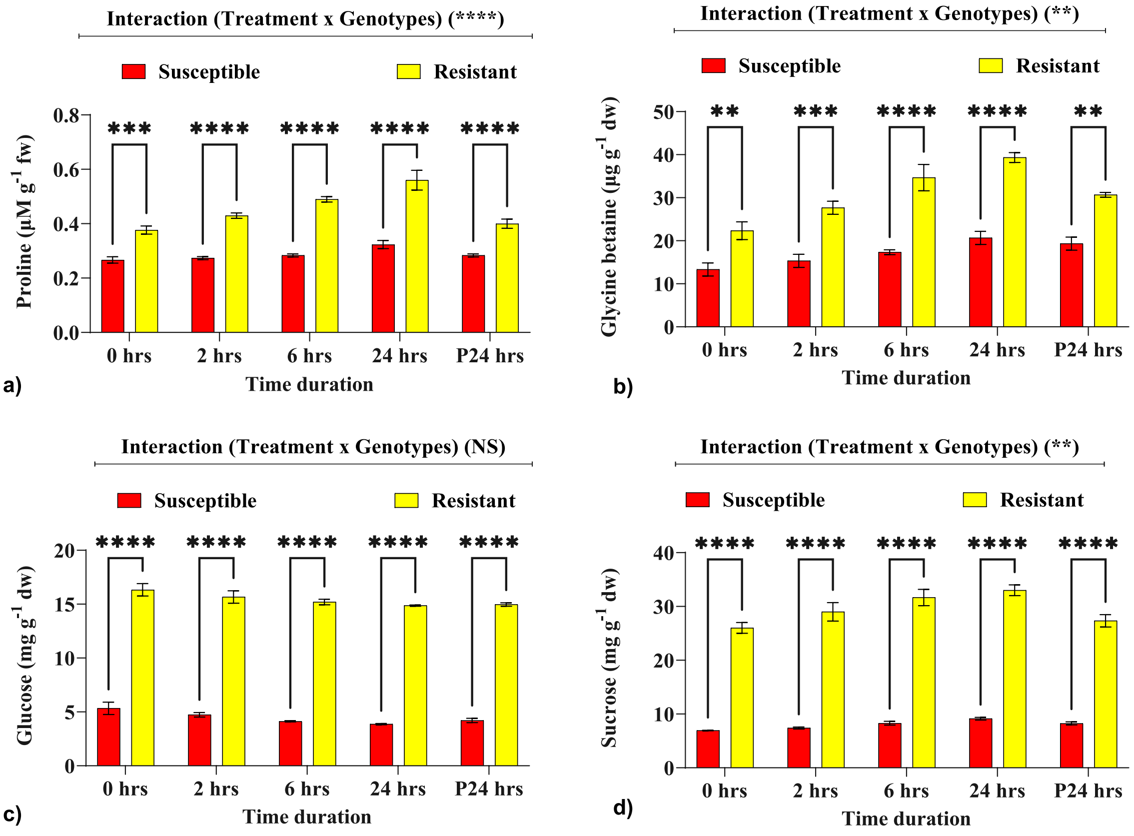


Fig. 2 Osmolyte and sugar contents evaluated in 2 distinct rice genotypes under cold stress. The selected resistant and susceptible rice genotypes subjected to cold stress (4 °C) at S3 stage in plant growth chamber and the leaf sample at 0 h, 2 h, 6 h, 24 h, and P24 (24 h recovery post chilling) from both genotypes (highly resistant and highly susceptible) showed differential response in osmolyte: **a** Proline, **b** Glycine betaine, **c** Glucose, **d** Sucrose

Table 1 Effect of cold stress on proline, glucose, sucrose and glycine betaine content

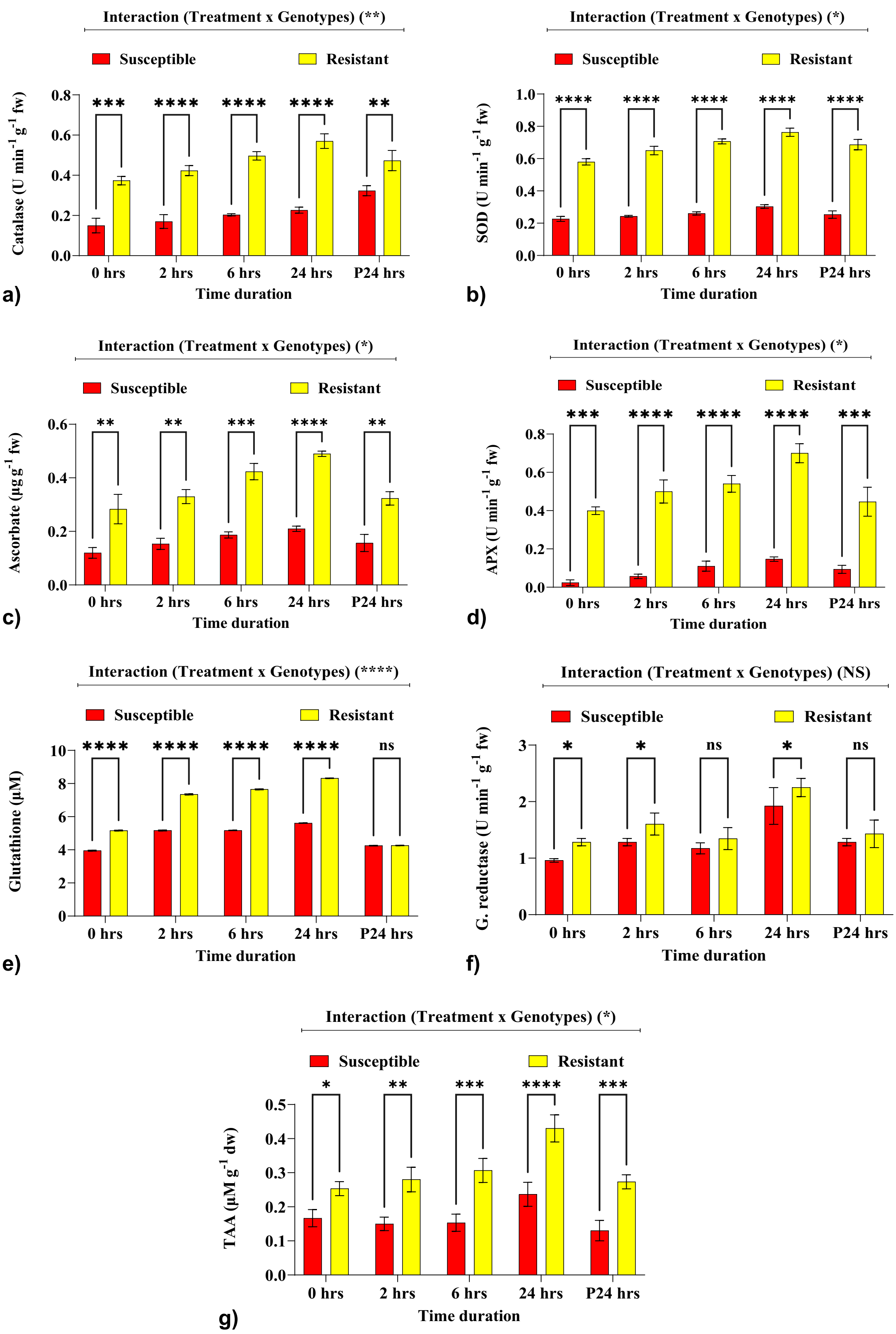
Treatment	Genotypes											
	Proline (μM g^{-1} fw)			Glucose (mg g^{-1} dw)			Sucrose (mg g^{-1} dw)			Glycine betaine (μg g^{-1} dw)		
	SR-4	GS-74	Mean	SR-4	GS-74	Mean	SR-4	GS-74	Mean	SR-4	GS-74	Mean
T1 (0 h)	0.26^{d}	$0.37^{e\#}$	0.32	5.33^{a}	$16.33^{a\#}$	10.83	6.93^{e}	$26.00^{e\#}$	16.46	13.33^{e}	$22.33^{e\#}$	17.83
T2 (2 h)	0.27^{c}	$0.43^{c\#}$	0.35	4.73^{b}	$15.66^{b\#}$	10.20	7.40^{d}	$29.00^{c\#}$	18.20	15.33^{d}	$27.66^{d\#}$	21.50
T3 (6 h)	0.28^{b}	$0.49^{b\#}$	0.38	4.13^{d}	$15.20^{c\#}$	9.66	8.30^{b}	$31.66^{b\#}$	19.98	17.33^{c}	$34.66^{b\#}$	26.00
T4 (24 h)	0.32^{a}	$0.56^{a\#}$	0.44	3.86^{e}	$14.86^{e\#}$	9.36	9.13^{a}	$33.00^{a\#}$	21.06	20.66^{a}	$39.33^{a\#}$	30.00
T5 (P24 hrs)	0.28^{b}	$0.40^{d\#}$	0.34	4.20^{c}	$14.96^{d\#}$	9.58	8.26^{c}	$27.33^{d\#}$	17.80	19.33^{b}	$30.66^{c\#}$	25.00
Mean	0.28	0.45		4.45	15.40		8.00	29.40		17.20	30.93	
CD ($p \leq 0.05$)				CD ($p \leq 0.05$)			CD ($p \leq 0.05$)			CD ($p \leq 0.05$)		
Genotype (A): 0.012				Genotype (A): 0.262			Genotype (A): 0.721			Genotype (A): 1.259		
Treatment (B): 0.019				Treatment (B): 0.414			Treatment (B): 1.140			Treatment (B): 1.990		
Interaction AXB: 0.026				Interaction AXB: NS			Interaction AXB: 1.613			Interaction AXB: 2.815		

The superscripts (a-e) or (a#-e#) within the respective genotype columns indicate that within these genotypes, treatments with the same superscript are statistically at par with each other (at the indicated *p*-value)

Table 2 Effect of cold stress on catalase, SOD, ascorbate and ascorbate peroxidase content

Treatment	Genotypes											
	Catalase (U min^{-1} g^{-1} fw)			Superoxide dismutase (U min^{-1} g^{-1} fw)			Ascorbate (μg g^{-1} fw)			Ascorbate peroxidase (U min^{-1} g^{-1} fw)		
	SR-4	GS-74	Mean	SR-4	GS-74	Mean	SR-4	GS-74	Mean	SR-4	GS-74	Mean
T1 (0 h)	0.15^{e}	$0.37^{e\#}$	0.26	0.22^{e}	$0.58^{e\#}$	0.40	0.12^{d}	$0.28^{e\#}$	0.20	0.02^{e}	$0.40^{e\#}$	0.21
T2 (2 h)	0.17^{d}	$0.42^{d\#}$	0.29	0.24^{d}	$0.65^{d\#}$	0.44	0.15^{c}	$0.33^{c\#}$	0.24	0.05^{d}	$0.50^{c\#}$	0.27
T3 (6 h)	0.20^{c}	$0.49^{b\#}$	0.35	0.26^{b}	$0.70^{b\#}$	0.48	0.18^{b}	$0.42^{b\#}$	0.30	0.11^{b}	$0.54^{b\#}$	0.32
T4 (24 h)	0.22^{b}	$0.57^{a\#}$	0.39	0.30^{a}	$0.76^{a\#}$	0.53	0.21^{a}	$0.49^{a\#}$	0.35	0.14^{a}	$0.70^{a\#}$	0.42
T5 (P24 hrs)	0.32^{a}	$0.47^{c\#}$	0.39	0.25^{c}	$0.68^{c\#}$	0.47	0.15^{c}	$0.32^{d\#}$	0.24	0.09^{c}	$0.44^{d\#}$	0.27
Mean	0.21	0.46		0.25	0.67		0.16	0.37		0.86	.51	
CD ($p \leq 0.05$)				CD ($p \leq 0.05$)			CD ($p \leq 0.05$)			CD ($p \leq 0.05$)		
Genotype (A): 0.022				Genotype (A): 0.015			Genotype (A): 0.020			Genotype (A): 0.030		
Treatment (B): 0.035				Treatment (B): 0.024			Treatment (B): 0.032			Treatment (B): 0.047		
Interaction AXB: 0.050				Interaction AXB: 0.034			Interaction AXB: 0.046			Interaction AXB: 0.067		

The superscripts (a-e) or (a#-e#) within the respective genotype columns indicate that within these genotypes, treatments with the same superscript are statistically at par with each other (at the indicated *p*-value)


Interaction (Treatment x Genotypes) (**)
Susceptible
Resistant
Catalase (U min^{-1} g^{-1} fw)
0.0
0.2
0.4
0.6
0.8
***
****
****
****
**
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
a)
Interaction (Treatment x Genotypes) (*)
Susceptible
Resistant
SOD (U min^{-1} g^{-1} fw)
0.0
0.2
0.4
0.6
0.8
1.0
****
****
****
****
****
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
b)
Interaction (Treatment x Genotypes) (*)
Susceptible
Resistant
Ascorbate (µg g^{-1} fw)
0.0
0.2
0.4
0.6
**
**
***
****
**
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
c)
Interaction (Treatment x Genotypes) (*)
Susceptible
Resistant
APX (U min^{-1} g^{-1} fw)
0.0
0.2
0.4
0.6
0.8
***
****
****
****
***
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
d)
Interaction (Treatment x Genotypes) (****)
Susceptible
Resistant
Glutathione (µM)
0
2
4
6
8
10
****
****
****
****
ns
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
e)
Interaction (Treatment x Genotypes) (NS)
Susceptible
Resistant
G. reductase (U min^{-1} g^{-1} fw)
0
1
2
3
*
*
ns
*
ns
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
f)
Interaction (Treatment x Genotypes) (*)
Susceptible
Resistant
TAA (µM g^{-1} dw)
0.0
0.1
0.2
0.3
0.4
0.5
*
**
***
****
***
0 hrs
2 hrs
6 hrs
24 hrs
P24 hrs
Time duration
g)

◀**Fig. 3** Antioxidant content evaluated in 2 distinct rice genotypes under cold stress. The selected resistant and susceptible rice genotypes subjected to cold stress (4 °C) at S3 stage in plant growth chamber and the leaf sample at 0 h, 2 h, 6 h, 24 h, and P24 (24 h recovery post chilling) from both genotypes (highly resistant and highly susceptible) showed differential response in: **a** Catalase, **b** SOD, **c** Ascorbate, **d** APX (Ascorbate Peroxidase), **e** Glutathione content, **f** Glutathione reductase, **g** Total anti-oxidant activity (TAA)

Soluble sugars

In the present study, the glucose and sucrose content was significantly higher in the resistant genotype than in the susceptible genotype. The average glucose content was nearly 3 times that of the tolerant genotype (15.4 mg/g dwt) compared to the susceptible genotype (4.40 mg/g dwt) (Table 1) (Fig. 2c). Up to 24 h under cold stress, both susceptible and tolerant genotypes showed a similar increasing trend in sucrose content (Table 1), which is consistent with earlier studies [57, 58]. However, there was a decline in the post-chilling recovery phase. Notably, the tolerant genotype maintained a higher sucrose concentration at all time points than the susceptible genotype (Fig. 2d).

Soluble sugars help plants withstand stress [59]. The present study demonstrated that sucrose and glucose contents were higher in the cold-tolerant genotype under cold stress, as these are used as substrates for cellular respiration and act as osmolytes to maintain cell homeostasis. Time duration had no significant effect on the soluble sugar content of rice plants (Table 3S; Fig. 2). In stressful situations, sucrose and glucose serve as cellular respiration substrates or osmolytes [60, 61]. Similar variations in the soluble sugar content were observed in earlier studies [57, 58]. Rice plants accumulate glucose in an increasing trend under cold stress, as reported by Ito et al. [48] and Tian et al. [62].

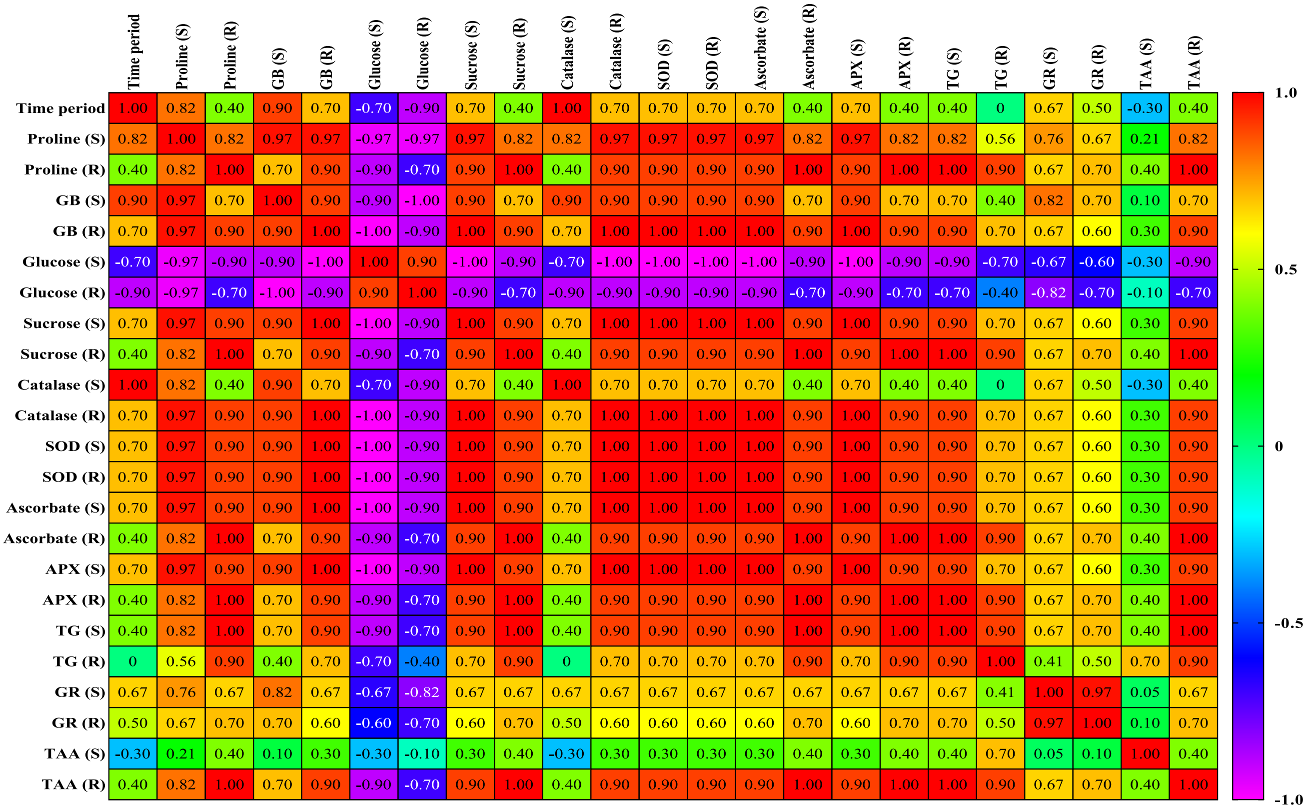
Oxidant/antioxidant status

In the present study, cold stress significantly increased catalase, superoxide dismutase, ascorbate and acorbate peroxidase activity in plants for 24 h of the chilling cycle in both susceptible and resistant genotypes (Table 2), and this change was significant for resistant compared to susceptible genotype at each time point (Table 4S; Fig. 3a–d). Post-24 h of the cold cycle, there was a significant decrease in catalase and SOD activity in both genotypes. Catalases and superoxide dismutase directly dismutase H_2O_2 into H_2O and O_2 [63]. A study by de Freitas et al. in rice showed that CAT activity increased fivefold during cold stress [58]. In the same study, SOD content increased more than 1.5-fold during cold stress (10 °C) for 72 h [58]. In another study on rice biochemical analyses at 15 °C for 4 days, SOD content increased during cold stress [64]. A previous study [65] on rice seedlings exposed to 4 °C for 7 days followed by a 2-day recovery phase found that APX activity increased rapidly in tolerant cultivars and continued to increase even after recovery, while APX activity decreased in susceptible cultivars both at the start of low temperature and during recovery.

Tripeptide glutathione (GSH) and Glutathione reductase (GR) play important roles in stress tolerance. GSH is an essential metabolite for intracellular defence against ROS damage in plants, and is abundant in reduced form in plant tissues [66]. GR is an ASH-GSH cycle enzyme which maintains the reduced status of GSH. GR reduces GSH, which is involved in many plant metabolic and antioxidant processes. In the present study, cold stress increased GR content significantly till 2 h of chilling in both the genotypes, which increased further at 24 h of chilling (Fig. 3e). The total glutathione increased significantly till 24 h of chilling in both genotypes, and then declined in the recovery phase (Fig. 3f). A previous study has reported that cold stress increases GR content in rice [64]. A previous study on sensitive and tolerant rice seedlings exposed to 4 °C for 7 days and then 2 days of recovery showed that the GR activity in tolerant cultivars increased during the chilling and recovery phases, while the GR activity in sensitive cultivars decreased during the chilling and recovery phases [65]. Another study [47] has reported that cold stress (8 °C) reduced GR content in two 7-day-old indica rice cultivars. These studies support our observations, demonstrating that when rice plants are exposed to cold stress, they release ROS detoxifying catalases and SODs, ascorbate and ascorbate peroxidase and that these antioxidant defence mechanisms become critical for their survival in the high altitude cold-temperate climates of the Himalayas (Fig. 4). There were significant differences between resistant and susceptible genotypes under cold stress in the total antioxidant activity (TAA) (Tables 3 and 4S). A sharp increase in TAA was recorded in both genotypes from 6 h, reaching a maximum at 24 h (Fig. 3g; Table 3). The TAA declined sharply during the post-chilling recovery phase. In an earlier study on the redox mechanism in rice under abiotic stress, TAA content increased during cold stress [47]. Similarly, in another study, DPPH assay revealed higher antioxidant activity in tolerant genotypes under cold stress [58]. The results in the present and earlier studies reveal that as a redox mechanism in rice, TAA content increases during cold stress and is critical for survival at lower temperatures.

Gene expression of some key transcription factors under cold stress

Transcription factors are proteins that activate and/or repress the transcription process. More than 2000 recognized and anticipated rice transcription factors are disbursed between 63 families [67]. Several families of these transcription



	Time period	Proline (S)	Proline (R)	GB (S)	GB (R)	Glucose (S)	Glucose (R)	Sucrose (S)	Sucrose (R)	Catalase (S)	Catalase (R)	SOD (S)	SOD (R)	Ascorbate (S)	Ascorbate (R)	APX (S)	APX (R)	TG (S)	TG (R)	GR (S)	GR (R)	TAA (S)	TAA (R)
Time period	1.00	0.82	0.40	0.90	0.70	-0.70	-0.90	0.70	0.40	1.00	0.70	0.70	0.70	0.70	0.40	0.70	0.40	0.40	0	0.67	0.50	-0.30	0.40
Proline (S)	0.82	1.00	0.82	0.97	0.97	-0.97	-0.97	0.97	0.82	0.82	0.97	0.97	0.97	0.97	0.82	0.97	0.82	0.82	0.56	0.76	0.67	0.21	0.82
Proline (R)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00
GB (S)	0.90	0.97	0.70	1.00	0.90	-0.90	-1.00	0.90	0.70	0.90	0.90	0.90	0.90	0.90	0.70	0.90	0.70	0.70	0.40	0.82	0.70	0.10	0.70
GB (R)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
Glucose (S)	-0.70	-0.97	-0.90	-0.90	-1.00	1.00	0.90	-1.00	-0.90	-0.70	-1.00	-1.00	-1.00	-1.00	-0.90	-1.00	-0.90	-0.90	-0.70	-0.67	-0.60	-0.30	-0.90
Glucose (R)	-0.90	-0.97	-0.70	-1.00	-0.90	0.90	1.00	-0.90	-0.70	-0.90	-0.90	-0.90	-0.90	-0.90	-0.70	-0.90	-0.70	-0.70	-0.40	-0.82	-0.70	-0.10	-0.70
Sucrose (S)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
Sucrose (R)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00
Catalase (S)	1.00	0.82	0.40	0.90	0.70	-0.70	-0.90	0.70	0.40	1.00	0.70	0.70	0.70	0.70	0.40	0.70	0.40	0.40	0	0.67	0.50	-0.30	0.40
Catalase (R)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
SOD (S)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
SOD (R)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
Ascorbate (S)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
Ascorbate (R)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00
APX (S)	0.70	0.97	0.90	0.90	1.00	-1.00	-0.90	1.00	0.90	0.70	1.00	1.00	1.00	1.00	0.90	1.00	0.90	0.90	0.70	0.67	0.60	0.30	0.90
APX (R)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00
TG (S)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00
TG (R)	0	0.56	0.90	0.40	0.70	-0.70	-0.40	0.70	0.90	0	0.70	0.70	0.70	0.70	0.90	0.70	0.90	0.90	1.00	0.41	0.50	0.70	0.90
GR (S)	0.67	0.76	0.67	0.82	0.67	-0.67	-0.82	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.41	1.00	0.97	0.05	0.67
GR (R)	0.50	0.67	0.70	0.70	0.60	-0.60	-0.70	0.60	0.70	0.50	0.60	0.60	0.60	0.60	0.70	0.60	0.70	0.70	0.50	0.97	1.00	0.10	0.70
TAA (S)	-0.30	0.21	0.40	0.10	0.30	-0.30	-0.10	0.30	0.40	-0.30	0.30	0.30	0.30	0.30	0.40	0.30	0.40	0.40	0.70	0.05	0.10	1.00	0.40
TAA (R)	0.40	0.82	1.00	0.70	0.90	-0.90	-0.70	0.90	1.00	0.40	0.90	0.90	0.90	0.90	1.00	0.90	1.00	1.00	0.90	0.67	0.70	0.40	1.00

Fig. 4 Heatmap of the coefficients of the Spearman correlation matrix between the variables time period and biochemical assays in susceptible and resistant genotypes

Table 3 Effect of cold stress on glutathione reductase, glutathione content and total antioxidant activity

Treatment	Genotypes								
	Glutathione reductase (U min^{-1} g^{-1} fw)			Glutathione (µM)			Total antioxidant activity (µM g^{-1} dw)		
	SR-4	GS-74	Mean	SR-4	GS-74	Mean	SR-4	GS-74	Mean
T1 (0 h)	0.96^{d}	1.28$^{e\#}$	0.26	3.95^{e}	5.16$^{d\#}$	4.55	0.16^{b}	0.25$^{b\#}$	0.21
T2 (2 h)	1.28^{b}	1.60$^{b\#}$	0.29	5.16^{c}	7.34$^{c\#}$	6.25	0.15^{b}	0.28$^{b\#}$	0.21
T3 (6 h)	1.17^{c}	1.34$^{d\#}$	0.35	5.17^{b}	7.65$^{b\#}$	6.41	0.15^{b}	0.30$^{b\#}$	0.23
T4 (24 h)	1.92^{a}	2.25$^{a\#}$	0.39	5.61^{a}	8.32$^{a\#}$	6.96	0.23^{a}	0.43$^{a\#}$	0.33
T5 (P24 hrs)	1.28^{b}	1.43$^{c\#}$	0.39	4.25^{d}	4.26$^{e\#}$	4.25	0.13^{b}	0.27$^{b\#}$	0.20
Mean	1.32	1.58		4.83	6.54		0.16	0.30	
CD ($p \leq 0.05$)				CD ($p \leq 0.05$)			CD ($p \leq 0.05$)		
Genotype (A): 0.129				Genotype (A): 0.017			Genotype (A): 0.023		
Treatment (B): 0.205				Treatment (B): 0.028			Treatment (B): 0.036		
Interaction AXB: NS				Interaction AXB: 0.040			Interaction AXB: 0.012		

The superscripts (a-e) or (a#-e#) within the respective genotype columns indicate that within these genotypes, treatments with the same superscript are statistically at par with each other (at the indicated *p*-value)

factors have been implicated in stress responses [68]. Based on the available literature, we selected five transcription factors (DREB1A, DREB1B, DREB1D, OSMYB-4 and AP-37) and studied their expression pattern at 5 °C in the two contrasting rice genotypes at different time points [0 h, 2 h, 6 h, 24 h, and P24 (24 h recovery post chilling)] at seedling stage (Table 4; Figs. 5, 3S).

Table 4 Effect of cold stress on relative gene expression of cold stress responsive transcription factors

Treatment	Genotype									
	OsMYB4		AP37		DREB 1A		DREB 1B		DREB 1D	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
T1 (0 h)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
T2 (2 h)	0.096	1.310	1.073	2.625	0.071	1.355	0.983	1.503	0.307	0.503
T3 (6 h)	0.088	1.793	1.343	3.610	0.014	4.207	0.012	2.783	2.377	5.860
T4 (24 h)	0.040	2.405	0.013	2.103	0.019	0.440	0.002	3.840	4.373	0.165
T5 (P24 hrs)	0.014	0.250	0.062	0.104	0.025	0.052	0.034	0.006	0.024	0.225
Mean	0.248	1.352	0.698	1.888	0.226	1.411	0.406	1.827	1.616	1.551
Statistical significance	Treatment: **** Genotype: **** Interaction: ****		Treatment: **** Genotype: **** Interaction: ****		Treatment: **** Genotype: **** Interaction: ****		Treatment: **** Genotype: **** Interaction: ****		Treatment: **** Genotype: ** Interaction: ****	

Asterisks indicate level of statistical significance: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$

Dehydration-responsive element binding proteins (DREB1A, 1B and DREB1D)

Several stress-responsive genes have been demonstrated to bind DRE (A/GCCGAC) and DRE-like cis regions and activate their expression using the DREB/CBF transcription factors [69, 70]. Overexpression of OsDREB1A, OsDREB1B, and OsDREB1D in rice and OsDREB1D in Arabidopsis promotes increased cold tolerance in transgenic plants [71–73]. The DREB1A gene showed early induction (2 h) and increased approximately 4-folds at 6 h in the resistant accession (GS-74) in our investigation on 4 weeks old rice seedlings under cold stress (at 5 °C), followed by a sharp reduction in its expression at 24 h after recovery. The susceptible accession (SR-4) had a non-significant modification in its expression (Table 4, Fig. 5). These findings align with a study [74] on 14-day-old rice seedlings exposed to cold stress (5 °C) for 24 h. In a previous investigation on 17-day-old rice seedlings exposed to cold stress (4 °C), the OsDREB1A gene began to express after 40 min, increased for 5 h, and then declined [71]. OsDREB1A was shown to be upregulated by cold at 6 h, reaching a maximum of 18-fold at 12 h, followed by a decrease in expression at 24 h in another investigation on transcriptional profiling of 14-day-old cold-tolerant rice seedlings under cold stress (5 °C for 48 h) [75]. Contrary to earlier studies, comparative transcriptome profiling in tolerant and susceptible genotypes during cold stress at the S3 stage revealed that OsDREB1A expression is induced at 2 h and peaks at 24 h [27].

In the present study, DREB1B gene expression enhanced at 2 h of cold stress and increased up to 24 h with a maximum at 24 h of 3.8-fold in resistant accession (GS-74), while in susceptible accession (SR-4) there is a mild expression under cold stress at 2 h which decreased with time (Table 4, Fig. 5). In an earlier study on transcriptional profiling of 14 days old cold-tolerant rice seedlings under cold stress (5 °C for 48 h), *OsDREB1B,* got induced by cold at 6 h, reaching a maximum of 20-fold at 24 h [75]. In a recent study, expression analysis of OsDREB1B in 2-week-old rice seedlings at 11 °C for 24 h showed around eightfold transcript induction in rice leaves. In the present study, DREB1D gene expression decreased at the beginning of cold stress (2 h), and later (at 6 h) increased sharply (nearly sixfold) in the resistant genotype. In the susceptible genotype, its expression reached a maximum value at 24 h under cold stress. In contrast, surprisingly, its expression dropped drastically in the resistant genotype (nearly 4-folds) (Table 4, Fig. 5). Expression analyses of 2-week-old rice seedlings at 11 °C for 24 h showed around fourfold OsDREB1D transcript induction in rice leaves [76], while in an earlier study, Dubouzet et al. [71] on 17 days old rice seedlings under cold stress (4 °C), OsDREB1D expression was not detected in plants under cold stress. The disparities in expression

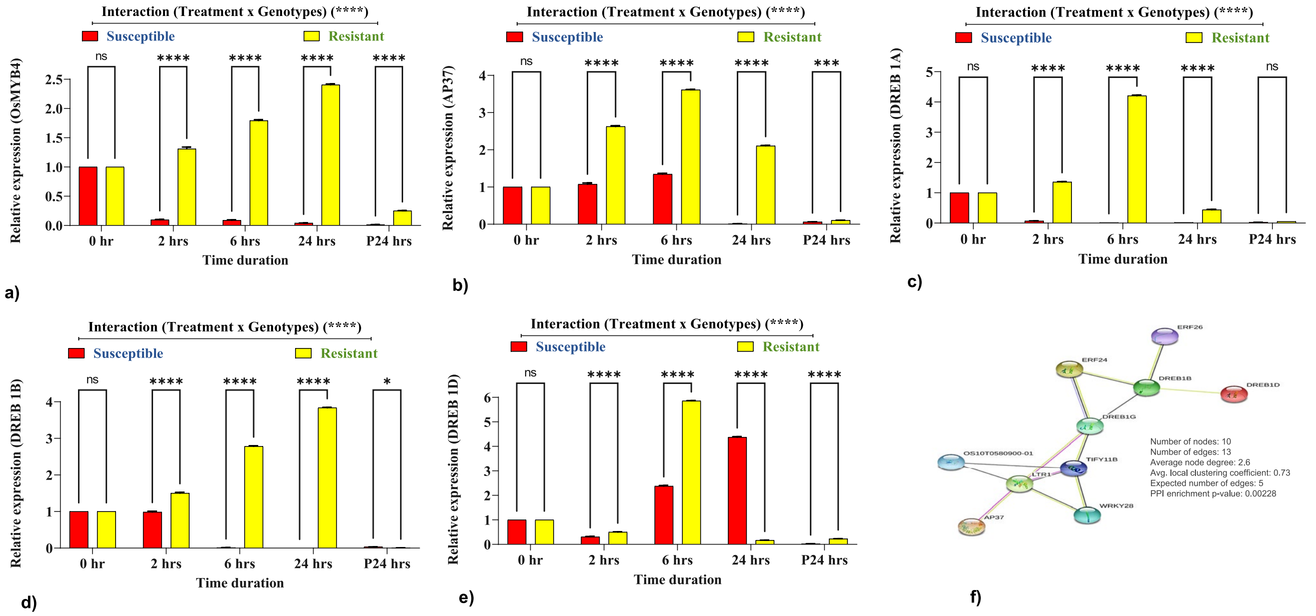


Fig. 5 Relative gene expression of transcription factors in response to cold stress treatment: **a** OsMYB; **b** AP37; **c** DREB1A; **d** DREB1B; **e** DREB1D. Leaves were collected at 0, 2, 6, 24 and P24 h. α-actin was used as internal standard. Values are means ± standard errors of three biological repeats; **f** *Association network of transcription factors* DREB1A (ERF24), DREB1B, DREB1D, OSMYB4 (LTR1) and AP37 in STRING**.** The thickness of the line indicates the degree of confidence prediction of the interaction. Network nodes represent proteins and edges represent protein–protein associations

profiles between studies can be attributed to the differences in cold stress temperature, cold stress duration, and time points of sample collection.

AP37

AP37, a member of the APETELA2 (AP2) subfamily is an anther-specific aspartic protease involved in tapetal programmed cell death (PCD). In the present study, AP37 expression increased in the resistant genotype till six hours of chilling (3.6 fold) and decreased at 24 h and in the recovery phase. Contrastingly, for the susceptible genotype, this change was subtle at six hours of chilling (1.36 fold) and declined after that (Table 4; Fig. 5).

In a study by Oh et al. it was observed that the expression of AP37 in rice at 4 °C up to 6 h, increased rapidly within 30 min upto 2 h, followed by an instant decrease [21]. In another study conducted on expression analysis of cold-induced transcription factor genes in 14 days old rice seedlings under cold stress (5 °C) for 24 h, the expression of OsAP37 gene was upregulated in the resistant genotype (8-folds) but remained unaltered in the susceptible genotype [74], which is consistent with our results.

OSMYB-4

The *OsMyb*4 gene from rice is a member of myeloblastosis protein family (MYB) of TFs, which has been introgressed into several plant species and its role in abiotic/biotic stress responses evaluated [77–80]. In the present study, transcription factor OsMYB4 showed early induction at 2 h reaching maximum expression at 24 h in the resistant genotype (GS-74) and after that, there was a sharp decrease in its expression. In contrast, in the susceptible genotype (SR-4) there was no expression under cold stress (Table 4, Fig. 5). In a study by Vannini et al. [81] on overexpression of rice OsMYB-4 gene at 4 °C, OsMYB-4 was induced in 3-day rice coleoptiles after 4 h of (4 °C) cold treatment. In another study by Vannini et al. on transgenic plants of tomato ectopically expressing the rice OsMYB4 gene at 4 °C, the OsMYB4 expression was elevated after 8 h of cold stress [77]. In a study by Soltesz et al. on rice, OsMYB4 gene enhanced germination in transgenic 10-day-old barley plants under cold stress (at 4 °C), and was upregulated after cold treatment [79]. In another study conducted by Baldoni et al. on the OsMYB4 gene family in rice seedlings placed at 4 °C in the light for 24 h, OsMYB-4 gene expression was upregulated at 4 h, reaching a maximum at 24 h [82]. In a study conducted by de Freitas et al. in rice under low temperature (10 °C) for a period of 72 h, OsMYB4 was induced with time in all the genotypes with maximum at 3 h in one genotype while at 48 h in some other genotypes [83].

Table 5 Effect of cold stress on different physiological and agronomic traits

Condition	Genotypes									
	Grain yield (g/plant)		Shoot height (cm)		Shoot biomass (g/plant)		Root biomass (g/plant)		Number of tillers	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
Control	31.39	15.87	109.22	127.00	24.56	21.16	15.70	19.31	25.00	19.33
Treated	26.45	14.42	100.75	124.46	17.68	18.54	8.43	14.47	18.00	16.00
Mean	28.92	15.14	104.98	125.73	21.12	19.85	12.06	16.89	21.50	17.67
Percent reduction	15.73%	9.09%	7.75%	2.00%	28.00%	12.37%	46.32%	25.06%	28.00%	17.24%
Statistical significance	Condition: NS		Condition: NS		Condition: NS		Condition: **		Condition: *	
	Genotype: ***		Genotype: NS		Genotype: NS		Genotype: *		Genotype: *	
	Interaction: NS		Interaction: NS		Interaction: NS		Interaction: NS		Interaction: NS	

Asterisks indicate level of statistical significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$

In conclusion, the disparities in expression profiles between studies could be due to differences in cold stress temperature (which ranged between 4–14 °C), duration (ranging from 15 min to several days), and time points at which samples were collected for analysis. The transcription factors under study have a significant role in regulating developmental and stress responses. DREB1 (A, B, D) transcription activators bind specifically to the C-repeat/DRE element DNA sequence 5′-[AG]CCGAC-3′ mediating high-salinity and dehydration-inducible transcription. Anther-specific aspartic protease (AP37), though involved in tapetal programmed cell death (PCD) showed interaction with MYB4, playing an indirect role in modulating cold tolerance (Fig. 5f). MYB4 is involved in cold stress response and positively regulates the expression of genes involved in scavenging reactive oxygen species (ROS). It transactivates a complex gene network affecting stress tolerance and panicle development [84].

Overall, the transcription factors DREB1A (ERF24), DREB1B, DREB1D, OSMYB4 (LTR1) and AP37 played a significant role based on Tukey's multiple comparisons test (Table 5S) which correlates with the typical association network of these transcription factors in STRING (as shown in Fig. 5f).

Phenotypic characteristics

Cold damage at the vegetative stage is significantly lower than at the reproductive stage, because the former has a lower threshold temperature (10–13 °C) while the latter has a higher threshold temperature for cold damage (18–20 °C) [85]. The present study showed that cold damage at the seedling stage caused a non-significant decrease in phenotypic characteristics like shoot height and shoot biomass in both cold tolerant and susceptible genotypes (Table 5, Fig. 6). However, there was a significant decrease in the number of tillers and root biomass due to cold stress at the S3 seedling stage, which ultimately affected the grain yield adversely. The decrease in grain yield of resistant genotype GS74 was 9.13%, which is statistically insignificant, while in susceptible type, it was 15.73% (statistically significant) (Fig. 6).

We conclude that rice seedlings exposed to cold stress induce the synthesis and accumulation of cryo-protectants and osmoprotectants like proline and sugars, thereby enhancing plant's cold tolerance through modulation of gene expression, osmotic adjustments, and ROS scavenging.

Broad outline of cold tolerance in rice

Plants perception and adaptation to adverse climatic conditions in influenced by stress hormones like abscisic acids, ethylene, salicylic acid, jasmonic acid, gibberellins, brassinosteroids, and cytokinins are induced under abiotic stress

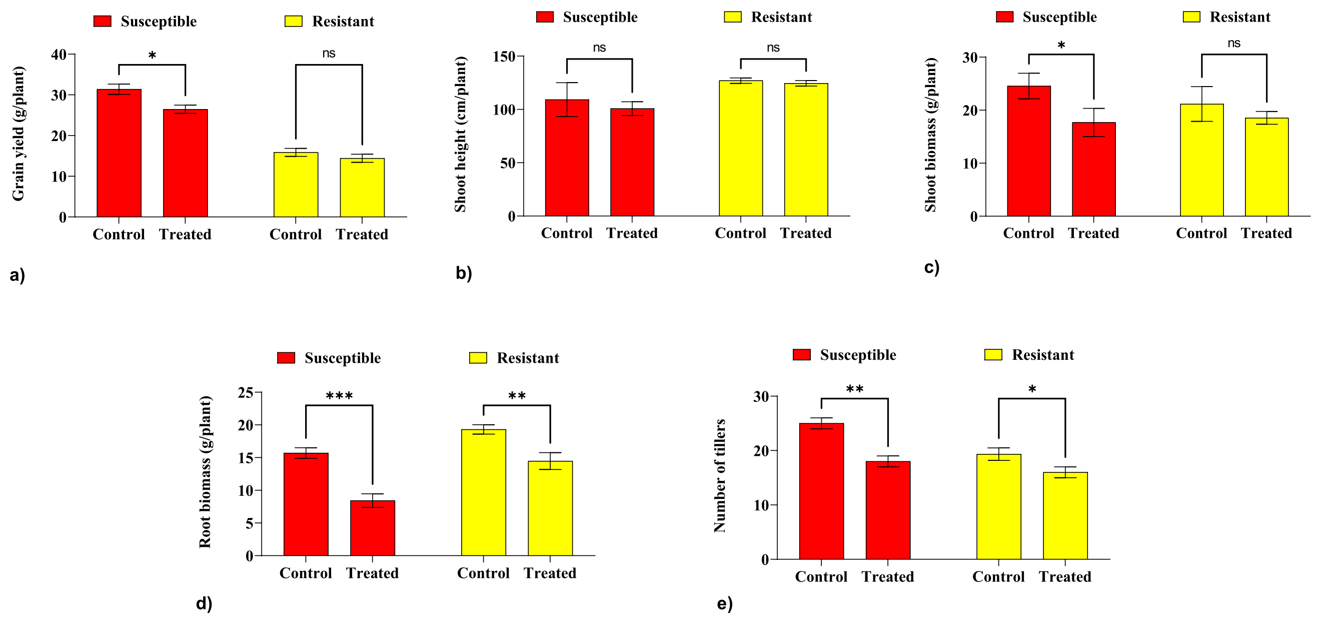


Fig. 6 Effect of cold stress treatment on phenotypic characteristics: **a** grain yield per plant, **b** shoot height, **c** shoot biomass, **d** root biomass, **e** number of tillers. Cold stress was given for 24 h at Stage 3 and data was taken at yielding stage. Data represents the mean of ten replicates ± SE

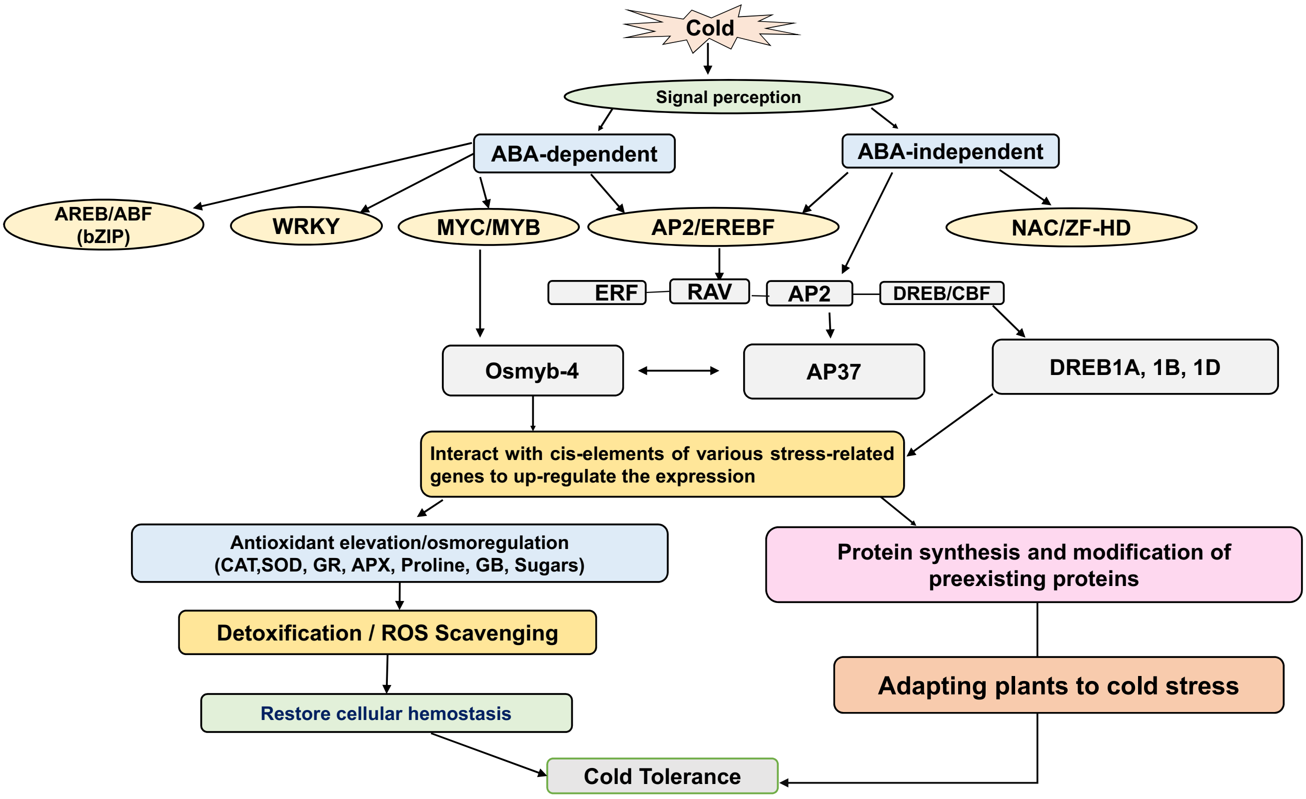


Fig. 7 Broad outline of the role of different factors in cold tolerance of rice

conditions to enable optimal responses [5, 86]. Among these, abscisic acid (ABA) is the central regulator of abiotic stress resistance and coordinates a range of functions [87]. Stress-responsive genes are expressed either via ABA-dependent or ABA-independent pathways [84]. Several transcription factor families play an important role in stress signal transduction, including APETALA2/ethylene-responsive factor (AP2/ERF), WRKY, NAC/ZF-HD, and MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene) [88]. ABA-dependent signalling systems mediate stress adaptation by inducing regulons like (i) the AREB/ABF bZIP regulon; and (ii) the MYC/MYB regulon [89–91]. ABA-independent signalling systems mediate stress adaptation through (i) the CBF/DREB regulon (AP2/EREBP (ERF); and (ii) the NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon [19, 91]. However, some studies show the existence of both ABA-dependent and independent pathways of stress response that function through AP2/EREBP (ERF) family [92, 93]. Rice ERF gene AP37, though not induced by ABA [21] interacts with OsMYB4, which regulates expression of genes involved in ROS scavenging [94]. DREB1 induces the expression of genes involved in ROS detoxification, membrane transport, osmolyte biosynthesis, and phosphoinositide metabolism [95–100]. The osmoprotectants and antioxidants stabilize cellular membranes and restore cellular homeostasis. Expression of genes involved in cellular regulation leads to protein synthesis and modification of preexisting proteins which help plants to repair the damaged protein machinery (Fig. 7).

Conclusions

Owing to climate change, rice-growing regions in the western Himalayas experience harsher, lower temperatures more frequently, especially during spring, coinciding with the early rice-growing season. Unlike most earlier studies worldwide, rice germplasm of the region was screened at a lower temperature of 5 °C at the S3 seedling stage. The screening results and molecular analyses suggested that the mechanisms triggered by cold stress in rice seedlings depend on the rice ecotype; and that most (more than 80%) of the *indica* types clustered into cold-sensitive class, while the *japonica* types were widely distributed across all cold-stress response categories.

Cold stress reduced the tiller number, root biomass and rice productivity, even when the rice plants were in the early vegetative stage (S3). Osmoprotectants and antioxidants were induced to alleviate the oxidant stress caused by ROS generation in plants witnessing cold-stress. Differential expression of transcription factors OsMYB-4, OsAP-37, OsDREB1A and OsDREB1B, presented a clear contrast between resistant and susceptible genotypes, implying that these transcription factors caused differential competence of redox-regulatory mechanism under cold stress in rice, and their expression was genotype-dependent. These transcription factors (OsMYB-4, OsDREB1A and OsDREB1B) are good candidates for developing cold-stress-resistant genotypes through genetic manipulation.

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Declarations

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