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Harnessing cytokinin biology in crop biofortification and enhanced food security

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Published in
Frontiers in Genetics

ISSN 1664-8714
ISBN 978-2-83251-680-5
DOI 10.3389/978-2-83251-680-5

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Harnessing cytokinin biology in crop biofortification and enhanced food security

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Citation

Gupta, S. K., Nadda, A. K., Bibi, S., Singh, J., Kumar, D., Komatsu, S., Mathur, J., eds. (2023). *Harnessing cytokinin biology in crop biofortification and enhanced food security*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-680-5

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frontiers in Genetics

ORIGINAL RESEARCH
published: 07 February 2022
doi: 10.3389/fgene.2021.780599

Identification of Potential Cytokinin Responsive Key Genes in Rice Treated With Trans-Zeatin Through Systems Biology Approach

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Rice is an important staple food grain consumed by most of the population around the world. With climate and environmental changes, rice has undergone a tremendous stress state which has impacted crop production and productivity. Plant growth hormones are essential component that controls the overall outcome of the growth and development of the plant. Cytokinin is a hormone that plays an important role in plant immunity and defense systems. *Trans*-zeatin is an active form of cytokinin that can affect plant growth which is mediated by a multi-step two-component phosphorelay system that has different roles in various developmental stages. Systems biology is an approach for pathway analysis to *trans*-zeatin treated rice that could provide a deep understanding of different molecules associated with them. In this study, we have used a weighted gene co-expression network analysis method to identify the functional modules and hub genes involved in the cytokinin pathway. We have identified nine functional modules comprising of different hub genes which contribute to the cytokinin signaling route. The biological significance of these identified hub genes has been tested by applying well-proven statistical techniques to establish the association with the experimentally validated QTLs and annotated by the DAVID server. The establishment of key genes in different pathways has been confirmed. These results will be useful to design new stress-resistant cultivars which can provide sustainable yield in stress-specific conditions.

Keywords: WGCNA, systems biology, co-expression, cytokinin, hub genes, QTLs

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *21 September 2021*
Accepted: *18 November 2021*
Published: *07 February 2022*

Citation:
Mishra DC, Arora D, Budhlakoti N, Solanke AU, Mithra SVACR, Kumar A, Pandey PS, Srivastava S, Kumar S, Farooqi MS, Lal SB, Rai A and Chaturvedi KK (2022) Identification of Potential Cytokinin Responsive Key Genes in Rice Treated With Trans-Zeatin Through Systems Biology Approach. Front. Genet. 12:780599. doi: 10.3389/fgene.2021.780599

Abbreviations: QTL, Quantitative trait locus; WGCNA, Weighted correlation network analysis; GEO, Gene expression omnibus; tz, trans-zeatin; DMSO, Dimethyl sulfoxide; dhga: Differential hub gene analysis; GSAQ, Gene Set Analysis with QTL; ER, Endoplasmic reticulum; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for annotation, visualization, and integrated discovery; TOM, Topological overlap matrix; GO, Gene ontology; PHT1, Phosphate transporter 1.

INTRODUCTION

Rice (*Oryza sativa*) is an important food grain crop that is consumed worldwide (Kubo and Purevdorj, 2004). The human population is estimated to reach approximately 10.7 billion by 2050 (Speidel, 2000) and accordingly, the demand for consumption of rice will also increase. On the other hand, the productivity of rice is not increasing at the same pace due to various reasons such as poor water management, soil depletion, abiotic stresses (e.g., drought, flooding, and salinity), biotic stresses (e.g., damage caused by bacteria, fungi, insects), etc. (Kumar et al., 2017). Therefore, it becomes necessary to understand the underlying mechanisms in crops to sustain production and fulfill the demand of the growing population (Ma and Michailides, 2007). Plants must be able to react rapidly with various abiotic and biotic stress signals and develop efficient defense responses to cope with adverse conditions arising in the field (Kumar et al., 2015). Understanding the crosstalk mechanisms in a stress response could help in the characterization of synergistic and antagonistic mechanisms (Kim et al., 2021). Plant hormones are the key components of these defense and adaptation mechanisms. To facilitate the adaptations to the stresses, various hormonal pathways are upregulated or downregulated. Change in hormonal influence usually affects the degree of resistance or susceptibility to different stresses (O'Brien and Benkova, 2013). Cytokinins (a class of phytohormones) are central regulators of plant growth and development. Cytokinins regulate numerous developmental and physiological processes such as cytokinesis of shoots and roots, reproductive behavior, leaf senescence, and responses to environmental cues, particularly to light and nutrients (Haberer and Kieber, 2002; Werner et al., 2003). Trans-Zeatin (tz) is an active form of cytokinin involved in managing environmental stress. Cytokinin pathway has been widely studied and a huge amount of gene expression data are available in public repositories (Edgar et al., 2002; Leinonen et al., 2011). These data can be better utilized for constructing gene regulatory networks and identifying key genes which will further help in developing improved rice varieties having the ability to produce high yield and resistance to such abiotic stress and adverse conditions (Stuart et al., 2003; Lelandais et al., 2011).

Key genes regulating plant growth and cytokinin responsive genes involved in development process will help in developing better stress tolerant varieties (Zhang et al., 2011, 2012; Li et al., 2019). Co-expression analysis is one of the important ways to construct such a network and identify the most relevant module (Heyer et al., 1999; Hudson et al., 2012; Gaiteri et al., 2014). A statistical approach, Weighted Gene Co-expression Network Analysis (WGCNA) is an effective way to perform such analysis (Tang et al., 2016; Che et al., 2018; Wu et al., 2018) and it has been successfully used previously in identifying important modules and key hub genes related to rice in different conditions (Tan et al., 2017; Zhang et al., 2018). This approach provides an analytical framework for the analysis of microarray and transcriptomic data and helps in relating the gene expression to external traits. Based on various co-expression analysis studies, we found WGCNA as a well-proven approach for the identification of functional modules and co-expressed genes from large biological datasets (Kost et al., 2017; Zhang et al., 2018; Mishra et al., 2021). WGCNA is available as an R package and works on the principle of "guilt by association" (Langfelder and Horvath, 2008), that is, a group of genes are more likely to be associated with each other when they have similar expressions (Gillis and Pavlidis, 2012; van Dam et al., 2018). It uses an unsupervised learning method where large-scale data is clustered by building a tree from bottom to top by connecting the two nearest branches or genes. Modules are formed using the hierarchical clustering method and comprised of genes with similar functions. The modules can be further utilized to identify the important key genes. These key or hub genes are centrally connected genes in different modules which are expected to have an important function and play a critical role in maintaining overall harmony within the cell and development of tissue (Sircar and Parekh, 2015).

In this paper, we have performed co-expression analysis on publicly available microarray data retrieved from the NCBI GEO database (Edgar et al., 2002; Barrett et al., 2013) for cytokinin-responsive genes. We have identified novel key genes in each module using sound statistical approaches of co-expression analysis. Furthermore, we have done the biological validation of the novel key genes using well-established and experimentally validated QTLs (quantitative trait locus) of rice.

MATERIALS AND METHODS

Data Summary

Microarray data related to cytokinin effect on root and leaves of rice were retrieved from the NCBI GEO database with reference series GSE6719 (Hirose et al., 2007). In this experiment, roots and leaves of rice seedlings were treated with *trans*-zeatin (an active form of type-A cytokinin hormone) for 30 and 120 min. Three independent replicate treatments were performed for roots and leaves at each time point. To identify cytokinin responsive genes, dimethyl sulfoxide (DMSO) treated roots and leaves for each time point (30 and 120 min) were used as a control. Three independent replicates of DMSO treatment were performed for each organ per time point. Then, the microarray data were generated by using the Affymetrix GeneChip® rice genome array which contains probes to query approximately 48,564 *japonica* and 1,269 *indica* transcripts. There are 24 samples having accession series (GSE6719). The number of transcripts (features) in the expression data was 57,381, of which annotation data was available for only 23,850 transcripts. We have used only the annotated transcripts for WGCNA-based co-expression analysis.

Weighted Gene Co-Expression Analysis

To perform the analysis, we first studied the distribution pattern of grown samples using transcript count data and performed principal component analysis. Following, uniform distribution pattern we have used the R package "WGCNA" for constructing the network (Langfelder and Horvath, 2008). The elements in the

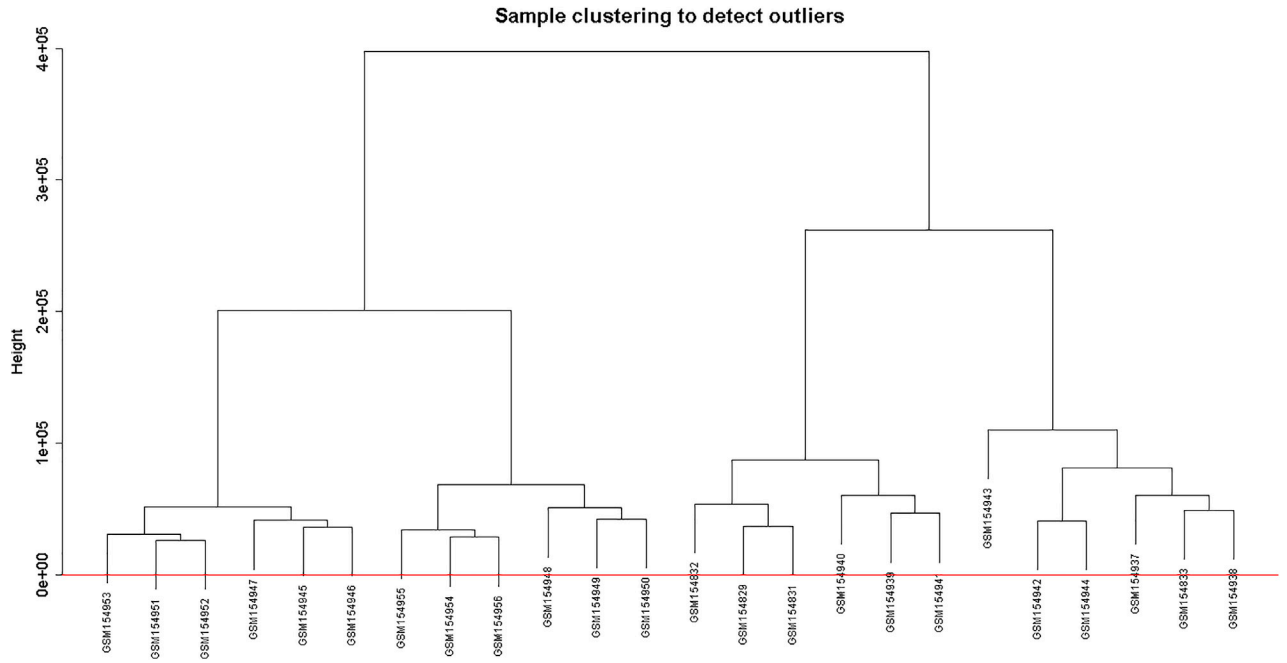

FIGURE 1 | Clustering dendrogram of samples based on their Euclidean distance.

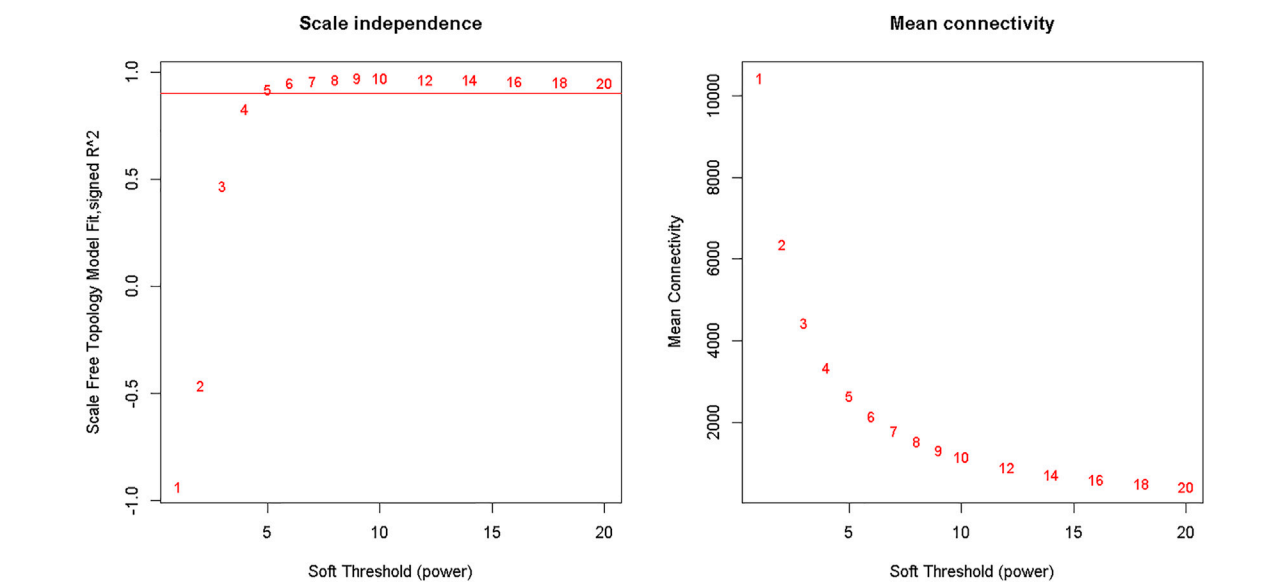

FIGURE 2 | Analysis of network topology for various soft-thresholding powers. The left panel shows the scale-free fit index (*y*-axis) as a function of the soft-thresholding power (*x*-axis). The right panel displays the mean connectivity (degree, *y*-axis) as a function of the soft-thresholding power (*x*-axis).

co-expression matrix are defined as the weighted value of correlation coefficients. Gene co-expression of a pair of genes i and j were calculated using **Eq. 1** of an unsigned network:

$$A_{ij} = \left|\mathrm{cor}\left(E_i, E_j\right)\right|^{\beta} \quad (1)$$

where E_i and E_j consist of expression vector profiles of genes i and j across multiple samples and A_{ij} is the adjacency of the unsigned network. Pearson's correlation coefficient was used to identify the similarity of genes. The absolute value of correlation is raised to a power β to create the adjacency matrix. WGCNA emphasizes high correlation by raising the absolute value of correlation to a

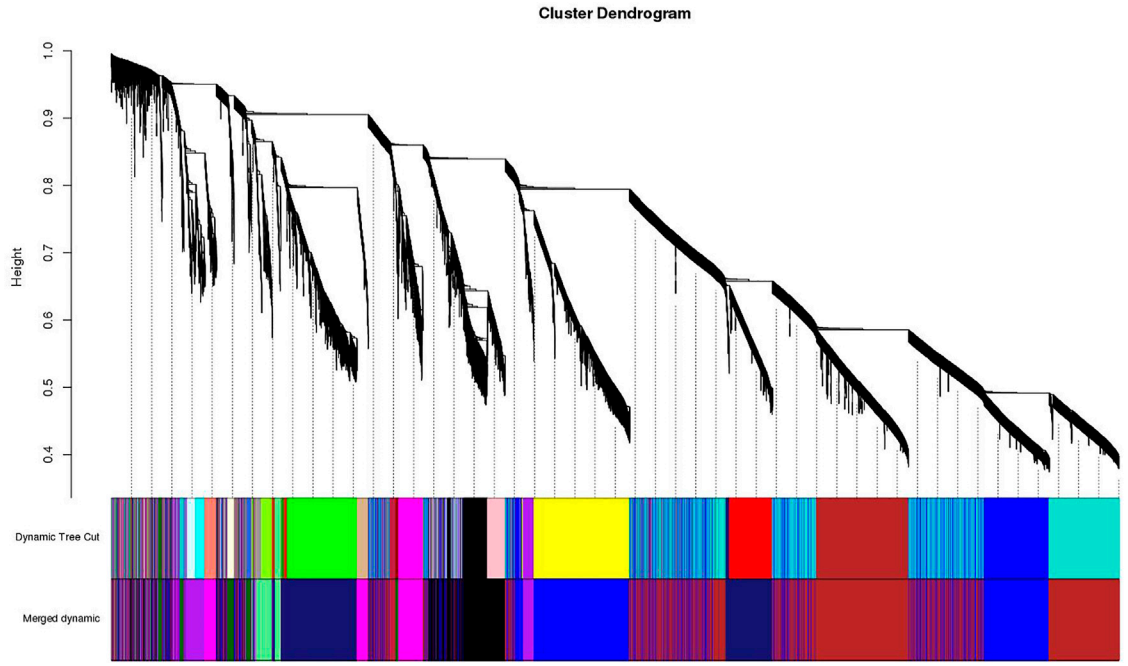

FIGURE 3 | Clustering dendrogram of genes, with dissimilarity based on topological overlap, together with assigned merged module colors and the original module colors.

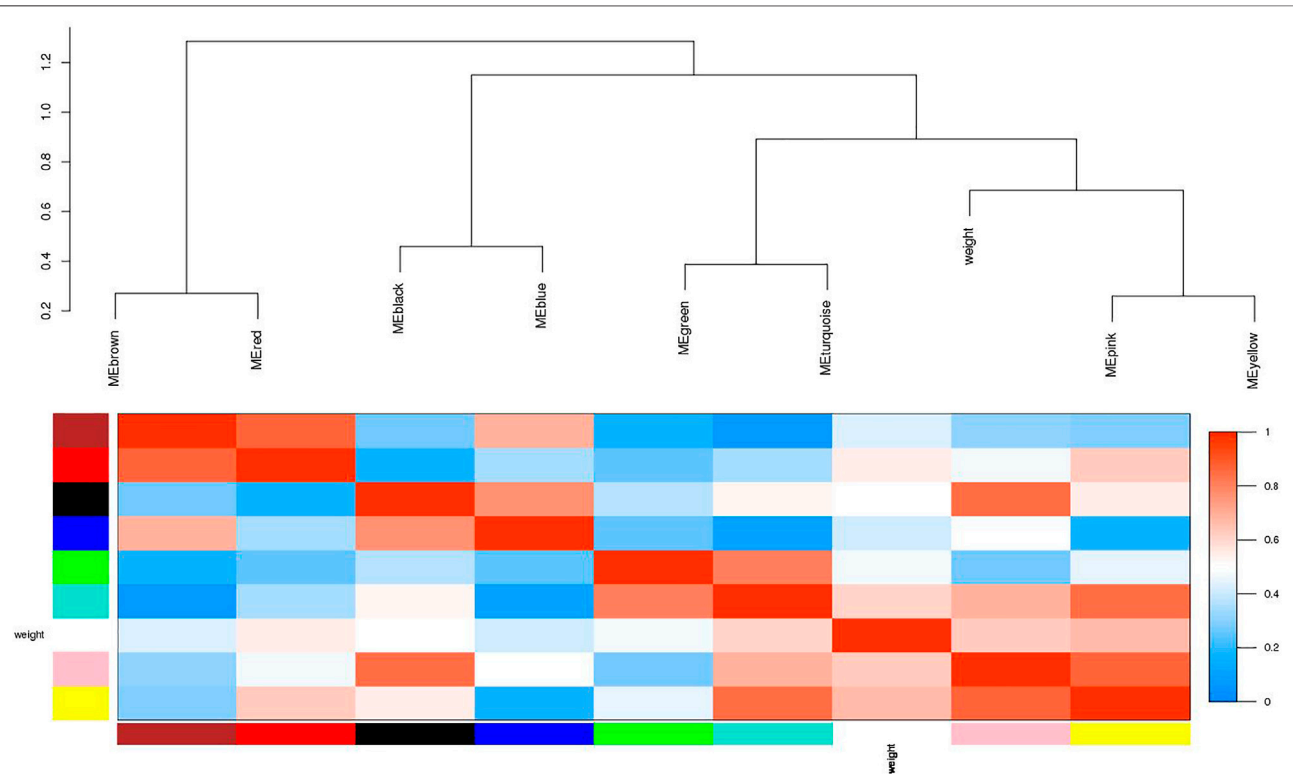

FIGURE 4 | Visualization of the eigengenes network representing the relationships among the modules. The top panel shows a hierarchical clustering dendrogram of the modules based on the dissimilarity score of E_i and E_j given by 1−cor (E_i, E_j). The heatmap in the bottom panel shows the eigengenes adjacency A_{ij}= (1 + cor (E_i, E_j))/2.

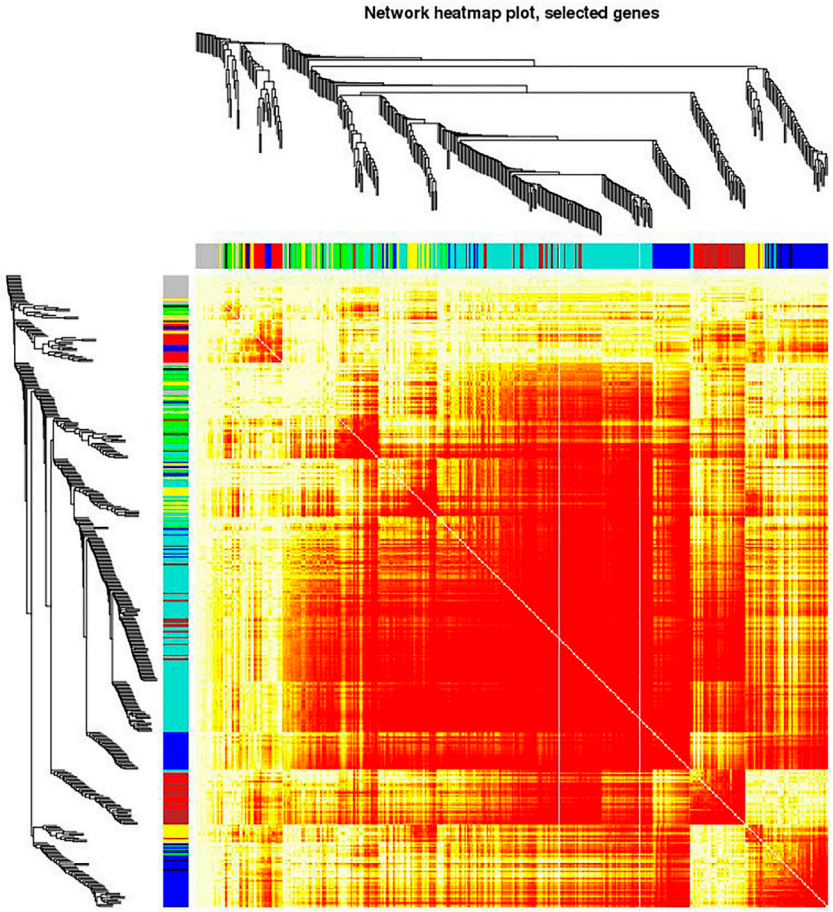

FIGURE 5 | Visualizing the gene network using a heatmap plot. The heatmap depicts the Topological Overlap Matrix (TOM). The light red color represents low overlap and progressively darker shades of red represent higher overlap. Blocks of dark colors along the diagonal depict modules. The gene dendrogram and module assignment are also shown along the left side and the top of the Figure.

power $\beta \geq 1$. The correlation network among the genes is built based on the adjacency matrix. Each gene is considered as a node and an edge between two genes is built if it passes a set threshold of co-expression strength. A soft threshold value of 6 was used to determine the significant edges for connecting pairs of genes. Furthermore, modules within the networks were identified by calling the R function "cutreeDynamic" available in the WGCNA package, which helps in identifying the minimum number of large modules with a strong association of genes. The function was applied after identifying the soft threshold of the adjacency matrix, and cut them respectively to get modules related to different functions. These identified modules were further used to identify the key or hub genes responsible for governing specific biological functions in cell development.

In order to identify the modules that were highly related, module similarity was quantified by eigengenes correlation (Shi et al., 2015). The eigengenes of a module are defined as the eigenvector associated with the first principal component of the expression matrix (Langfelder and Horvath, 2007). Highly related modules were identified using module significance. Module significance is defined as the average gene significance of all the genes in the modules to access the association of a module to the phenotype.

Visualization of Network and Hub Genes Identification

Various methods have been used for the identification of hub genes from a large dataset (Bader and Hogue, 2003; Chin et al., 2014). These methods mainly focused on hub gene identification, based only on gene connection degrees in the gene co-expression network. A heat map was generated to compare the expression pattern across the samples and assigned different colors to genes with similar values (Zhao et al., 2014). Genes are interconnected in each module and possess specific functions. After identifying the most significant module, identification of key or hub genes was

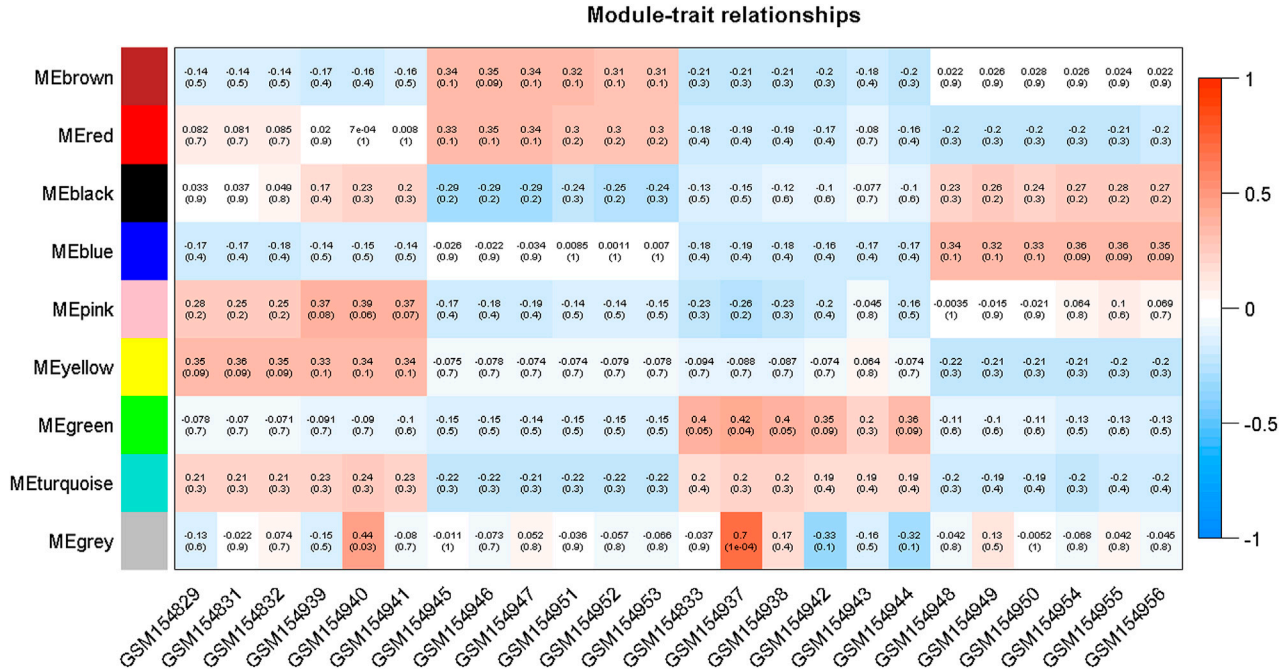

FIGURE 6 | Module-trait associations where each row corresponds to a module or eigengene and a column to a trait. Each cell contains the corresponding correlation and *p*-value. The table is color-coded by correlation according to the color legend mentioned in the Figure.

TABLE 1 | Identified hub genes with respect to their level of connectivity in the turquoise module.

S.No	Hub gene	Function/Annotation	Locus location	Acc. No
1	Os04g0295400	Horcolin/Jasmonate-induced protein, putative, expressed	LOC4335407/Chr 4, NC_029259.1 (12989808.12993215)	AK067477
2	Os03g0425800	uncharacterized LOC4333157	LOC4333157/Chr 3, NC_029258.1 (17780383.17784105)	AK100427
3	Os02g0731200	MADS-box transcription factor 57	LOC4330621/Chr 2, NC_029257.1 (30456659.30462758)	AY177702
4	Os01g0558800	uncharacterized LOC4324676	LOC4324676/Chr 1, NC_029256.1 (21151568.21155639, complement)	AK068120

TABLE 2 | Identified hub genes with respect to their level of connectivity in the blue module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os07g0424400	probable cellulose synthase A catalytic subunit 3 [UDP-forming]	LOC4343049/Chr 7, NC_029262.1 (13741571.13747205, complement)	AK120236
2	Os02g0511800	uncharacterized LOC4329457	LOC4329457/Chr 2, NC_029257.1 (18343563.18347153, complement)	AK069817
3	Os08g0505200	uncharacterized LOC4345975	LOC4345975/Chr 8, NC_029263.1 (24986078.24990484)	AK067190
4	Os01g0276800	Glucosidase 2 subunit beta	LOC4324264/Chr 1, NC_029256.1 (9702711.9709700, complement)	AK108476

carried out using an R package, "dhga" (Differential Hub Gene Analysis) (Das et al., 2017).

Gene Annotation and Gene Ontology Analysis

The annotation of expressed genes was performed using the Institute for Genomic Research (TIGR) database which includes information regarding biological processes (BP), molecular function (MF), and cellular components (CC) (Ouyang et al., 2007). Furthermore, the identified genes from identified modules were submitted to the OryzaExpress database (Hamada et al., 2011) and converted the ids into probeID (Affymatrix). These ids were subsequently converted into geneid using DAVID (the database for

TABLE 3 | Identified hub genes with respect to their level of connectivity in the brown module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os01g0743600	ATP-dependent protease La domain containing protein, expressed	LOC4326165/Chr 1, NC_029256.1 (31078612.31088072, complement)	AK102317
2	Os06g0319800	Os01g0512300	Os01g0512300/Chr 1, NC_008394.3 (18047906.18050788, complement)	AK107048
3	Os07g0636600	dirigent protein 5	LOC4344033/Chr 7, NC_029262.1 (26445635.26446517, complement)	AK106022
4	Os05g0128100	uncharacterized LOC4337691	LOC4337691/Chr 5, NC_029260.1 (1652011.1653860)	AK108556

TABLE 4 | Identified hub genes with respect to their level of connectivity in the green module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os07g0171300	uncharacterized LOC4342515	LOC4342515/Chr 7, NC_029262.1 (3773297.3779772)	AK100663
2	Os03g0610800	serpin-ZXB	LOC4333434/Chr 3, NC_029258.1 (23054420.23068346)	AK107194
3	Os01g0827600	exocyst complex component EXO70B1	LOC4327525/Chr 1, NC_029256.1 (35412698.35416714)	AK122173
4	Os08g0200400	KH domain-containing protein At4g18375	LOC4344900/Chr 8, NC_029263.1 (5810049.5816102, complement)	AK067859

TABLE 5 | Identified hub genes with respect to their level of connectivity in the yellow module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os09g0442400	protein GAMETE EXPRESSED 1	LOC4347181/Chr 9, NC_029264.1 (16443693.16448158)	AK106970
2	Os08g0522400	putative L-ascorbate peroxidase 6	LOC4346078/Chr 8, NC_029263.1 (2,5971775.25974968, complement)	AK065893
3	Os03g0654600	chlorophyll (ide) b reductase NOL, chloroplastic	LOC4333604/Chr 3, NC_029258.1 (25520290.25525342, complement)	CB669633
4	Os07g0550600	benzyl alcohol O-benzoyltransferase	LOC4343545/Chr 7, NC_029262.1 (21854513.21856895)	AK109553

TABLE 6 | Identified hub genes with respect to their level of connectivity in the red module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os12g0566000	boron transporter 1	LOC4352546/Chr 12, NC_029267.1 (23248819.23253256)	AK100510
2	Os04g0658300	ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	LOC4337267/Chr 4, NC_029259.1 (33575149.33579656, complement)	AK067399
3	Os05g0358200	DNA primase small subunit	LOC9267485/Chr 5, NC_029260.1 (17006498.17011733, complement)	AK073973
4	Os11g0707000	ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	LOC4351224/Chr 11, NC_029266.1 (28932976.28936094, complement)	CB673145

TABLE 7 | Identified hub genes with respect to their level of connectivity in the black module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os11g0491400	uncharacterized LOC4350546	LOC4350546/Chr 11, NC_029266.1 (17379762.17382920)	AK068341
2	Os07g0271500	bisdemethoxycurcumin synthase	LOC4342896/Chr 7, NC_029262.1 (10018732.10020733)	AK109558
3	Os09g0482740	uncharacterized LOC9271634	LOC9271634/Chr 9, NC_029264.1 (18576964.18581257)	AK061852
4	LOC4338611	lichenase-2	LOC4338611/Chr 5, NC_029260.1 (18106236.18110996, complement)	CB628871

TABLE 8 | Identified hub genes with respect to their level of connectivity in the pink module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os07g0187700	SEC12-like protein 1	LOC4342601/Chr 7, NC_029262.1 (4682485.4687647)	AK111777
2	Os05g0568800	bradykinin-potentiating and C-type natriuretic peptides	LOC4339650/Chr 5, NC_029260.1 (28310927.28311938)	AK059883
3	Os04g0334700	aspartic proteinase-like protein 2	LOC4335504/Chr 4, NC_029259.1 (15623634.15653865, complement)	AK120870
4	Os03g0359000	uncharacterized LOC4332880	LOC4332880/Chr 3, NC_029258.1 (13927427.13935150, complement)	AK064512

TABLE 9 | Identified hub genes with respect to their level of connectivity in the grey module.

S.No	Hub gene	Function	Locus location	Acc. No
1	Os07g0131600	hexose carrier protein HEX6	LOC4342334/Chr 7, NC_029262.1 (1669273.1671384)	AK068296
2	Os03g0704100	probable plastid-lipid-associated protein 4, chloroplastic	LOC4333849/Chr 3, NC_029258.1 (28304914.28307925)	AK070474
3	Os06g0130400	probable aminotransferase ACS12	LOC4340002/Chr 6, NC_029261.1 (1629690.1633597)	AK065212
4	Os03g0322500	14 kDa zinc-binding protein	LOC4332685/Chr 3, NC_029258.1 (11672780.11677395)	AK121029

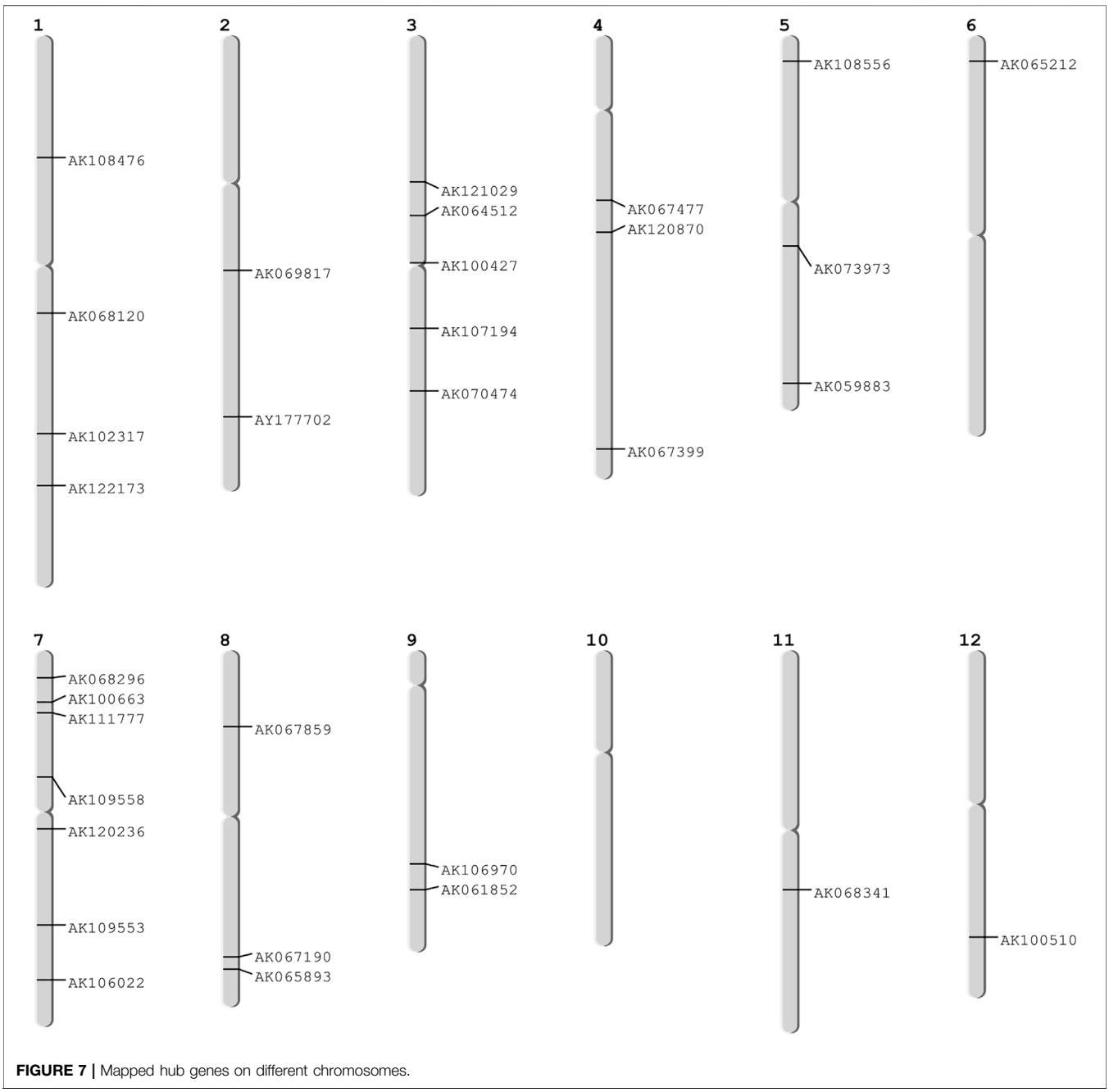

FIGURE 7 | Mapped hub genes on different chromosomes.

annotation, visualization, and integrated discovery) (Huang et al., 2007) for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis web server to decipher the role of genes varies from BP, CC, MF, and KEGG pathway analysis respectively against *oryza sativa* database and observed in REVIGO database (Supek et al., 2011). We found the

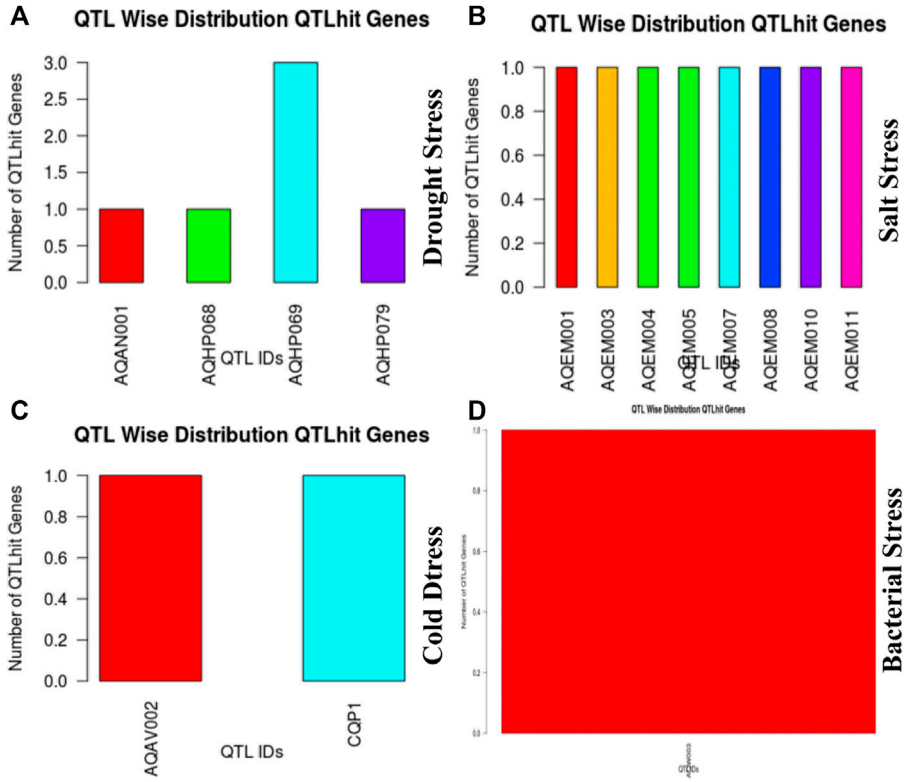

FIGURE 8 | Hub gene distribution on chromosome to the corresponding QTL ids.

involvement of these hub genes by observing the changes in the expression level of treated and control (normal) conditions [S1_file].

Validation of Identified Hub Genes

We validated the identified hub genes using *in-silico* approach. In the *in-silico* approach, we identified the chromosome-wise distribution of the hub genes and mapped these genes on chromosomes using web server oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/) (Kurata and Yamazaki, 2006). Further, we used a statistical framework approach to test the association of these hub genes with well-known experimentally validated and genetically rich QTLs data reported for biotic and abiotic stress conditions (Jaiswal et al., 2002). We have used an R package "GSAQ" (https://cran.r-project.org/web/packages/GSAQ) (Das et al., 2018) for mapping the identified hub genes with QTLs on respective chromosomes. GSAQ provides a platform to associate the selected hub genes to the corresponding overlapped QTL-IDs with their genomic positions. Furthermore, the identified hub genes were also validated through pathway analysis.

RESULTS

Weighted Co-expression Network Construction and Module Identification

Clustering of the samples (**Figure 1**) suggests that there is no outlier present in the data. Power β was obtained through two types of graphs given in **Figure 2**: 1) soft threshold values of β (x-axis) *vs* scale-free topology model fit scaled R^2 (y-axis) and 2) soft threshold values of β (x-axis) vs mean connectivity scores (y-axis). The optimal value of β obtained using these graphs is 6 with R^2value 0.8. This value of β was further used to produce hierarchical clustering (**Figure 3**).

A dynamic hierarchical tree algorithm was used to divide the clustering tree constructed from the differentially expressed genes, resulting in 24 different co-expression modules in the data which were named as blue (3,972 genes), brown (3,064 genes), green (1801 genes), yellow (2,387 genes), red (1,272 genes), and turquoise (5,670 genes), Black (1,115 genes), Cyan (275 genes), dark green (85 genes), dark red (113), dark turquoise (83 genes), green yellow (338 genes), grey (35 genes), grey60 (162 genes), light cyan (246 genes), light green (144 genes), light yellow (141

FIGURE 9 | The classification of identified hub genes represented by heat map using DAVID software. It is noted that eight genes (*y*-axis) are classified into four classes (*x*-axis).

genes), magenta (644 genes), midnight blue (252 genes), pink (835 genes), purple (442 genes), royal blue (122 genes), salmon (296 genes), and tan (326 genes) (**Figure 4**). After clustering, the genes were grouped into modules (subnetworks) depicted in different colors for easy identification (**Figure 5**).

Moreover, in order to explore the relationship between identified modules and the experimental samples (traits), we calculated and tested the correlation coefficients. These correlation coefficients along with their *p*-values for module-trait relationship were depicted (**Figure 6**). In **Figure 6**, the red color shows a strong positive correlation and the blue color displays a strong negative correlation. Furthermore, the centralized hub genes were identified from these modules through statistical analysis with the help of R-package "dhga" using a weighted gene score. A total of 36 hub genes were identified in 9 modules and their detailed description (function/annotation, location, and accession number) are mentioned in **Table 1**; **Table 2**; **Table 3**; **Table 4**; **Table 5**; **Table 6**: **Table 7**; **Table 8**; **Table 9**.

Validation of Identified Hub Genes

These 36 identified hub genes were mapped on rice chromosomes and 32 genes were found to be located at various rice chromosomes (**Figure 7**). In the process of finding the association of these genes with the well-known QTLs related to salt, cold, drought, bacterial stress, we found that 17 out of the 36 identified hub genes were associated with these QTLs mapped on various chromosomes (**Figure 8**). We also performed pathway analysis and found that the expression of these genes either increased or decreased during the period of treatment in five different pathways. Heat map analysis was conducted through DAVID to produce a matrix of enriched GO terms with the identified genes. The green and black color shading on the heat map matrix indicate a positive and negative correlation between the enriched GO term and identified DMSO and tz-treated hub genes, respectively (**Figure 9**).

DISCUSSION

The productivity of rice is severely affected due to various biotic and abiotic stresses. Therefore, to develop a variety that is resistant to these stresses, there is an urgent need to identify important hub genes governing the whole production process. Rice genome consists of around 58,000 genes (Cao et al., 2012)

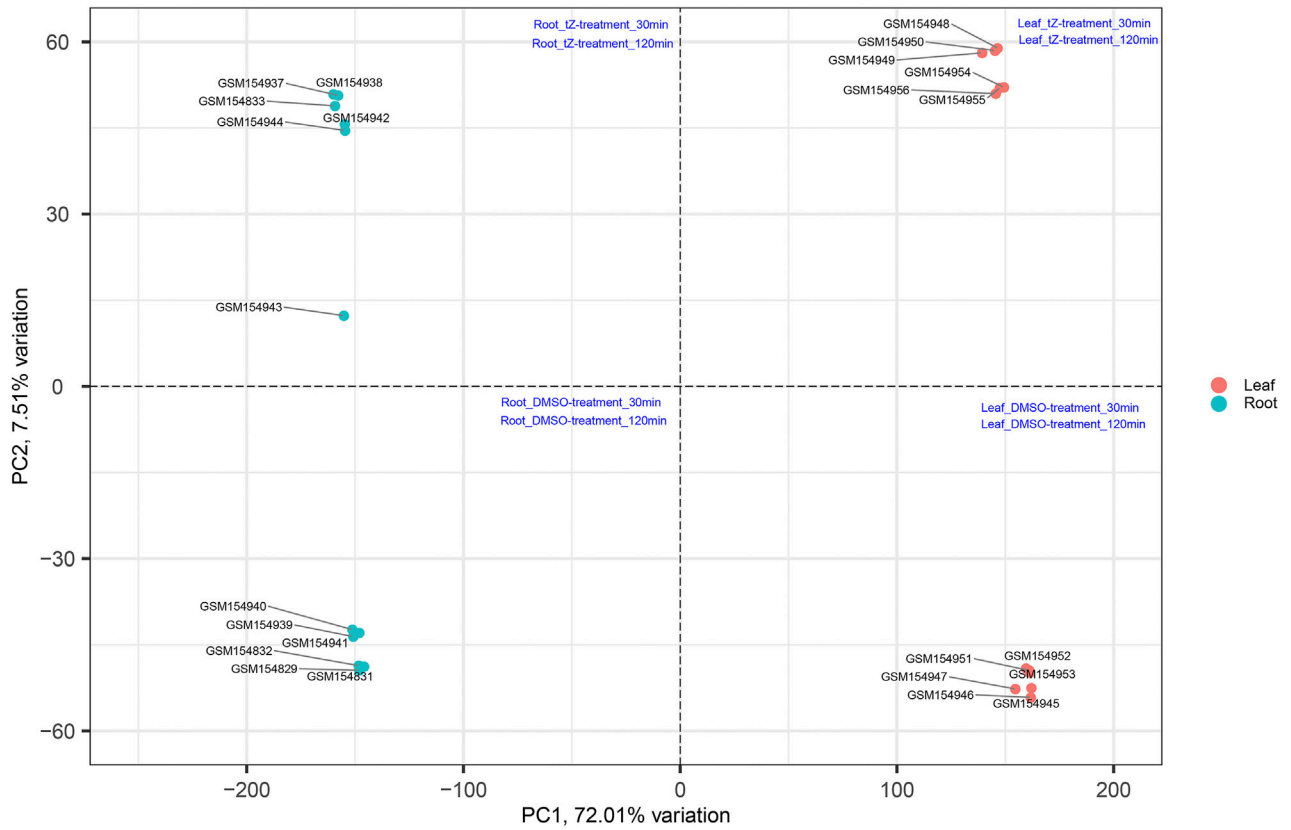

FIGURE 10 | Distribution of samples based on different stress at different time intervals and obtained uniform distribution of data in all four coordinates (Szklarczyk et al., 2017).

and conventional approaches can identify several hundred genes related to these stresses. However, by using conventional approaches, it becomes difficult to identify the few centrally important genes that play important roles in cellular functions to cope with these stresses. This problem can be solved by applying systems biology approaches (Arora et al., 2019). In this study, we have performed a comprehensive analysis on existing data retrieved from NCBI to understand the potential genes and mechanisms involved in such processes by first performing PCA to validate the distribution and uniformity of data evenly (**Figure 10**) and subsequently obtained significant modules associated with the biological functions regulating the growth and development of the plant. Moreover, hub genes in these modules were also identified that play an important role during cytokinin signaling and are crucial in plant growth and development.

Amongst the identified modules, the hub genes identified in these modules were observed to be involved in various processes. For example, in the turquoise module, the top hub gene (Os04g0295400) is located in chr4 and its important function is to encode Jasmonate-induced protein. Though little is known about this function in rice, Jasmonate-induced proteins are already reported for immunity and development in other plants (Wasternack and Hause, 2013; Campos et al., 2014). In-depth characterization of this gene is further required as this family of genes are reported to play important roles such as in the defense systems against lethal disease and bacterial blight (Yamada et al., 2012; Taniguchi et al., 2014) and it may also be involved in stress management as both these stresses (abiotic and biotic stress) are interlinked with each other (Cao et al., 2017). Similarly, in the blue module, we identified the Os07g0424400 hub gene in chr7 that played an important role in cellulose synthase A catalytic subunit 3 [UDP-forming] that governs a major mechanism of cell wall formation (Taylor et al., 2000) and ultimately helps in supporting the plant growth against abiotic stress. In the brown module, the top potential hub gene, Os01g0743600, is located on chr1 with the reported function of ATP-dependent protease La domain containing protein (Koodathingal et al., 2009) and it is one of the key components in providing protection against the harmful effects of unfolded proteins. It is activated by stress conditions in the endoplasmic reticulum (ER) and it supports plant defense as well as response to abiotic stresses (Bao and Howell, 2017).

The top potential key gene in the green module, Os07g0171300, is still not fully characterized, but annotation results suggest it has a key influence in the protein kinase-like domain superfamily which is believed to be a conserved protein domain mainly involved in most signaling and regulatory

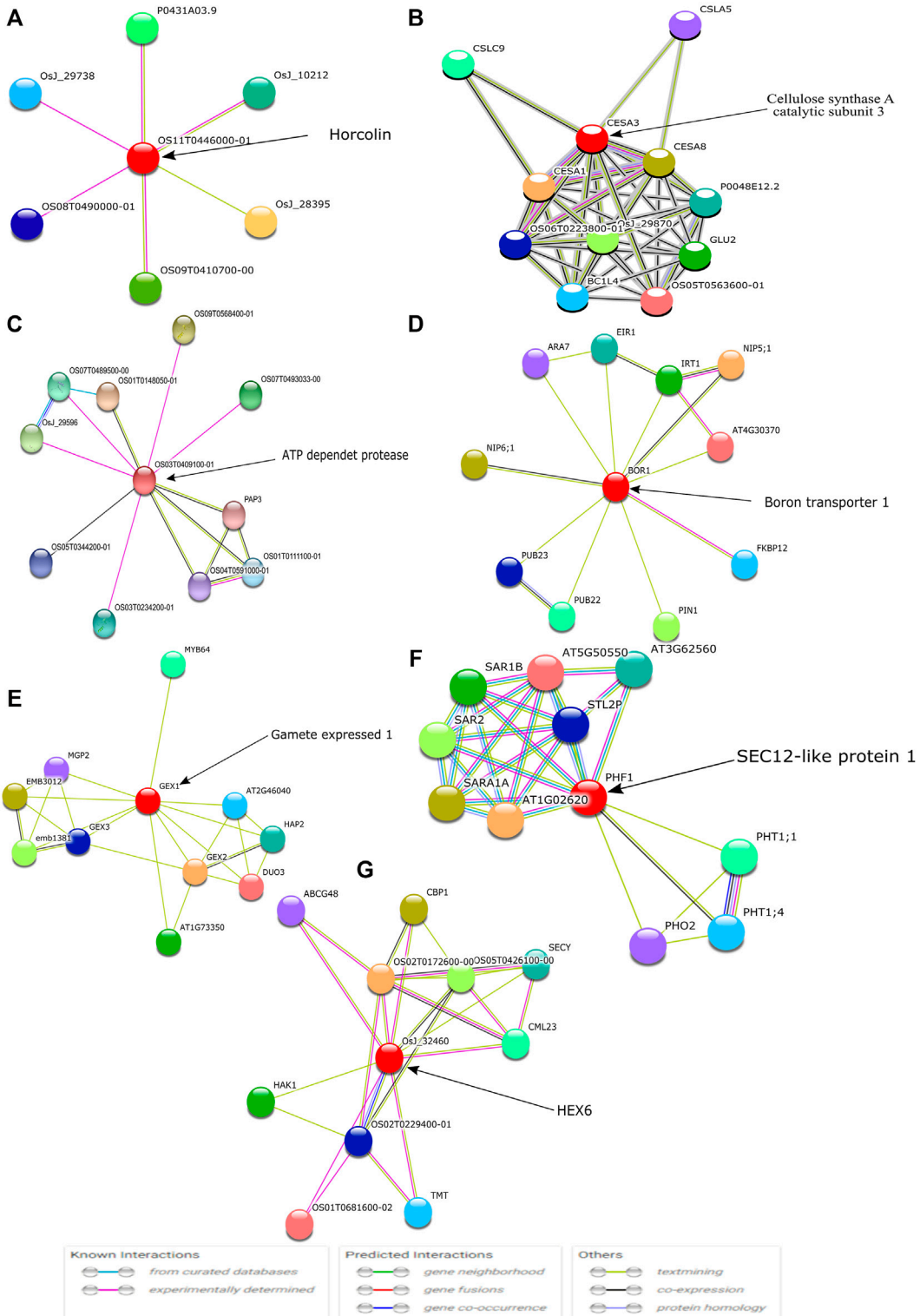

FIGURE 11 | The identified top hub genes are denoted with circles and are known as the first shell of interactors. Each color symbol signifies a specific interaction either known or predicted, as mentioned in previous studies.

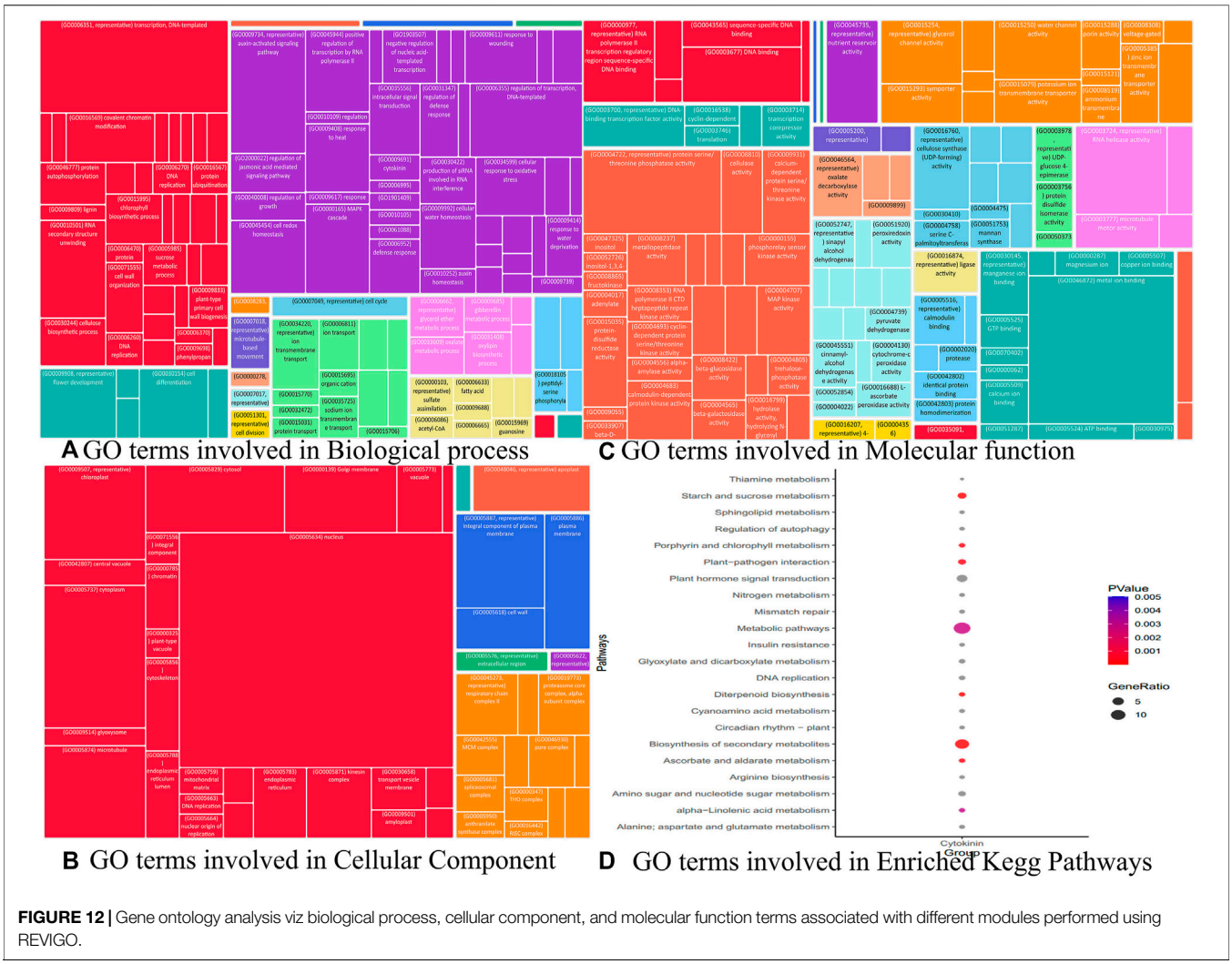

FIGURE 12 | Gene ontology analysis viz biological process, cellular component, and molecular function terms associated with different modules performed using REVIGO.

processes in the eukaryotic cell, and as a switch in controlling biological processes such as metabolism, transcription, cell moment, apoptosis, etc.

In the yellow module, the identified key gene Os12g0566000 codes for boron transporter 1, and is located at LOC4352546 of Chr12 and mapped with QTL identification number AK100510. Boron is essential for maintaining the integrity of plants cell walls. It exhibits an important structural role in shaping the cell by providing mechanical strength via cross-linking of cell wall rhamnogalacturonan II (RG-II) to form a stable three-dimensional pectic network which contributes to the mechanical properties of cell wall structure (Funakawa and Miwa, 2015). It is also reported that boron expression deficiency inhibits plant photosynthetic capacity (Zhao and Oosterhuis, 2002; Kastori et al., 2008) and directly impacts the total yield of the crop.

In the red module, the identified key gene Os09g0442400 codes for protein Gamete expressed 1, and is located at LOC4347181 of Chr9 and mapped with QTL identification number AK106970. Gamete expressed 1 protein is mainly responsible for fertilization. It has a dual function during gametophyte development and early embryogenesis and it is required for correct pollen maturation (Engel et al., 2005). In the pink module, the identified key gene Os07g0187700 codes for protein SEC12-like protein1, and is located at LOC4342601 of Chr7 and mapped with QTL identification number AK111777. Phosphate Transporter 1 (PHT1) is a plant-specific SEC12 gene that encodes phosphate transporter involved in phosphate uptake by facilitating the trafficking of PHT1-1/PHT1; 1 from the ER to the plasma membrane that enables the ER exit of a high-affinity phosphate transporter (González et al., 2005). The top key hub gene identified in the grey module, Os07g0131600, codes for HEX6 protein which is one of the hexose carrier proteins, and is located at LOC4342334 of Chr7 mapped with QTL identification number AK068296. HEX6 protein has an active uptake of hexose with an important role in glucose/hydrogen symport (Boles and Hollenberg, 1997). These different hub genes directly or indirectly govern the main function of the positive build-up of overall cell growth but there are leftover top key genes Os07g0171300 in the green module and Os11g0491400 in the black modules that need to be further characterized to understand their role at different stages of crop development.

Further, the identified key hub genes were visualized using the STRING database for protein-protein interaction (Szklarczyk et al., 2017). The STRING database helped in the identification of direct (physical) interactions and indirect (functional) interactions as long as the interactions were specific and biologically meaningful. Out of the 9 obtained modules, seven genes were found to be associated with neighboring proteins (**Figure 11**).

Similarly, we identified other important hub genes in each module (**Table 1** to **Table 9**) which are not yet fully explored with respect to cytokinin signaling to maintain the harmony of cell and rice growth mechanisms. Although we identified 36 key genes, we were interested in understanding the role of genes in various processes such as BP, CC, and MF to further delineate the role of these genes in playing different roles in the development of cytokinin-related responses. The Gene Ontology terms in these processes include transcription (GO:0006351), auxin-activated signaling pathway (GO:0009734), MAPK cascade (GO:0000165), and regulation of transcription in BP (GO:0006355) (**Figure 12A**), whereas the GO terms mostly constituted in CC include nucleus (GO:0005634), cytoplasm (GO:0005737), chloroplast (GO:0009507), and microtubule (GO:0005874) (**Figure 12B**) and composed of molecular functions metal ion binding (GO:0046872), protein serine/threonine phosphatase activity (GO:0004722), ATP-dependent RNA helicase activity (GO:0004004), and RNA polymerase II regulatory region sequence-specific DNA binding (GO:0000977) are some (**Figure 12C**). Likewise, the Kegg pathway also revealed the enrichment of GO terms such as plant hormone signal transduction (osa04075), starch and sucrose metabolism (osa00500), and diterpenoid biosynthesis (osa00904) (**Figure 12D**). In the absence of wet lab experiments, these identified hub genes were validated with the help of well-known QTLs and pathways using an *in-silico* approach. The analysis indicates the involvement of identified hub genes in these stress conditions as these genes are found to be associated with biotic and abiotic stress-related QTLs and pathways. These results indicate the role of identified hub genes in the regulation of plant growth and development. However, these hub genes need further attention at the molecular level through wet lab experiments to improve the traits which will be useful in enhancing the productivity of the crop.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DM, DA, NB, AS, SM, AK, and SS conducted the experiments. DA, NB, and SS analyzed the data, DM and DA drafted the manuscript. MF, SL, AR, PP, and KC proofread the manuscript. AR and KC revised the manuscript. All authors agreed to the final manuscript.

ACKNOWLEDGMENTS

Authors duly acknowledge the support for fund and manpower received from ICAR sponsored CABin scheme network project entitled "Agricultural Bioinformatics and Computational Biology" and also the computational support received from Advanced Supercomputing Hub for OMICS Knowledge in Agriculture (ASHOKA).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.780599/full#supplementary-material

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frontiers in Genetics

MINI REVIEW
published: 24 March 2022
doi: 10.3389/fgene.2022.877510

Cytokinin Oxygenase/Dehydrogenase Inhibitors: An Emerging Tool in Stress Biotechnology Employed for Crop Improvement

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In order to meet the global challenges of food security, one of the foremost solutions lies in enhancing the crop productivity. This can be attained by considering key plant hormones such as cytokinins as agrochemicals as cytokinins in particular are known to control the essential processes of the plants. Even though, it has already been established since 1980s that the enzyme, cytokinin oxidase/dehydrogenase (CKO/CKX) deactivates cytokinins; the potential applications of manipulating these enzymes have mostly been speculated to have a high potential in the biotechnology industry and spreads to agriculture, horticulture and agroforestry. The enzyme is critical in maintaining a balanced level of cytokinins in plants. However, it is yet to be fully established that inhibiting this enzyme can be the constant source of improvement in the productivity of plants, even though success has been obtained in some economically important plant species. Furthermore, the impact efficiency of this enzyme may vary from plant to plant, which needs to be evaluated employing tissue culture and other extrinsic applications. This review intends to cover the relevant studies addressing any biological activity of this enzyme in the current context and any associated biotechnological applications specific to enhanced grain yield, abiotic stress tolerance, delayed senescence and *in vitro* organogenesis among various plants and not only cereals. Moreover, our study will identify the present gaps in research with respect to many important food crops, which will be useful for researchers who are actively involved in providing a foundation for a variety of genetically improved plants achieved through this manner. In addition to this, other ways of engineering the amount of cytokinin levels appropriate for signaling also needs to be analyzed in order to extend the benefits of cytokinin biology to other crops too. The application of these inhibitors can be considered among the best alternates as well as addition to genetically modified plants for overcoming the gaps in crop demand.

Keywords: cytokinins, cytokinin oxygenase/dehydrogenase, inhibitors, stress tolerance, overexpression

OPEN ACCESS

Edited by:
Jyoti mathur,
Banasthali University, India

Reviewed by:
Md Mahmudul Hassan,
Patuakhali Science and Technology University, Bangladesh

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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *16 February 2022*
Accepted: *04 March 2022*
Published: *24 March 2022*

Citation:
Arora K and Sen S (2022) Cytokinin Oxygenase/Dehydrogenase Inhibitors: An Emerging Tool in Stress Biotechnology Employed for Crop Improvement. Front. Genet. 13:877510. doi: 10.3389/fgene.2022.877510

INTRODUCTION

The exponential rise in human population over the last few decades has forced many ultimate challenges at the basic level in terms of "food, feed, and bioenergy" (Gupta et al., 2021; Nisler et al., 2021), especially for the developing countries, such as India. Moreover, constant human interference has led to environmental imbalance causing poor crop yield. Along with this, various types of abiotic

stresses such as drought, salinity, etc., have marred the agricultural production (Aremu et al., 2015). All this has led to scarcity of agricultural land, leaving almost no scope for its expansion to keep pace with the burst to meet the population needs. In order to maintain a sustainable balance between the supply chain of food and demand, it has been strongly realized by the scientists that the solution to this problem lies in focusing on developing ways of enhancing crop productivity of the "existing" agricultural land (Nisler et al., 2021). There are many facets through which the crop yields can be improved; one of such aspect involves controlling the level of plant growth regulators (PGRs) in the crops. It is well known fact that amongst the common PGRs, cytokinins play an indispensable function in plant growth and morphogenesis (Pavlů et al., 2018; Hai et al., 2020). Extensive research on cytokinins have revealed that appropriate levels of cytokinin are necessary for cytokinin governed essential physiological and regulatory responses in different cell types (Gupta et al., 2021) through the "complex network" of cytokinin signaling (Li et al., 2019). These include, controlling the "cell division" involving the expansion, proliferation and development of foliage, branches, root as well as the reproductive organs through "photomorphogenic cell differentiation" (Chiang et al., 2012; Efroni et al., 2013; Bishopp, et al., 2011); non-initiation of lateral roots (Bielach et al., 2012), prolongs stomatal closure (Pospíšilová et al., 2005) and seed fill (Kieber and Schaller, 2014). It has been realized that most of these morphogenetic responses can be directed towards enhancing crop production. Therefore, cytokinins can be employed as "potential agrochemicals" (Koprna et al., 2016; Nisler et al., 2021) for inducing the physiological advantages that can be achieved through enhancing the levels of cytokinins in the plants. Moreover, it has been reported that the increase in cytokinin levels in a plant can enhance seed/crop yields (Bartrina et al., 2011; Jameson and Song, 2016), increase positivity in tillering, improve setting of flowers and seeds (Koprna et al., 2016), impede senescence of the leaf (Zwack and Rashotte, 2015) and mediate their stress tolerance especially in case of drought (Hai et al., 2020; Devireddy et al., 2021), salinity adaptation (Joshi et al., 2018; Li et al., 2019), etc. This review focuses on the current understanding of cytokinin biology in relation to crop improvement. It has been divided into four further sections, commencing with the ways through which the level of cytokinins can be enhanced in the plants, followed by the understanding of the types of the cytokinin inhibitors, their mode of action, then summarizing the various biotechnological responses, especially related to various forms of stress.

CYTOKININ AUGMENTATION IN PLANTS

The enhancement of cytokinins in the plants can be achieved through two possible ways, either by the addition of cytokinins that are natural or synthetic in nature or by restricting the cytokinin inhibitors. Strong natural cytokinins such as zeatin can only be applied to the plant as a "single dose at one time point", which typically gets diluted after some days (Nisler et al., 2021). The positive impact is visible, however, as a short-term effect rather than a long term one and causes variations that are unreproducible and are therefore, unacceptable from the commercial point of view (Koprna et al., 2016). In contrast, synthetic ones such as thidiazuron (TDZ), N-(2-Chloro-4-pyridyl)-N′-phenylurea (CPPU), etc., are ineffective in their signaling aspects and may induce undesirable side effects.

Besides these, another way to increase the cytokinin levels can be through inhibiting the action of cytokinin regulation. Physiologically within the plants, the levels of cytokinins are controlled through the balance of four enzymes; out of which isopentenyl transferase (IPT), which employs the mevalonate as well as methylerythritol phosphate pathway (Wang et al., 2014), is primarily responsible for the cytokinin metabolism in nature (Jameson and Song, 2016), while deactivation of cytokinin is the sole responsibility of the enzyme called cytokinin oxidase/dehydrogenase, CKO/CKX (Chatfield and Armstrong, 1986; Jiang et al., 2016). As the part of the mechanism of action, CKO/CKX enzyme irreversibly inactivates the cytokinins through the removal of N^6-isoprene side chain from the cytokinin molecules (Mok and Mok, 2001). It can also be suggested that the CKX enzyme, being a flavoprotein (Gupta et al., 2021), is also involved in the balance as well as regulation of cytokinins, thereby helps in maintaining cytokinin homeostasis (Thu et al., 2017; Hai et al., 2020). This regulatory function has mostly been reported from major cereals such as *Hordeum vulgare* (Zalewski et al., 2014), *Zea mays* (Brugière et al., 2003), *Oryza sativa* (Ashikari et al., 2005) and *Triticum aestivum* (Song et al., 2012; Zhang et al., 2012; Ogonowska et al., 2019). At the genetic level, the prevalence of *CKX* gene families in plants has varied from species to species (Nisler et al., 2021) with isoforms differing in "spatial and temporal expression patterns and subcellular localization" with some being localized in the apoplast, vacuoles and cytosols (Joshi et al., 2018; Nisler et al., 2021). The number of genes involved in cytokinin inhibition ranges from seven as found in *Arabidopsis thaliana* (Werner et al., 2003) and *Medicago sativa* (Li et al., 2019) to eight in *Fragaria vesca* (Jiang et al., 2016), eleven in *Oryza sativa* (Tsai et al., 2012) and *Triticum aestivum* (Chen et al., 2020), twelve in *Malus domestica* (Tan et al., 2018), thirteen in *Zea mays* (Morris et al., 1999) and 23 in *Brassica napus* (Liu et al., 2013). These genes can be targeted for production of genetically modified plants, which will induce the overexpression of CKX enzyme and can cause drastic changes in the "organ proportions" especially root morphology in barley plants as observed by Mrí;zová et al. (2013). The negative regulation of the cytokinins leads to enhanced crop yield and mediation towards tolerance of abiotic stresses as reported in rice (Yamburenko et al., 2017), *Arabidopsis* (Werner et al., 2003; Prerostova et al., 2018), barley (Pospíšilová et al., 2016; Holubová et al., 2018). Besides the up regulation of this gene, its down regulation or knocking off has also caused increased yield in rice due to the increase in the quantity of reproductive organs (Ashikari et al., 2005) even during salinity stress (Joshi et al., 2018). Apart from the traditional forms of genetic modification such as selective breeding and crossbreeding, genetic engineering and genome editing are some of the mechanisms through which

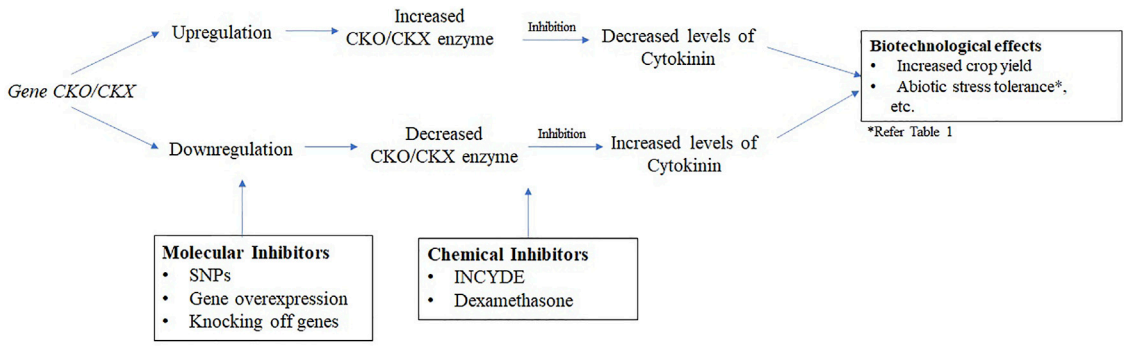

FIGURE 1 | Mechanism of *CKO/CKX* gene control.

gene manipulation can be done (US Food and Drug, 2022). It was reported that controlling this enzyme can lead to "tailor made" improvements in the productivity of plants (Ashikari et al., 2005). Moreover, newer techniques for genome editing such as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat) have been recently used for knocking out of *CKX/CKO genes* in barley (Holubová et al., 2018; Gasparis et al., 2019) and rice (Mao et al., 2020; Rong et al., 2021). However, none of the mechanism of action has not been fully understood till now (Joshi et al., 2018), even though success has been obtained in some economically important plant species such as apple (Liao et al., 2017), tobacco (Macková et al., 2013), etc. Furthermore, the effectivity of the impact of this enzyme may vary from plant to plant, which needs to be evaluated employing tissue culture and other extrinsic applications (Gupta et al., 2021).

CYTOKININ OXYGENASE/ DEHYDROGENASE INHIBITORS: TYPES AND MODE OF ACTION

The primary approaches to decrease the expression of CKX enzyme can either be through chemical means (Kopecný et al., 2010; Nisler et al., 2021) and molecular approaches (Gouda et al., 2020a; Nguyen et al., 2021). **Figure 1** represents a schematic diagram on the mechanism of *CKO/CKX* control. Nisler et al. (2021) points that inhibition of CKX enzyme by chemicals had been reported long time back which is predated even before the engineering of the genetically modified plants. These chemicals are classified as synthetic cytokinins such as TDZ and its variants (Nisler et al., 2016, Nisler, 2018), diphenyl urea (DPU), chloropyridin phenyl urea (CPPU), N-(2-amino-pyridin-4-yl)-N′-phenylurea (APPU) (Kopecný et al., 2010) or new potent inhibitors derived from CPPU, DPU, and DCPU (Nisler et al., 2021). The findings from Nisler et al. (2016) showed a 15-times decrease in half-maximal inhibitory concentration (IC50) with TDZ for *AtCKX2* in *Arabidopsis* and *ZmCKX1* and *ZmCKX4a* in *Zea mays*. Along with this, derivatives of 2-X-6-anilinopurine along with 2-chloro-6-(3-methoxy- phenyl) aminopurine (INCYDE) have also been found to be effective inhibitors of CKX enzyme in *Arabidopsis* (Zatloukal et al., 2008; Prerostova et al., 2020) and tomato (Aremu et al., 2014), respectively. The antioxidant defense mechanism and efficiency of photosynthesis got elevated by the use of these potent compounds (Aremu et al., 2014). The potency of inhibition was found to be higher in the variant of DPU in comparison to DCPPU and the inhibition occurred at the concentration of 10^{-8} M (Nisler et al., 2021). Similarly, APPU was found to be a better inhibitor as compared to CPPU, TDZ and their derivatives (Kopecný et al., 2010). Moreover, the chemical use of CKX enzyme inhibitors was found to be more advantageous than the application of cytokinin exogenously as a moderate level but "long-term" enhancement in the endogenous levels of cytokinins was observed. Among the molecular approaches, heterogenous nuclear RNA (hRNA-CX3 and -CX5) were used to suppress expression of CKX enzyme in rice (Yeh et al., 2015). An increase in growth, chlorophyll content and grain yield were observed in this case. Recently, one of the molecular approaches applied specific missense single nucleotide polymorphisms (SNPs), namely SNP42, SNP43, SNP44, and SNP46 to reduce the expression of CKX enzyme in rice that led to increase in grain numbers (Gouda et al., 2020a), while another nine SNPs from five genes were demarcated in soybean for enhanced seed yield (Nguyen et al., 2021). In a new approach, computational means has also been followed to study the "structure, function and interaction" of the CKX enzyme from rice plants for the first time (Gouda et al., 2020b). A hypothetical 3-D structure of this enzyme was predicted, which showed the presence of 24 α helix and 13 β strands. This can be extremely useful in understanding the cause of enhanced yield in these plants.

BIOTECHNOLOGICAL RESPONSES

The decrease in CKX enzyme using various form of inhibitors has manifested a series part of the biotechnological application response or effects. **Table 1** summarizes the various studies conducted on the understanding the influence of CKX enzyme inhibitors over abiotic stress tolerance. One of the most common manifestations observed in the genetically modified plant includes the reduction of abiotic stresses and adaptations to drought in *Arabidopsis* (Prerostova et al., 2018), barley

TABLE 1 | Biotechnological responses of plants targeted with CKX inhibitors through chemical and molecular approaches.

Sl	Plant name	Chemical/Molecular approaches	CKO/CKX family member or gene targeted	Biotechnological applications/ response/ effects	Reference
1	Soybean	Molecular- SNPs	*GmCKX GFMs,*	Increased yield and proposed abiotic stress resistance	Nguyen et al. (2021)
2	Maize, *Arabidopsis*, Spring barley, Winter wheat, winter oilseed rape	Chemical- new inhibitors derived from DPU	*AtCKX 2, Zm CKX1, ZMCKX4q* and *ZmCKX8*	Stress resistance and increased seed yield in *Arabidopsis*	Nisler et al. (2021)
3	*Arabidopsis*	Chemical- INCYDE	Not mentioned	Heat tolerance	Prerostova et al. (2020)
4	*Arabidopsis*	Molecular- Overexpression of genes in the genetically modified plant	Introduced *MsCKX from Alfalfa*	Salt tolerance	Li et al. (2019)
5	*Arabidopsis*	Chemical- Dexamethasone	*AtCKX1*	Drought tolerance	Prerostova et al. (2018)
6	Barley	Molecular- Overexpression of genes in genetically modified plant	Introduced *AtCKX1*	Drought tolerance	Ramireddy et al. (2018)
7	Rice	Molecular – Knocking off in genetically modified plant	*OsCK2*	Yield increase and salinity tolerance	Joshi et al. (2018)
8	Apple	Molecular- Overexpression of genes in genetically modified plant	*MdCKX4a*	Drought tolerance	Liao et al. (2017)
9	Tomato	Chemical: 2-chloro-6-(3-methoxy-phenyl) aminopurine (INCYDE)	Not mentioned	Salt tolerance, vegetative and reproductive growth	Aremu et al. (2014)
10	Medicinal plants- *Bulbinea* and Curly dock	Chemical- INCYDE	Not mentioned	Adaptation towards cadmium stress	Gemrotová et al. (2013)
11	Tobacco	Molecular- Genetically modified plant	Introduced *AtCKX1*	Drought and heat tolerance	Macková et al. (2013)

(Pospíšilová et al., 2016; Ramireddy et al., 2018), tobacco (Werner et al., 2010; Macková et al., 2013; Lubovská et al., 2014) and apple (Liao et al., 2017); heat tolerance in *Arabidopsis* (Prerostova et al., 2020); cold as well as salinity tolerance in tomato (Aremu et al., 2014), in alfalfa (Li et al., 2019), in *Arabidopsis* (Nisler et al., 2021), etc. Moreover, tolerance towards stresses from heavy metals such as cadmium also can be observed as a result (Gemrotová et al., 2013). Most importantly, there is an increase of antioxidant enzymes (Devireddy et al., 2021). Other outcomes include inducing shoot regeneration, roots and morphogenesis in Chinese water chestnut (Wang et al., 2015), *in vitro* responses such as organogenesis (Aremu et al., 2015; Werbrouck, 2016; Chen and Wei, 2018; Mazri et al., 2018), callus culture bioassays (Kopecný et al., 2010), delayed senescence (Nisler et al., 2016; Prernostava et al., 2018) and as basic as increasing yield of the cereal crops (Ashikari et al., 2005; Nisler et al., 2021). Moreover, 44% increased zinc levels were present in the seeds of the transgenic barley plant along with drought tolerance. It was construed that the overexpression of CKX enzyme made the plant more nutrient efficient (Ramireddy et al., 2018). In contrast, Gasparis et al. (2019) reported that knocking out the *CKX* genes may not enhance the grain yield in barley.

CONCLUSION, PERSPECTIVES AND FUTURE SCOPE OF RESEARCH

The application of inhibitors of CKX enzyme as a successful and capable tool for tolerance of abiotic stresses is evident from this study, which has a great potential for crop improvement in a variety of crops, including cereals. The present study reviews relevant research pertaining to the biological activity of the CKX enzyme in the context of adapting towards abiotic stresses along with improved grain yield. This can also be extended as the source of providing benefits to various crops through cytokinin biology. Other biotechnological responses of this enzyme also include delayed senescence and inducing organogenesis through tissue culture. In addition to this, other ways of manipulating the level of cytokinin suitable for signaling was also explained and the present gaps in this research area has been identified from this study.

The comparison of CKX enzyme inhibitors reveals that the use of chemicals is more popular over the molecular approaches. Therefore, it is anticipated that these chemicals can work as an alternate to genetically modified crops (Nisler et al., 2021). This will be extremely advantageous for mankind as any legal hassles towards acceptance of genetically modified organisms (GMOs) can be easily avoided using this approach, implying a wider reach among many varieties of plants across countries. However, an appropriate dosage level as well as the "cost effectiveness" of these chemicals is yet to be assessed at a commercial level, thereby warranting immediate attention from the researchers in this field. Recent studies demonstrate the emergence of successful genetic approaches (Wang et al., 2020; Nguyen et al., 2021; Nisler et al., 2021; and many others), emphasizing that modulating CKX enzymes can open up multiple paths for developing "tailor made" stress resistant and nutrition rich crops which will be useful in the long-term breeding programs (Ramireddy et al., 2018). These will be developed as a means of sustainable agriculture through unravelling the signaling network of the cytokinins (Pavlů et al., 2018). From this review, it was also realized that both up- and

downregulation of the *CKX* gene can be instrumental in improving the economic needs, even though it seems to vary from plant to plant and even within a plant species. This ambiguity opens up a wide scope for further molecular research. In future, CKX inhibitors can be treated as part of plant defense regulators and studies can focus on comprehending the molecular mechanism of the interaction of CKX enzyme with other plant defense regulators such as jasmonic acid, salicylic acid, ethylene, abscisic acid (ABA) and others in order to develop a better understanding towards abiotic stresses.

AUTHOR CONTRIBUTIONS

KA and SS were equally involved in the conception of the review, literature search, drafting the article and critical revision of the manuscript.

ACKNOWLEDGMENTS

We would like to thank the entire Frontiers editorial team for providing us the opportunity to write this review.

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frontiers | Frontiers in Genetics

ORIGINAL RESEARCH
published: 05 April 2022
doi: 10.3389/fgene.2022.859676

SNP Discovery Using BSR-Seq Approach for Spot Blotch Resistance in Wheat (*Triticum aestivum* L.), an Essential Crop for Food Security

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: 21 January 2022
Accepted: 17 February 2022
Published: 05 April 2022

Citation:
Saxesena RR, Mishra VK, Chand R, Kumar U, Chowdhury AK, Bhati J, Budhlakoti N and Joshi AK (2022) SNP Discovery Using BSR-Seq Approach for Spot Blotch Resistance in Wheat (Triticum aestivum L.), an Essential Crop for Food Security. Front. Genet. 13:859676. doi: 10.3389/fgene.2022.859676

The pathogenic fungus, *Bipolaris sorokiniana*, that causes spot blotch (SB) disease of wheat, is a major production constraint in the Eastern Gangetic Plains of South Asia and other warm, humid regions of the world. A recombinant inbred line population was developed and phenotyped at three SB-prone locations in India. The single nucleotide polymorphism (SNP) for SB resistance was identified using a bulked segregant RNA-Seq-based approach, referred to as "BSR-Seq." Transcriptome sequencing of the resistant parent (YS#24), the susceptible parent (YS#58), and their resistant and susceptible bulks yielded a total of 429.67 million raw reads. The bulk frequency ratio (BFR) of SNPs between the resistant and susceptible bulks was estimated, and selection of SNPs linked to resistance was done using sixfold enrichments in the corresponding bulks (BFR >6). With additional filtering criteria, the number of transcripts was further reduced to 506 with 1055 putative polymorphic SNPs distributed on 21 chromosomes of wheat. Based on SNP enrichment on chromosomal loci, five transcripts were found to be associated with SB resistance. Among the five SB resistance-associated transcripts, four were distributed on the 5B chromosome with putative 52 SNPs, whereas one transcript with eight SNPs was present on chromosome 3B. The SNPs linked to the trait were exposed to a tetra-primer ARMS-PCR assay, and an SNP-based allele-specific marker was identified for SB resistance. The *in silico* study of these five transcripts showed homology with pathogenesis-related genes; the metabolic pathway also exhibits similar results, suggesting their role in the plant defense mechanism.

Keywords: wheat, food security, spot blotch, RILs, bulk segregant analysis, SNPs

INTRODUCTION

As a primary staple crop, the importance of wheat is well-documented in food security and provides nutrition for more than 35% of the world's population (FAO, 2018). Great progress has been achieved in wheat production since the Green Revolution; however, due to climate change and the popularization of dwarf and semidwarf varieties, many of the biotic factors, including the pathogen

of spot blotch (SB) disease have gained importance in countries such as India (Joshi et al., 2007a). The prevalence of SB is more common in the wheat-producing countries, notably in the Eastern Gangetic Plains of India, Nepal, Bangladesh, China, and South America (Joshi et al., 2007b; Gupta et al., 2018; Singh et al., 2018). A hemi-biotrophic fungus, *Bipolaris sorokiniana* (Sacc.), causes SB disease in wheat, seedling blight, common root rot, seedling rot, and seed rot (Acharya et al., 2011). Crop yield losses in the Indian subcontinent alone are estimated to be in the range of 15%–25% (Dubin and Van Ginkel, 1991; Pandey et al., 2021), but the level of loss in individual fields can be much higher. In South Asia, the disease is expected to inflict a 15%–20% average yield loss (Duveiller and Sharma, 2009); however, under favorable conditions, more than 85% of losses are reported in Zambia during the summer season (Raemaekers, 1987). Thus, SB disease might have a significant impact on global food security. Seed treatments and foliar fungicidal sprays are both recommended for the treatment of SB disease. Although strong-efficacy fungicides are available to manage SB disease, their application may have adverse effects on human health and the environment (Monyo et al., 2009; Castro et al., 2018). Other than health and environmental constraints, the use of fungicides led to an increase in the cost of cultivation and a reduction in farmers' income. Therefore, the most successful, cost-efficient, and environmentally friendly strategy to control the disease is to use cultivars with host resistance (Gupta et al., 2018; Zhang et al., 2020). However, breeding for SB resistance has been slow due to the quantitative nature of inheritance (Joshi et al., 2004) and is often influenced by the environment (Joshi et al., 2007b). In such a situation, genetics, and genomics-based technology aid in developing resistant plants more efficiently. Classic methods of mapping QTLs/genes, involve phenotyping and genotyping of segregating mapping populations with polymorphic markers identified between parents. However, the identification of polymorphic markers between contrasting parents is a time-consuming and tedious task (Schneeberger and Weigel, 2011; Jaganathan et al., 2020). So far, SSRs, or microsatellite markers, are used most widely to map wheat genomes for SB resistance (Singh and Singh, 2015). Presently, several QTLs and four genes (*Sb1-Sb4*) have been identified as having major effects on SB resistance (Gupta et al., 2018). Gene *Sb1* is present on chromosome 7DS, where it shares space with *Lr34* (Lillemo et al., 2013), *Sb2* on chromosomes 5BL (Kumar et al., 2015), *Sb3* on 3BS (Lu et al., 2016), and a recently discovered *Sb4* are present on 4BL (Zhang et al., 2020).

The identification of genetic regions and the development of robust molecular markers in wheat has long been hampered by its hexaploidy nature (AABBDD), which has the large sizes of the subgenomes and more than 85% repeated sequences (Paux et al., 2012; Wicker et al., 2018). It is difficult to design single-copy markers because the level of polymorphism is quite low in wheat compared with other cereals (Paux et al., 2012). Liu et al. (2012) propose a new genetic mapping strategy called "BSR-Seq," which combines bulked segregant analysis with RNA-Seq. BSR-Seq is a technique that involves sequencing RNAs from extreme bulks for the trait of interest. The method is especially important for crops with large and complex genomes, such as wheat, where resequencing is still prohibitively expensive (Liu et al., 2012; Xie et al., 2020). BSR-Seq can also be used to fine-map crops that do not yet have a reference genome sequence (O'Neil and Emrich, 2013). RNA-Seq captures the full range of dynamic spectrum of the transcriptome, advantageous over array platforms that are restricted to the predefined set of variants incorporated into the array design. SNPs can be identified either by aligning to a known transcriptome or by *de novo* assembly over the transcriptome (Grabherr et al., 2011; O'Neil and Emrich, 2013). RNA-Seq is more likely to discover functional SNPs than other SNP discovery methods (Pootakham et al., 2014). Genotyping by RNA-Seq can detect much more variation compared with array-based technology because it covers 70%–90% of the total genes based on the tissue and development stage of the sample. For the development of constitutive markers, the combination of advanced sequencing technology with BSA provides a powerful tool for the rapid identification of genes or causal mutations (Xu and Bai, 2015; Zou et al., 2016).

BSR-Seq has been applied successfully to localize the candidate gene for grain protein content (GPC) gene *GPC-B1* in wheat to 0.4 cM from 30 cM (Trick et al., 2012). The glossy 3 *(gl3)* gene of maize was allocated to a ~2 Mb area by BSR-Seq, and a single gene, MYB transcription factor, was found (Liu et al., 2012). Ramirez-Gonzalez et al. (2015) identified putative single nucleotide polymorphisms (SNPs) for the *Yr15* locus using BSR-Seq and mapped this gene to a 0.77-cM interval that imparts resistance to yellow rust in wheat. Pearce et al. (2016) used RNA-Seq to analyze and compare the transcriptomes of phyB-null and phyC-null TILLING mutants and identified 82 genes that are significantly upregulated or downregulated in both types of mutants. In a more recent study on BSR-Seq (Klein et al., 2018), cloned mutant genes in maize were involved in plant growth by delineating mapping intervals. Compared with the entire population analysis, BSA provides a shortcut to identifying and developing markers for a trait. The substantial reduction in the cost of sequencing, particularly with the introduction of BSR-Seq, may be accomplished by genotyping several bulks from a large-sized population, and the power of detection can be significantly improved, particularly for alleles of interest or rare alleles (Hiebert et al., 2014; Zou et al., 2016). As a result, BSR-Seq is widely used for the quick finding of genes and markers associated with the target gene.

To meet future food demands, climatic resilience and disease-resistant wheat combined with good agronomic value can potentially improve its productivity (Mondal et al., 2016). To date, SB resistance of wheat is quantitative, involving genes and many QTLs with low-coverage linkage maps. To reduce the loss of wheat productivity and grain quality caused by SB, new resistance genes must be identified. Here, the present investigation was initiated with an objective to identify putative SNPs for SB resistance by the "BSR-Seq" approach.

MATERIALS AND METHODS

Plant Materials

A total of 211 single seed descent (SSD)-derived recombinant inbred lines (RILs) were generated from the cross YS#24 × YS#58.

The parental lines YS#24 and YS#58 are stable RILs selected from the Yangmai6 × Sonalika cross and advanced to the F_{12} generation. Yangmai6 is a Chinese source of SB resistance, and Sonalika is a susceptible cultivar of Mexican origin that has been under cultivation in India for more than five decades/ during the green revolution. The parents of the RILs used in this study are similar with respect to the agronomical and phenological traits but harbor different SB resistance QTLs (Kumar et al., 2009). To develop RILs, field trials in the crop season were conducted on the Agricultural Research Farm, BHU, Varanasi, whereas off-season nurseries were raised for generation advancement at Wellington, Tamil Nadu, India. The whole procedure of RIL development and its evaluation is mentioned in the flow chart (**Supplementary Figure S1**).

Phenotypic Evaluation of RILs for SB Resistance

The 211 RILs (F_5 and F_6) were evaluated at three hot spots in India, namely, Agricultural Research Farm, BHU, Varanasi (25°18′N, 83°03′E); Borlaug Institute for South Asia, Samastipur, Pusa (25°57′N, 85°40′E), Bihar; and Uttar Banga Krishi Vishwavidyalaya, Coochbehar (26°19′N, 89°27′E), West Bengal, during two consecutive crop seasons 2013–14(F_5) and 2014–15(F_6). The F_7 generation was evaluated only at BHU in crop season 2015–16. At each location, all RILs were planted along with their parents (YS#24 and YS#58) in two replications following a randomized complete block design (RCBD). Each line was sown in two rows of 2 m, keeping row-to-row and plant-to-plant distances of 20 and 5 cm, respectively. To ensure disease build-up and spread, two rows of the susceptible genotype Sonalika was planted after every 20th row and in alleys along with the plots. The susceptible spreader rows served as an inoculum source for epidemic development in addition to the direct inoculation on the RILs (Saxesena et al., 2017). Planting at all sites was done between the 1st and 10th of December each year to coincide with the post-anthesis with higher temperatures, conducive to disease development (Chaurasia et al., 2000). The experimental plots were fertilized at 120 kg N_2, 60 kg P_2O_5, and 40 kg K_2O per hectare. A complete dose of K_2O and P_2O_5 was given at the time of sowing. The N_2 was given in three splits of 60, 30, and 30 kg per ha at sowing, 21 days after sowing (at first irrigation), and 45 days after sowing (at second irrigation), respectively. A total of five irrigations were given to maintain sufficient moisture in the field.

Inoculation of the Pathogen, Disease Assessment, and Estimation of Area Under the Disease Progress Curve (AUDPC)

The highly aggressive *B. sorokiniana* isolate HDBHU (NABM MAT1; NCBIJN128877) was used to create an artificial epiphytotic. The pathogen was multiplied on sorghum grains, suspended in water (10^4 spores per ml), and sprayed in the evening at the time of flag leaf emergence as described earlier (Joshi and Chand, 2002). Disease severity (DS) was scored using a double-digit scale, first at the beginning of anthesis (GS 63) and then at the end of anthesis (GS 69) and third at the late milk stage (GS 77) (Saari and Prescott, 1975). The percentage DS was calculated as D1/9 × D2/9 × 100, where D1 is the first digit referring to the vertical progress of disease and D2 is the second digit indicating the extent of leaf area affected. The AUDPC based on DS recorded at three growth stages was calculated following Shaner and Finney (1977).

$$\text{AUDPC} = \sum_{i=1}^{n-1}\left[\{(Y_i + Y_{i+1})/2\} \times (t_{(i+1)} - t_i)\right],$$

where Yi = disease level at time t_i, $t_{(i+1)}-t_i$ = time (days) between two disease scores, n = number of dates when disease was recorded.

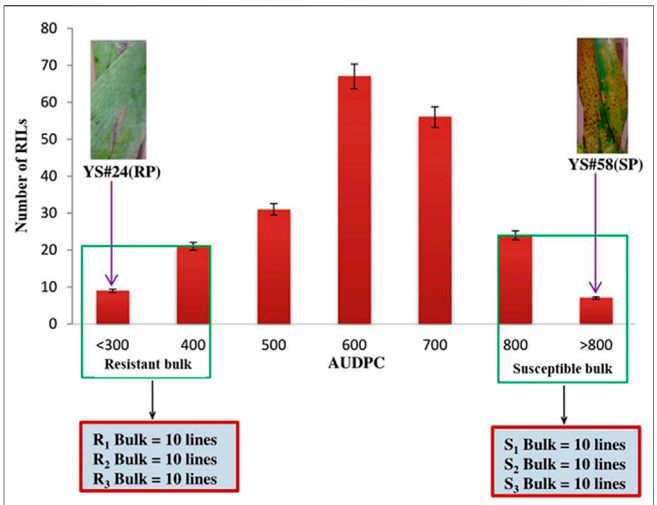

FIGURE 1 | Frequency distribution of the AUDPC of the wheat RIL population and parents. Resistant parent (RP), YS#24, and susceptible parent (SP), YS#58 clearly show the phenotypic differences for SB disease severity. Resistant bulk (R1-bulk, R2-bulk, and R3-bulk) with lower AUDPC and susceptible (S1-bulk, S2-bulk, and S3-bulk) with higher AUDPC were selected from each tail.

Grouping of RILs and Construction of Bulk Samples

To increase the statistical power and reduce false positives, multiple bulks were selected independently from each of the three locations in both years (Zou et al., 2016). AUDPC was analyzed from the pooled data of disease severity in all the screened environments, and three resistant bulks with lower AUDPC (R-bulk1, R-bulk2, and R-bulk3) and three susceptible bulks with higher AUDPC (S-bulk1, S-bulk2, and S-bulk3) were prepared (**Figure 1**). Each group bulked for resistance and susceptibility is composed of 10 RILs.

RNA Extraction and Transcriptome Sequencing

Seeds of each RIL of the resistant and susceptible bulks (30R + 30S RILs) and their parents (YS#24 and YS#58) were grown

separately in a greenhouse; seedling leaves were harvested 14 days after sowing for RNA isolation. Total RNA was extracted from the leaf tissues of each RIL using a standard TRIzol method (Catalog # 15596018) and treated with DNase I to remove residual DNA contaminants. RNA samples were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the quantitative and qualitative estimation was performed using the NanoDrop 1000 (Thermo Fisher Scientific) and the Agilent Bioanalyzer 2100, respectively. An equimolar concentration of RNA from 10 resistant individuals of RILs was pooled together to make resistant bulk 1, and the same was done to prepare the other resistant and susceptible bulks. Before cDNA library preparation, we enriched the RNA samples for transcripts using the absolute mRNA Purification Kit (Agilent Technologies, Santa Clara, CA, United States). The cDNA libraries were constructed, and Illumina paired-end adapters and barcode sequences were ligated onto the cDNA fragments (Pootakham et al., 2014). The pooled libraries were sequenced at QTLomics Technologies (Bengaluru, India) using the Illumina Next Seq 500 platform (Illumina, San Diego, CA, United States) to generate 76 bp paired end (PE) sequence reads for parents and bulked samples (resistance and susceptible) in three biological replicates.

Sequence Analysis; SNP Calling and Bulk Frequency Ratio Calculation

The data from Illumina Next Seq 500 was passed through Fast QC to check the quality of the reads (Babraham Bioinformatics - FastQC A, 2012). The ends of the reads with low quality, adaptor contamination, and low-quality regions were trimmed using the fastx-tool kit (http://hannonlab.cshl.edu/fastx_toolkit). Identification of SNPs was done; using a reference-based approach, the UniGene build63 was used as reference transcriptome (NCBI:ftp://ftp.ncbi.nhi.gov/repository/UniGene/Triticum_aestivum/Ta.seq.uniq.gz). Since UniGene represents only the genic portion, the complexity of the wheat genome was further reduced (Sidhu et al., 2015). The reads were aligned to transcript sequence build 63 using the Burrows–Wheeler Aligner (BWA) (Li and Durbin, 2009) using default parameters for PE libraries. Misaligned reads were removed from the unigene alignment and the unigenes with a high bulk frequency ratio (BFR) shortlisted. The allele frequency ratio of the resistant allele (allele of the resistant parent) in the resistant bulk compared with the resistant allele frequency in the susceptible bulk is known as the BFR. However, the genome locations of the SNPs were derived by alignment of shortlisted unigenes to the reference genome. We ran BLAST alignment and took the best hit throughout the transcript rather than the short read. The whole transcript alignment would give the best alignment to their respective genomes rather than the short read. The alignments were converted to one binary alignment/map (BAM) file per sample. On average, 80% of the RNA-Seq reads from each sample were able to align to the reference transcriptome (Pootakham et al., 2014). The SNPs for each sample were collected using the SAMtool sv0.1.18 as described by Li et al. (2009).

To identify SNPs linked to SB resistance, polymorphic markers between the parents were identified using a custom Perl script followed by BFR calculation for the bulks following Trick et al. (2012). The algorithm was implemented in Perl and R software. BFR values were calculated independently for the comparisons of three bulks (Bulk 1:S-bulk1, R-bulk1; Bulk 2: S-bulk2, R-bulk2; Bulk 3: S-bulk3, R-bulk3). BFR values and depth of each SNP for all the bulks and parents were calculated in the R program. Potential SNPs linked to the trait were selected based on SNPs with a BFR ratio of six as the minimum threshold in all three bulk replicates. The unigene that contain putative SNPs were aligned to a repeat masked reference genome (transcriptome) of wheat using BLASTN, and the best hit for each transcript was recorded. The BLAST result was segregated according to the chromosome number, and the chromosomal regions based on SNP density (twice or greater than the average SNP density) were further shortlisted. To identify SNPs that were enriched for the corresponding parental allele, BFR was calculated in the appropriate bulk, YS #24 derived SNPs for the resistant bulks, and YS#58 derived SNPs for the susceptible. Finally, to classify and prioritize the SNPs across each bulk, the frequency of the allele (SNP index) calculated at each SNP position (Takagi et al., 2013) was estimated, and then the ratio between the bulks (BFR) for each SNP was determined (Trick et al., 2012). Thus, a high BFR in the resistant parent (YS#24) derived SNP was indicative of an allele that is very frequent in the resistant bulk while depleted in the susceptible. In addition, a threshold of BFR >6 was set to select putative SNPs for the presence or absence of polymorphism between bulks for further validation as done previously in wheat (Ramirez-Gonzalez et al., 2015).

SNP-Based Primer Designing

The tetra-primer amplification refractory mutation system-PCR (ARMS-PCR) is a simple and economical technique that produces an allele-specific reaction (Ye et al., 2001; Ruiz-Sanz et al., 2007) The principle behind this technique is that the allele-specific primer amplifies a region specific to the base present at the 3′terminus, thus making it allele-specific (Newton et al., 1989; Ye et al., 2001). The genomic region flanking 300 bp on both ends of putative SNPs was extracted and formatted using the Perl script, and the SNPs with the highest score were selected to design primers. The batch primer 3 web tools (http://probes.pw.usda.gov/batchprimer3/) were finally used to create the tetra-primer ARMS-PCR (You et al., 2008).

DNA Isolation and PCR Conditions for Allele-Specific Primer

The genomic DNA of RILs used for BSR-Seq was extracted from the leaves of young seedlings using a cetyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof et al., 1984). The DNA pellet was vacuum dried, dissolved in DNase-free water, and stored at −20°C. A target-specific tetra-primer ARMS PCR amplification of all the resistant and susceptible bulks along with the parents was performed. The PCR was performed in a total volume of 10 μl reaction mixture, containing 30 ng DNA, 1X

TABLE 1 | Disease response to SB of parents and RILs measured in different environments.

Env.	AUDPC					
	YS#24	YS#58	RILs			
	Mean ± SD	Mean ± SD	Range	Mean ± SD	h^2	CV
BHU14	288.90 ± 53.25	796.50 ± 81.52	142.20–906.80	583.30 ± 141.12	0.87	8.80
BHU15	401.50 ± 96.46	1062.30 ± 61.10	235.80–1016.00	701.00 ± 174.00	0.81	11.41
BHU16	360.90 ± 18.75	896.70 ± 32.89	145.20–925.40	610.00 ± 172.00	0.83	11.02
BISA14	229.80 ± 8.43	545.00 ± 39.84	219.00–588.20	424.00 ± 77.18	0.65	11.97
BISA15	330.50 ± 9.16	680.60 ± 21.38	248.40–846.90	546.39 ± 127.40	0.83	10.05
UBKV14	321.40 ± 18.58	886.00 ± 18.43	161.90–928.80	469.80 ± 173.59	0.81	16.92
UBKV15	431.80 ± 39.72	966.30 ± 39.73	192.30–1085.80	546.50 ± 178.21	0.77	16.55
Over Env	336.29 ± 68.07	820.55 ± 183.28	231.70–836.84	554.00 ± 183.60	0.92	13.61

Env, environment; AUDPC, area under disease progress curve; h^2, broad sense heritability; SD, standard deviation; CV, coefficient of variation.

PCR buffer [75 mM Tris-HCl (pH9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$], 0.33 pmol of each outer primer (forward and reverse), 0.5 pmol of inner forward primer, 0.83 pmol of inner reverse primer, 2.5 mM $MgCl_2$, 0.125 mM of each dNTPs, 1U of Taq Polymerase (3B DNA polymerase, 3B Black Bio Biotech India Ltd.). PCR reactions were performed in an Agilent SureCycler 8800 using the following program: initial denaturation at 95°C for 5 min, 38 cycles consisting of denaturation at 95°C for 1 min, annealing at 62°C for 90 s, extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR product was resolved on 3.5% agarose gel and stained with ethidium bromide to visualize.

Functional Identification and Annotation of Transcripts

To perform the functional analysis of the transcripts, Blast2GO v 2.5 was used (Conesa et al., 2005). It is a Gene Ontology–based annotation tool and was found effective in the functional characterization of sequence data (Conesa and Götz, 2008). For functional characterization of the transcripts, we performed NCBI-BLASTX analysis using transcripts homologous to annotated proteins in the nr database with the criterion of E-value (threshold of 1e-03) and alignment size (threshold length 33). Furthermore, the transcript sequences were categorized for gene ontology (i.e., GO terms) into three groups: molecular function, biological process, and cellular component. The pathways for the selected transcripts were also delineated using the Kyoto encyclopaedia of genes and genomes (KEGG) database.

RESULTS

Phenotyping of RIL Population and Construction of Bulk Samples

In all environments, the AUDPC of the resistant parent YS#24 was consistently and considerably higher than that of the susceptible parent YS#58 (**Table 1**). The mean AUDPC values of the resistant (YS#24) and susceptible parents (YS#58) were 336.29 and 820.55, respectively, over the environment (**Table 1**). Based on the pooled data, the RIL population for SB AUDPC revealed considerable phenotypic variation, ranging from 231.70 to 836.80. The RILs also exhibited a high coefficient of variation (CV) for AUDPC across environments, ranging from 8.80% (BHU14) to 16.92% (UBKV14), whereas the broad-sense heritability (h^2) values of AUDPC were lowest at BISA14 (0.65) and highest at BHU14 (0.87). Therefore, resistant and susceptible bulks were constituted by taking the samples independently using extreme phenotypes (resistant and susceptible) from both tails as illustrated in **Figure 1**.

Transcriptome Alignment and Variant Identification

The bulk samples utilized for BSR-Seq produced 429.40 million raw reads, containing 32,634.40 million base pairs. The number of raw reads in the sequenced samples ranged from 30.40 million (7.08%) in the resistant bulk-3 to 171.70 million (39.99%) in the susceptible parent (**Table 2**). The highest mapping of reads to the reference genome was obtained for the susceptible parent (171.70 million reads), followed by susceptible bulk-3 (43.90 million reads), resistant parent (42.50 million reads), resistant bulk-1 (37.90 million reads), and susceptible bulk-2 (36.50 million reads), and the lowest values were obtained for resistant bulk-3 (30.40 million reads) (**Figure 2A**). All the raw sequence reads have been deposited at NCBI, and the provisional Sequence Reads Archive (SRA) identifiers were obtained under the accession codes SRR5948908.

Each sample yielded an average of 80% high-quality (free of adaptor contamination and low-quality areas) RNA-Seq reads that were matched to the wheat reference transcriptome. Susceptible bulk-1 had the highest percentage alignment (80.38% with 535390 SNPs), followed by susceptible bulk-3 (80.09% with 613480 SNPs), and resistant bulk-1 had the lowest percentage alignment (79.01% with 669279 SNPs) (**Table 2**). The resistant parent had the highest number of SNPs (843836), whereas the minimum number (535390) was in the susceptible bulk-1 with the highest percentage of alignment **(Figures 2B,C)**.

BFR and Polymorphic SNPs Associated With SB Resistance

A total of 1,379,122 SNPs were identified on 91,855 transcripts of wheat samples used in the present investigation (**Table 3**). The

TABLE 2 | Number of reads generated, alignment percentage, and number of SNPs in parents, resistant bulk, and susceptible bulk of the "YS#24 × YS#58" in wheat.

Samples used for BSR-seq	Number of reads (76 bp/read pair)	% of total number of reads	Number of base pairs	Percent alignment	Number of SNPs
Resistant parent	42.50 × 10^6	9.90	3230.0 × 10^6	79.11%	843,836
Susceptible parent	171.70 × 10^6	39.99	13,049.2 × 10^6	79.55%	656,548
Resistant bulk-1	37.90 × 10^6	8.83	2880.4 × 10^6	79.07%	629,129
Resistant bulk-2	35.10× 10^6	8.17	2667.6 × 10^6	79.01%	669,279
Resistant bulk -3	30.40 × 10^6	7.08	2310.4 × 10^6	79.27%	694,316
Susceptible bulk-1	31.40 × 10^6	7.31	2386.4 × 10^6	80.38%	535,390
Susceptible bulk-2	36.50 × 10^6	8.50	2774.0 × 10^6	79.43%	635,868
Susceptible bulk-3	43.90 × 10^6	10.22	3336.4 × 10^6	80.09%	613,480
Total	429.40× 10^6	100	32,634.4 × 10^6	—	5277846

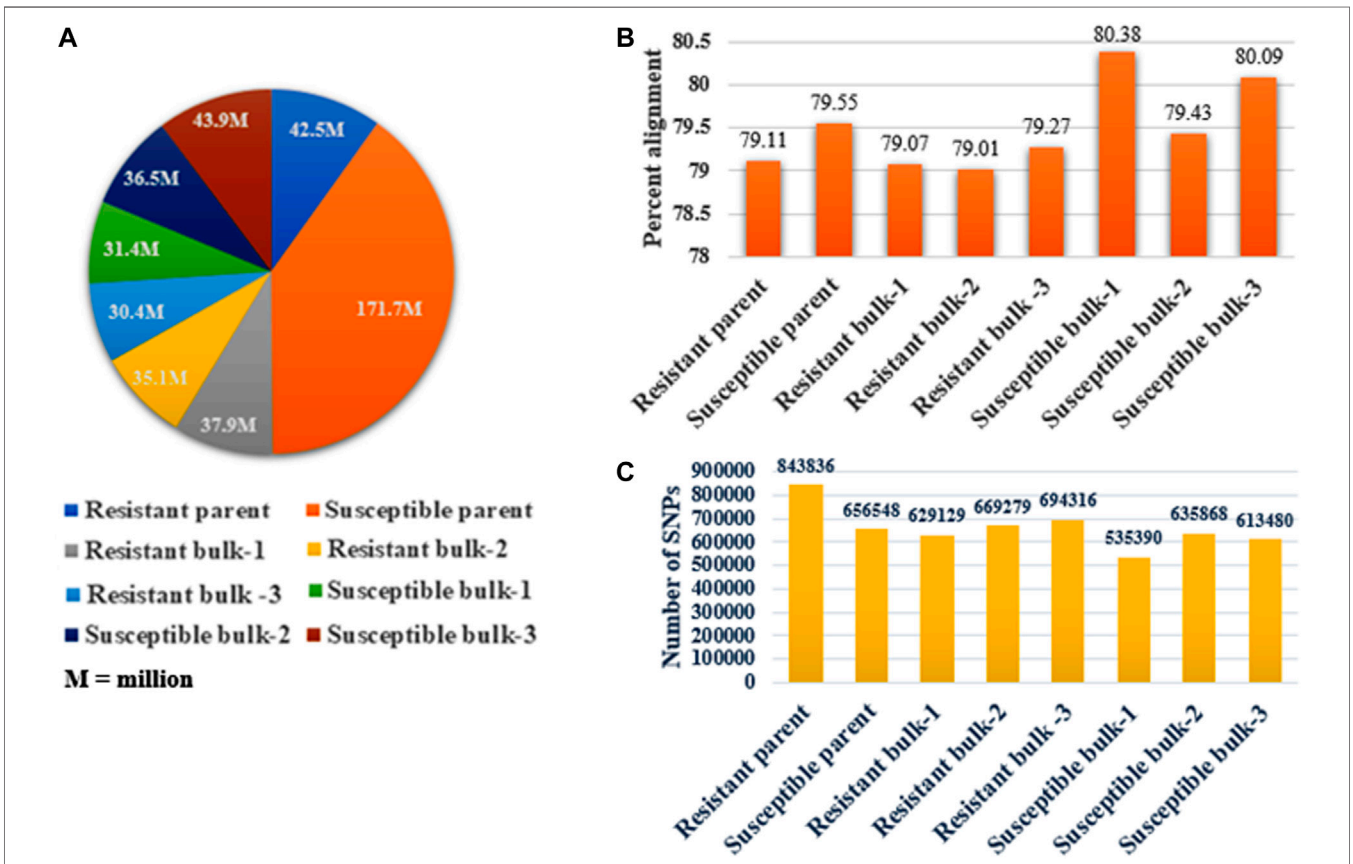

FIGURE 2 | RNA-Seq data study in parents and bulk samples. **(A)** The number of raw sequence reads (76 bp/read pair) generated in resistant and susceptible parents and bulk samples. **(B)** The percentage alignment of the read to the wheat reference sequence. Susceptible bulk-1 shows the highest alignment. BWA was used to align the reads. **(C)** The total number of SNPs obtained in each sequenced sample and the maximum number of SNPs present in the resistant parent.

TABLE 3 | Number of transcripts containing SNPs having BFR >6 in bulked samples.

Description of samples	SNPs count	Number of transcripts
Raw SNP count	1379122	91855
Bulk 1 BFR >6	3666	1837
Bulk 2 BFR >6	3635	2056
Bulk 3 BFR >6	2130	1443
Bulk total BFR >6	7860	3950
Bulk total with parental polymorphism	1055	506

putative SNPs linked to SB were selected based on SNPs with BFR >6 in all three bulk samples, i.e., 3666 SNPs present on 1837 transcripts in Bulk-1 (S-bulk1: R-bulk1), 3635 SNPs on 2056 transcripts in Bulk-2 (S-bulk2: R-bulk2), and 2130 SNPs on 1443 transcripts in Bulk-3 (S-bulk3: R-bulk3). A total of 7860 SNPs with >6 BFR were found across all bulks (Bulk-1: Bulk-2: Bulk-3) on 3950 transcripts. Out of 7860 SNPs, only 1055 SNPs were present on 506 transcripts detected as homozygous and polymorphic between parents (**Table 3**). The transcripts

TABLE 4 | Distribution of polymorphic SNPs markers of SB with BFR >6 across the A, B, and D genomes of wheat.

Chromosome	Number of SNPs	Number of transcripts	% of total number of SNPs	Number of SNPs in homoeologous group	Percentage
1A	30	14	2.84	90	8.53
1B	36	17	3.41		
1D	24	10	2.27		
2A	63	32	5.97	135	12.79
2B	56	36	5.30		
2D	16	11	1.52		
3A	49	16	4.64	207	19.62
3B	136	67	12.89		
3D	22	13	2.09		
4A	34	22	3.22	79	7.49
4B	27	22	2.56		
4D	18	14	1.70		
5A	50	26	4.74	313	29.67
5B	198	74	18.77		
5D	65	34	6.16		
6A	44	14	4.17	126	11.94
6B	53	18	5.02		
6D	29	13	2.75		
7A	41	27	3.88	105	9.95
7B	26	8	2.46		
7D	38	18	3.60		
Average	50.23	24.09	4.76	150.71	14.29
Total	1055	506	100	1055	100

carrying homozygous and polymorphic SNPs with other details are listed in **Supplementary Table S1**.

Distribution of Polymorphic SNPs for SB in Wheat Genome

The distribution of trait-linked SNP markers in the A, B, and D genomes for each homoeologous group and percentage of the total number of SNPs is given in **Table 4**. The 1055 polymorphic SNP markers, bar plotted on 21 wheat chromosomes, formed seven unevenly distributed homoeologous groups (**Figure 3**). The highest number of SNP markers were identified in the B genome (532 SNP; 50.42%), followed by A (311 SNP; 29.46%) and D (212 SNP; 20.09%). The number of SNPs per linkage group ranged from 16 (2D) to 198 (5B) (**Figure 4**). The homologous group 5 had the highest markers (313 SNP, 29.67%), followed by group 3 (207 SNP, 19.60%), and group 4 had the lowest (79 SNP, 7.49%) (**Table 4**).

Analysis of Polymorphic SNPs on Chromosomes 3B and 5B

The maximum number of polymorphic SNP with BFR >6 was found on chromosome 5B (198 SNP), followed by 3B (136 SNP) (**Figure 4**). Thus, a total of 334 SNPs of 3B and 5B chromosomes were present on 142 transcripts, out of which 60 SNPs were found only on five transcripts. Out of these five, one transcript of the 3B chromosome had eight SNPs, and four transcripts of the 5B chromosome had 52 SNPs (**Table 5**). Among the four transcripts of 5B, a maximum of 27 SNPs were present on transcript gnl/UG/Ta#S61812294, followed by transcripts gnl/UG/Ta#S17985740 (19 SNPs) and gnl/UG/Ta#S61830716 (4 SNPs), while transcript gnl/UG/Ta#S65715070 had the lowest number (2 SNPs) (**Table 5**). The chromosomal distribution of 60 polymorphic SNP present on five different transcripts of the 3B and 5B chromosomes associated with SB resistance is shown in **Figure 5**, which indicates their relative position.

Development of an Assay From Allele-Specific Primers in Bulk Samples

In this study, allele-specific tetra-primer ARMS PCR primers were designed to develop an assay for SB resistance. The SNPs on transcript gnl/UG/Ta#S61799095 of 3B chromosomes and transcript gnl/UG/Ta#S61830716 and gnl/UG/Ta#S17985740 of chromosome 5B were used to design the tetra-primers for ARMS PCR (**Supplementary Table S2**). A primer (*Ta_S61830716_1262_3*) from chromosome 5B amplified 142 bp fragments in both the resistant parent and resistant bulk-1, whereas a 142 bp fragment was not amplified in the susceptible parent, but in a few samples of susceptible bulk-1, a faint band appeared in lane 10–12 (**Figures 6A,B**). The primer (Ta_S61830716_1262_3) showed clear differentiation between resistant and susceptible genotypes.

Pathway Enrichment Analysis Using GO and KEGG

The transcript of 3B chromosomes (gnl/UG/Ta#S61799095) shared homology with acetyl-CoA acetyltransferase, whereas two transcripts of 5B (gnl/UG/Ta#S17985740 and gnl/UG/

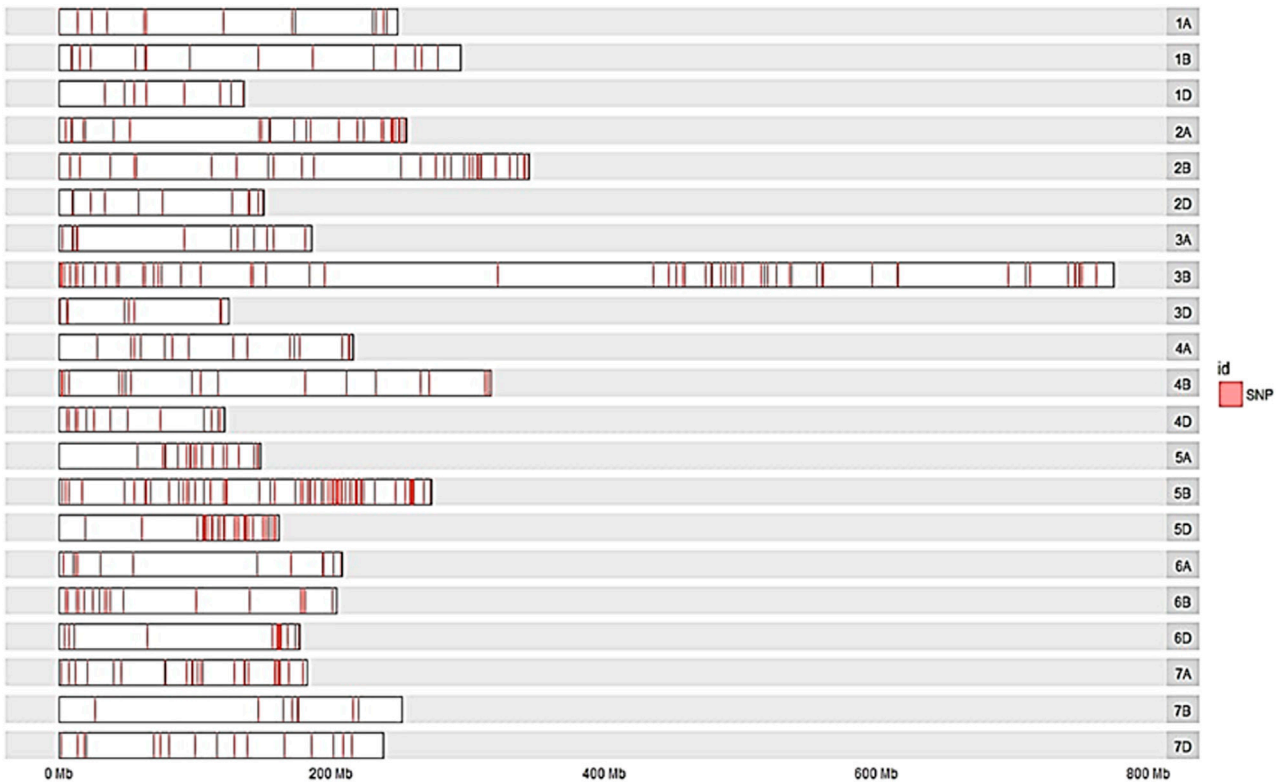

FIGURE 3 | Bar plot showing the densities of polymorphic SNPs marker with bulk frequency ratio greater than 6 (BFR >6) on the 21 wheat chromosomes of the cross "YS#24 × YS#58" RIL population. The locations of the SNPs were determined by the best alignment to the wheat genome with the transcript containing the SNPs.

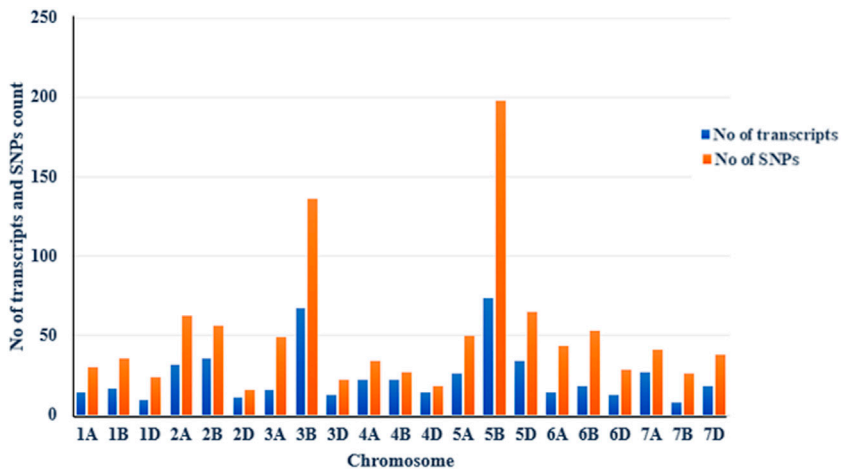

FIGURE 4 | Chromosomal distribution of SB-associated transcripts (blue bar) and polymorphic SNPs (orange bar) on wheat chromosomes with a bulk frequency ratio greater than 6. The figure shows that the 5B chromosome has the maximum number of SNPs, followed by the 3B.

Ta#S61830716) shared homology with proteinase/protease and the remaining two with phospholipase C1 and exohydrolase proteins (**Supplementary Table S3**). A total of 346 GO terms were identified for the selected transcripts and further categorized into molecular function, biological process, and cellular component (**Supplementary Figure S2**). The maximum number of GO terms fall into the molecular function category (231 terms), which were further grouped into hydrolase activity, transferase activity, peptidase activity, and catalytic activity. There were 70 terms associated with the biological process,

TABLE 5 | Transcripts of 3B and 5B chromosomes having SNPs markers of SB resistance with BFR >6.

Chromosome	No. of transcripts	Total number of SNPs	Transcript ID	Number of SNPs
3B	1	8	gnl/UG/Ta#S61799095	8
5B	4	52	gnl/UG/Ta#S17985740	19
			gnl/UG/Ta#S61812294	27
			gnl/UG/Ta#S61830716	4
			gnl/UG/Ta#S65715070	2

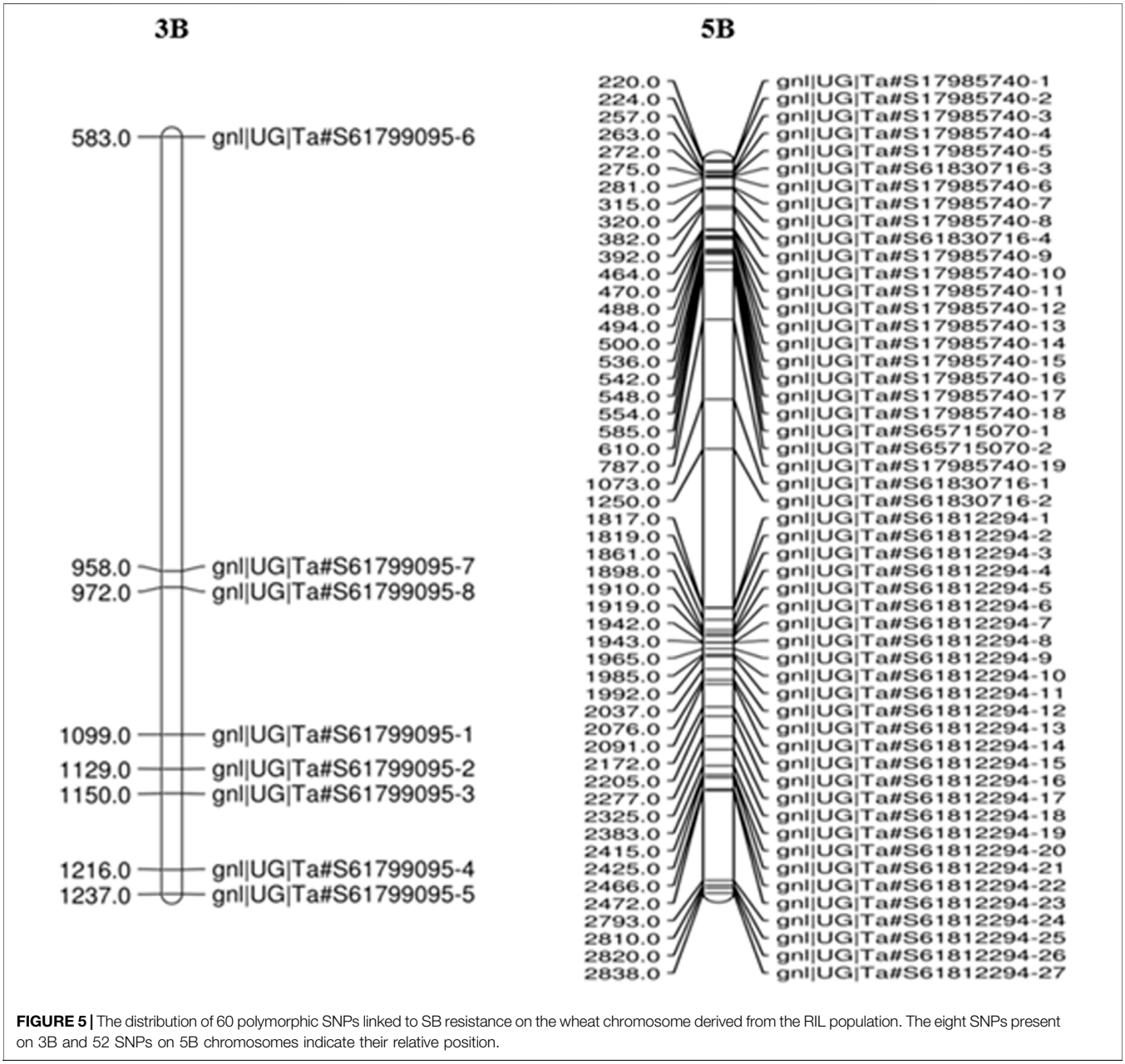

FIGURE 5 | The distribution of 60 polymorphic SNPs linked to SB resistance on the wheat chromosome derived from the RIL population. The eight SNPs present on 3B and 52 SNPs on 5B chromosomes indicate their relative position.

which fall into the category of protein catabolic process, carbohydrate metabolic process, metabolic process, phospholipid catabolic process, and fatty acid beta-oxidation (**Supplementary Figure S2**). A few terms were associated with cellular components, viz., peroxisome, membrane, an integral component of the membrane, lysosome, and extracellular space.

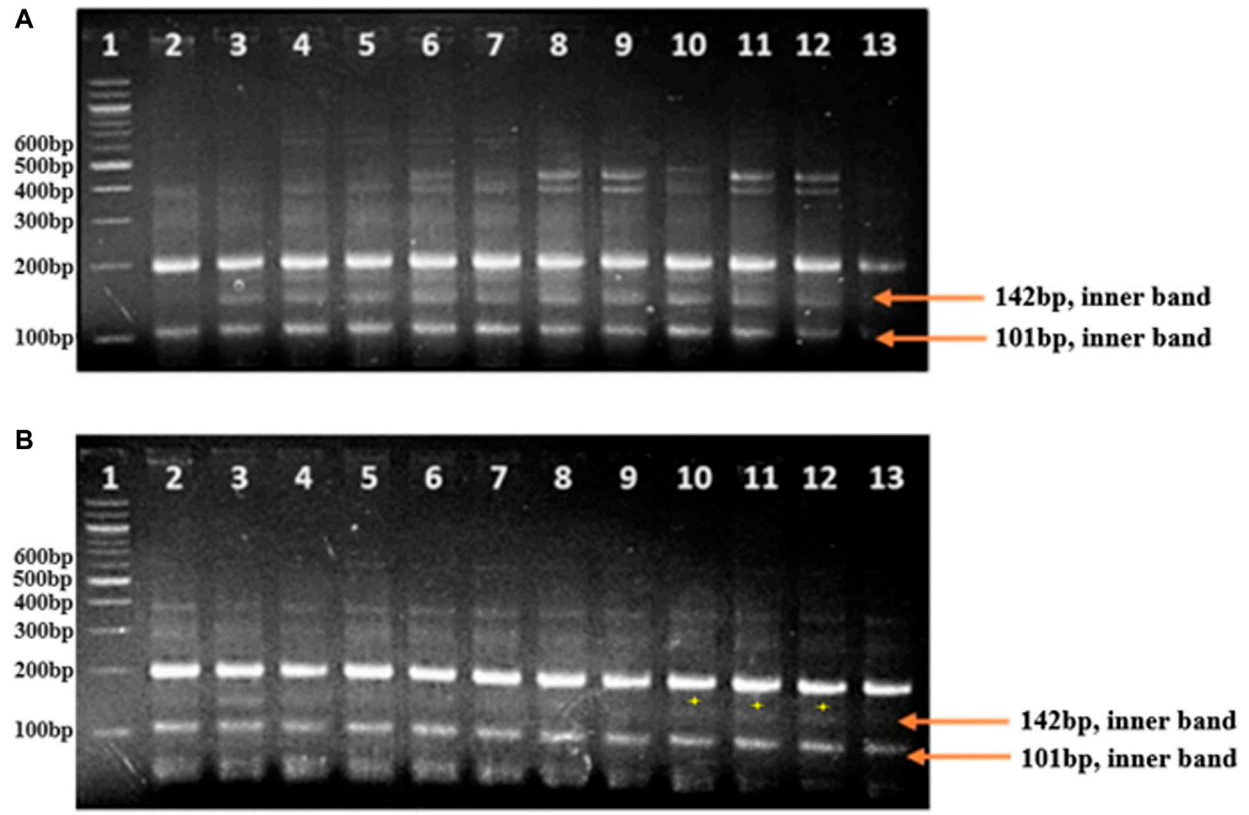

FIGURE 6 | (A) Lane 1: 100 bp ladder, Lane 2: susceptible parent, Lane 3: resistant parent, Lane 4–13: resistant lines. The resistant lines amplify the inner allele-specific primer region with a product size of 142 bp, which is absent in the susceptible parent. **(B)** Lane 1: 100bp ladder, Lane 2: susceptible parent, Lane 3: resistant parent, Lane 4–13: susceptible lines. The inner allele-specific primer region amplifies in the resistant parent at 142 bp and is absent in the susceptible lines except for lanes 10–12.

Furthermore, as shown in **Supplementary Figure S3**, the most highly identified pathways in the study were fatty acid metabolism, valine, leucine, isoleucine metabolism, and benzoate degradation.

DISCUSSION

In the 21st century, wheat is placed as one of the world's most productive and essential crops (Curtis and Halford, 2014), contributing significantly to global food security by providing nutrition for 35% of the world population (FAO, 2018; Tomar et al., 2021). The continuous threat of SB fungus in major wheat-growing areas of the world results in a significant yield loss that affects future food security. Wheat cultivars with host resistance are employed to minimize crop yield loss, which is the most effective and economical way to manage SB (Gupta et al., 2018). However, the conventional breeding approaches to develop cultivars with disease resistance have certain limitations due to the genetic complexity of wheat (Zhang et al., 2020). Several molecular breeding tools and techniques have been developed to study the genetics and genomics of plants having simple and complex genomes. For a significant period, researchers primarily relied on SSR markers, but their sparse distribution across the genome rendered them less ideal for a large-scale genotyping assay (Pootakham et al., 2014). Recently, SNP markers have become increasingly popular in molecular genetics and breeding studies due to their abundance. However, SNP discovery in organisms with highly repetitive DNA and polyploid nature, such as wheat, remains difficult (Ganal et al., 2009; Trick et al., 2012). The SNP discovery *via* transcriptome sequencing is an attractive strategy to reduce genome complexity in wheat (Westermann et al., 2012; Edae and Rouse, 2019). To reduce the complexity of the data, we focused on sequencing the wheat transcriptome using RNA-Seq instead of genomic DNA.

Due to the introduction of next-generation sequencing, combining BSA and RNA-Seq (BSR-Seq) reduces the cost remarkably when repetitive sequences are enriched in the genome and enabled a rapid and detailed understanding of a near-complete set of transcripts and SNPs linked to the trait (Garg et al., 2011; Zou et al., 2016). In this context, the RIL population was used for making bulks with extreme phenotypes for SB disease. The RIL population's AUDPC showed a continuous distribution across environments, indicating that SB resistance components behave like quantitative traits (**Figure 1**) as quantitative traits generally

show a continuous phenotypic distribution. The estimated broad-sense heritability for AUDPC was high (0.92) over the environments, indicating good reproducibility of the phenotypic data (**Table 1**). The high heritability over environments revealed the genetic control of AUDPC. The numbers of bulks for pooling were selected in multiples (in replicate) independently from each of the two tails by following Navabi et al. (2009) for small- to moderate-sized populations; the optimum tail size should be 20%–30% of the entire population. The replicated number of bulks for pooling provides high accuracy in SNP predictions by reducing false positives, increasing the likelihood of obtaining reliable markers by many orders of magnitude. The parents were used to define the SNPs rather than to make quantitative estimates; therefore, replications to detect SNPs were omitted (Ramirez-Gonzalez et al., 2015). In diploid crops of the medium genome, approximately 57–65 million reads were generated and successfully achieved higher (>90%) genome coverage in cucumber (Lu et al., 2014) and pigeon pea (Singh et al., 2016). In this study, 429.40 million reads were generated for the bulk samples; these excess reads provided better coverage to the genome. In a bulked sample of groundnut (allotetraploid) for rust and late leaf spot resistance, 423.70 million reads were generated by Pandey et al. (2017). Because bread wheat is hexaploid, having three subgenomes (A, B, and D), more sequence data should be generated than the other diploid species, to achieve maximum genome coverage and read depth. Hence, the generated sequencing data with maximum genome coverage and read depth allowed for detailed sequence analysis. The alignment of reads to the reference transcriptome revealed that the maximum number of SNPs were present in the resistant parent, whereas the minimum was in the susceptible bulk-1 with the highest percentage of alignment. The higher percentage alignment indicated that the sample was closer to the reference being aligned. In the present study, a total of 1,379,122 genome-wide, high-quality SNPs were identified in parents and resistant and susceptible bulks of wheat after sequence alignment of filtered reads, and only 1055 SNPs were detected as polymorphic between the parents using BFR >6, i.e., associated with SB resistance. Among the 1055 SNPs, 198 (18.77%) and 136 (12.89%) were mapped on chromosomes 5B and 3B, suggesting the SB resistance gene might be located on chromosome 5B or 3B. Among the three genomes, there were polymorphic SNPs present in the B genome (50.42%) compared with A (29.46%) and D (20.09%) (**Table 4**). A similar result was reported by Kumar et al. (2009) in wheat when parents were screened with SSR markers. However, the density of polymorphic SNPs throughout the B genome was not uniform; only the 5B and 3B chromosomes were found saturated considerably with SNP markers having a magnitude of BFR >6. Kumar et al. (2009, 2010) also report two QTLs for SB resistance on the 5B chromosome in two different populations that explained around 38.62% and 10.70% of the phenotypic variation, respectively.

In the recent past, three major SB resistance genes, *Sb2* (Kumar et al., 2015), *Sb3* (Lu et al., 2016), and *Sb4* (Zhang et al., 2020), were identified in the B genome of wheat. Thus, the previous (SSR, SNP) and present SNP studies indicate the effectiveness of the B genome for SB resistance, especially 5, 4, and 3B. The average number of SNPs mapped to each linkage group was 50.23, whereas the highest number of markers was mapped to 5B. In this study, the D genome was found less saturated compared with A and B because a lesser number of polymorphic SNP markers (>6 BFR) was identified on it, which is commensurate with microsatellite markers reported in wheat (Ganal and Röder, 2007). However, the first resistance gene (*Sb1*) for SB was reported on the 7D chromosome by Lillemo et al. (2013); as a result, the importance of the D genome on a saturation basis cannot be overstated. It appears to indicate that, despite the lesser number of SNPs identified on the D genome, we should pick only those SNPs having the highest magnitude of BFR. Because the higher the BFR, the more likely the SNP is genetically linked to the *R*-gene, the putative SNPs with enriched BFR can then be converted into high-throughput SNP assays and genotyped across the individuals that were used to assemble the bulk (Ramirez-Gonzalez et al., 2015). Finally, the key issue is to move from *in silico* SNPs into a high-throughput SNP assay with allele-specific markers that can score on agarose gel electrophoresis. The allelic-specific primer *XTaSb_S61830716_1262_3*, designed from SNPs present on transcript id *Ta_S61830716*, was found polymorphic in parents and validated in each line of resistant bulk-1, but only in 70% lines of the susceptible bulk-1 and was named as a marker of SB resistance. The marker *XTaSb_S61830716_1262_3* is characterized by a few individuals of susceptible bulks as resistant but is otherwise susceptible phenotypically, limiting the efficiency of the marker.

Because plant immunity is regulated by the expression of pathogenesis-related genes (PRs), transcription is de-repressed by pathogen-induced signals. The studied transcripts here are shown to have homology with pathogenesis-related genes. The transcript gnl/UG/Ta#S61799095 of 3B identified in this study showed homology with acetyl-CoA acetyltransferase, which is conferred for its pathogenesis-related activity in rice (Zhong et al., 2015). It is a vital starting molecule for the biosynthesis of various metabolites. The transcripts of 5B (gnl/UG/Ta#S17985740 and gnl/UG/Ta#S61830716) also show homology to cysteine proteinase and proteases. Because different families of proteases manage the extracellular defense, which contributes to effector-triggered immunity (ETI), others help in the induction of microbe-associated molecular pattern–triggered immunity. A few proteases/proteinases are associated with systemic acquired resistance and the establishment of induced systemic resistance (Kim and Hwang, 2015; Balakireva and Zamyatnin, 2018). The other transcripts of 5B (gnl/UG/Ta#S61812294 and gnl/UG/Ta#S65715070) were found homologous to phospholipase and exohydrolase. It is reported that phospholipase affects the translocation of nonexpressor pathogenesis-related (NPR) proteins to the nucleus in *Arabidopsis thaliana*. The structural

changes and localization of this protein in plant cells are responsible for the plant defense signaling (Kinkema et al., 2000; Janda et al., 2015). The major pathways identified in the study are fatty acid degradation and valine, leucine, and isoleucine degradation, along with other pathways. Fatty acid degradation is the process by which fatty acids break down into their metabolites, which finally generates acetyl-CoA, the entry molecule for the citric acid cycle. In the case of *Brassica napus*, when infected with the pathogen, it significantly enriched in fatty acid oxidation activities in the upregulated gene sets on both susceptible and resistant lines (Chittem et al., 2020). Thus, the study of five wheat transcripts shows that they are closely related to genes involved in pathogenesis and metabolism, suggesting their prominent role in the plant defense mechanism.

CONCLUSION

In this study, BSA combined with RNA-Seq (BSR-Seq) appears to be useful for SNP discovery in bread wheat for SB resistance, later used to develop a new marker assay. The marker *XTaSb_S61830716_1262_3* could be productive in screening wheat germplasm for SB resistance. In future projects, the SNPs discovered for SB resistance across the wheat genome will be visualized by converting them into Kompetitive Allele Specific PCR (KASP) markers for establishing a high-throughput genotyping platform, useful for MAS of the target genes. The newly developed SNPs marker could also be converted to a qPCR-based assay for large-scale application in crop improvement and study of the molecular biology of SB resistance.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

VM and AJ conceived and designed the experiments. RS performed the experiments as part of his Ph.D. research. RS, RC, AC and UK handled field phenotyping of SB disease. UK evaluated the lines for spot blotch at BISA, Bihar, and edited the manuscript. RS and VM performed the molecular work. RS, VM, JB and NB analyzed the data. RS and VM wrote the manuscript. AJ revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

We gratefully acknowledge the partial financial support provided by donor organizations Bill and Melinda Gates Foundation and USAID CSISA project of CIMMYT as well as the University Grants Commission (UGC), New Delhi, India. A part of this research was also supported by the Delivering Genetic Gain in Wheat (DGGW) project (funded by the Bill and Melinda Gates Foundation and the Foreign, Commonwealth and Development Office (FCDO) and managed by Cornell University) under the terms of Contract No. OPP1133199.

ACKNOWLEDGMENTS

Authors are thankful to IARI, Wellington, India for providing off-season facilities, and to Borlaug Institute for South Asia (BISA), Samastipur, Bihar, India, and UBKV, Coochbehar, West Bengal, India for the main season experimental facilities. QTLomics Technologies (Bengaluru, India) is also acknowledged for providing the sequencing facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.859676/full#supplementary-material

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The reviewer SCB declared a past co-authorship with the author(s) NB to the handling editor.

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REVIEW
published: 26 April 2022
doi: 10.3389/fgene.2022.883930

Cytokinins: A Genetic Target for Increasing Yield Potential in the CRISPR Era

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: 25 February 2022
Accepted: 21 March 2022
Published: 26 April 2022

Citation:
Mandal S, Ghorai M, Anand U, Roy D, Kant N, Mishra T, Mane AB, Jha NK, Lal MK, Tiwari RK, Kumar M, Radha, Ghosh A, Bhattacharjee R, Proćków J and Dey A (2022) Cytokinins: A Genetic Target for Increasing Yield Potential in the CRISPR Era. Front. Genet. 13:883930. doi: 10.3389/fgene.2022.883930

Over the last decade, remarkable progress has been made in our understanding the phytohormones, cytokinin's (CKs) biosynthesis, perception, and signalling pathways. Additionally, it became apparent that interfering with any of these steps has a significant effect on all stages of plant growth and development. As a result of their complex regulatory and cross-talk interactions with other hormones and signalling networks, they influence and control a wide range of biological activities, from cellular to organismal levels. In agriculture, CKs are extensively used for yield improvement and management because of their wide-ranging effects on plant growth, development and physiology. One of the primary targets in this regard is cytokinin oxidase/dehydrogenase (CKO/CKX), which is encoded by CKX gene, which catalyses the irreversible degradation of cytokinin. The previous studies on various agronomically important crops indicated that plant breeders have targeted CKX directly. In recent years, prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been increasingly used in editing the CKO/CKX gene and phenomenal results have been achieved. This review provides an updated information on the applications of CRISPR-based gene-editing tools in manipulating cytokinin metabolism at the genetic level for yield improvement. Furthermore, we summarized the current developments of RNP-mediated DNA/transgene-free genomic editing of plants which would broaden the application of this technology. The current review will advance our understanding of cytokinins and their role in sustainably increase crop production through CRISPR/Cas genome editing tool.

Keywords: cytokinin, cytokinin oxidase/dehydrogenase, IPT, CRiSPR/Cas, crop improvement

INTRODUCTION

As a result of climate change and decreasing biodiversity, recent efforts in crop breeding programmes have focused on increasing tolerance to disease and environmental stress (Bellard et al., 2012; Mills et al., 2018). Furthermore, little progress is being made in terms of increasing the yield potential and agricultural productivity (Fugile, 2018). One of the most significant consequences of a growing global population is the need to provide ever-increasing amounts of food, fibre, and fuel while also acknowledging the need for sustainable production methods that operate in a changing global environment. In order to meet this growing requirement, the seed production is critical as it provides the raw material for fibre, fuel and food (Raza et al., 2019). Moreover, it has been noted that there is frequently a conflict between the seed production and the final end-product (food). Seed yield optimization must be done while maintaining the quality of the fibre, fuel, or food (vegetable, forage, cereal).

Cytokinins have been linked to seed development as high levels were detected during the process of seed development which led to the discovery of the first naturally occurring cytokinin, zeatin, from *Zea mays* (Letham, 1963) (**Figure 1**). It is possible to use modern biotechnology tools along with the traditional breeding for crop improvement. With the invent of new biotechnological tools (NBTs), such as marker-assisted selection (MAS), next-generation sequencing (NGS), genome editing (GE), and genetic transformation protocols, can be used as an additional tool along with the traditional breeding methods.

In these efforts, novel GE tools could be particularly useful. In the field of GE, CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR associated Cas) is considered a breakthrough technology, primarily due to its high efficacy and ease of implementation. As a foundation, CRISPR/Cas technology allows for the introduction of precise knockout mutations at specific genomic locations. Additionally, this system can be used to introduce defined mutations (gene repair), stack genes, or regulate gene expression in more advanced approaches (Bortesi and Fischer, 2015; Nidhi et al., 2021). Moreover, large number of crops are successfully edited through CRISPR/Cas tool because of its precision, simplicity and relatively cheaper (Jaganathan et al., 2018). Furthermore, induced mutations were found to be stably inherited in progeny plants. Additionally, with the availability of genome sequences for many crops and efficient genetic transformation protocols in recent years, various advanced forms of CRISPR have become readily available for use (Hensel et al., 2009; Mascher et al., 2017). Increased crop productivity is one of the major goals in agriculture, alongside adaptation to various biotic and abiotic stress. Manipulation of cytokinin (CK) metabolism is a promising tool for increasing plant productivity.

A wide range of physiological processes are regulated by the naturally occurring cytokinins, that stimulate cell division, root formation and regulate shoot, and are involved in the growth and development of leaves, flowers and fruits (Kieber and Schaller, 2018; Vankova, 2014). The regulators of cytokinin metabolism and signalling are particularly interesting among these genes because the effects of cytokinin activity on seed development is well established (Jameson and Song, 2016). Cytokinins are a sign of high physiological activity, and as a result, plants have evolved a variety of mechanisms to regulate the levels of active cytokinin at different developmental stages. Members of several multigene families are responsible for cytokinin biosynthesis (isopentenyl transferase, IPT), activation (LONELY GUY, LOG), irreversible degradation (cytokinin oxidase/dehydrogenase, CKX), reversible inactivation (zeatin O-glucosyltransferase, ZOG), and reactivation (glucosidase, GLU). Cytokinin oxidase/dehydrogenase enzymes (CKX) catalyse the degradation of cytokinin by cleaving the active cytokinin form's unsaturated isoprenoid side chains. A family

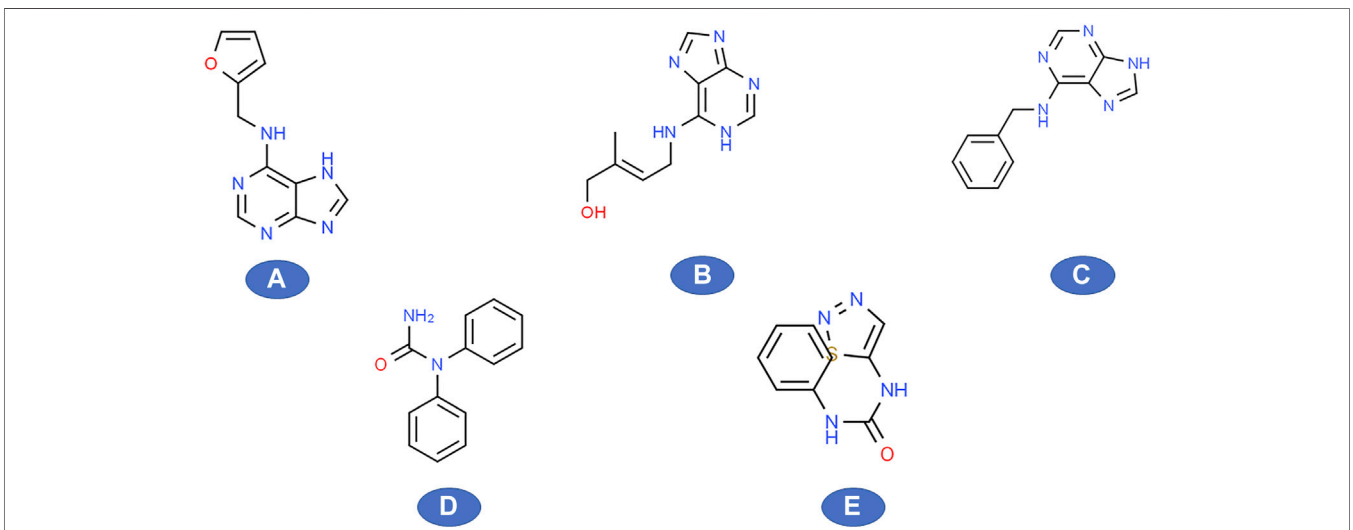

FIGURE 1 | Various natural and synthetic cytokinins (structures obtained from www.ChemSpider.com) **(A)** kinetin, **(B)** zeatin, **(C)** 6-benzylaminopurine, **(D)** Diphenylurea, **(E)** thidiazuron (TDZ).

of CKX genes encodes CKX enzymes with tissue specificity and developmentally dependent expression patterns (Werner et al., 2006). The CKX genes, which are important regulators of cytokinin levels, have attracted the attention of researchers, who have noted their potential for crop improvement as a result of their ability to manipulate cytokinin homeostasis in plants. Evidently, several studies have demonstrated that suppressing the expression of CKX can improve the quality of some crop yield attributes in some cases. In rice, barley, cotton, and Arabidopsis, for example, downregulation of CKX genes via mutation, RNAi-based silencing, or genome editing (CRISPR) resulted in higher seed numbers and/or seed weight (Bartrina et al., 2011; Zalewski et al., 2012; Li et al., 2013; Zhao et al., 2015; Li et al., 2016). Additionally, it has been demonstrated that CKX genes have an effect on root growth and morphology (Werner et al., 2003; Mrízová et al., 2013).

Alternatively, homeostasis of cytokinins can be manipulated by overexpression of IPT genes. IPT is a member of a small family of genes that encode isopentenyl transferases, which are directly involved in the *de novo* synthesis of cytokinin hormones. In barley, IPT genes, like CKXs, indicate tissue specificity and development-dependent expression patterns (Mrízová et al., 2013). Numerous crop plants have benefited from ectopic IPT gene expression during seed production (Jameson and Song, 2016). This review provides an overview of molecular aspect of cytokinin actions that regulates various physiological process in the plant. Furthermore, we also highlight the current progress on the applications of CRISPR/Cas9 and various other molecular technique in increasing grain yields by manipulated the CKX gene. Finally, we also discussed the CRISPR/Cas9 DNA-free editing in crops that has a great prospect of being commercialized.

MOLECULAR BASIS OF CYTOKININ REGULATION

Physiological and developmental processes include apical dominance release, axillary shoot branching, root meristem cell patterning, and the formation of lateral roots, are controlled by CKs in plants through a complex network of cross-talk between signaling pathways (Koprna et al., 2016). In addition to playing a critical role in the regulation of plant cell proliferation and differentiation, they are involved in the regulation of a variety of processes in plant growth and development. These include the inhibition of root development, the promotion of shoot growth, development of seed and fruit, delaying of the onset of senescence, the transduction of nutritional signals, and the response to both biotic and abiotic stresses (Koprna et al., 2016; Cortleven et al., 2019). To maintain cytokinin homeostasis and regulate a variety of physiological processes, bioactive molecules are synthesised, activated, degraded, and conjugated (**Figure 2**).

Cytokinins are produced by nearly every living organism. Cytokinins are naturally occurring adenine derivatives with isoprenoid or aromatic side chains at the adenine ring's N6 position (Kiba et al., 2013). Thus, natural cytokinins can be classified into isoprenoid and aromatic CKs, with the former being more abundant in plants (Sakakibara, 2006). The most important components of isoprenoid cytokinins are trans-zeatin (tZ), isopentenyl adenine (iP) and cis-zeatin (cZ), with iP and tZ being the most active (Sakakibara, 2006; Kudo et al., 2010). The aromatic cytokinins entail orthotopolin (oT), mesotopolin (mT), their methoxy derivatives (MemT and MeoT), benzyladenine (BA), and more. These, on the other hand, are found only in a few plant species, such as *Nicotiana tabacum* and poplar (Sakakibara, 2006; Kudo et al., 2010). The terpenoid pathway is responsible for the synthesis of cytokinin. The enzymes LONELY GUY (LOG) and isopentenyl transferase (IPT) are two of the most important players in cytokinin biosynthesis (Kuroha et al., 2009). Adenosine phosphate-isopentenyl transferases and tRNA-isopentenyl transferases (tRNA-IPTs) make up the majority of IPTs, which serve as the primary rate-limiting enzymes in cytokinin biosynthesis. The IPPT-binding domain of these two types of IPTs is conserved, which is a characteristic shared by both the enzymes (Sakakibara, 2006). The adenosine phosphate-isopentenyl transferases can catalyze dimethylallyl diphosphate (DMAPP) to form iP-ribotides, which are precursors of iP that initiates cytokinin biosynthesis (Sakakibara, 2006; Kudo et al., 2010). The cytochrome P450 monooxygenase CYP735As catalyses the conversion of iP-ribotides to tZ-ribotides, which is also important in promoting shoot growth of *Arabidopsis thaliana* (Kiba et al., 20013). Furthermore, iP- and tZ-ribotides are the precursors of the majority of iP- and tZ-type cytokinins (Kudo et al., 2010).

The synthesis of cZ starts with tRNA-IPTs, which use DMAPP to help with the prenylation of tRNA and then generats cZ-ribotides (Sakakibara, 2006). In some plants, multiple gene families encode IPTs; for example, *A. thaliana* has 9 IPTs (*AtIPT1-AtIPT9*), *Oryza sativa* has 10 IPTs (*OsIPT1-OsIPT10*), and the *Fragaria* × *ananassa* has 7 *FvIPTs* (Frebort et al., 2011; Zurcher and Muller, 2016). The lysine decarboxylase-binding domain of all the proteins expressed by LOGs which is conserved across the plant kingdom and a new discovered cytokinin-activating enzyme (Mi et al., 2017). In the next step, LOGs convert inactive cytokinin ribotides into biologically active free base form through their cytokinin-specific phosphohydrolase activity (Kudo et al., 2010). The first LOGs were discovered in *O. sativa*, and the nine LOGs (*AtLOG1-AtLOG9*) discovered in *A. thaliana* were later thought to be rice homologs (Kuroha et al., 2009). The *Fragaria* × *ananassa* genome recently revealed the presence of nine *FvLOGs*. Under osmotic stress, high temperature treatment, and exogenous abscisic acid (ABA), the expression of most *FvLOGs* and *FvIPTs* changes, suggesting that these genes may play a role in plant resistance to abiotic stresses (Mi et al., 2017).

Plants require precise control of biosynthesis and metabolic enzymes to maintain cytokinin levels. Biosynthesis and metabolic enzymes must be tightly regulated in plants in order to maintain cytokinin levels and regulate numerous physiological and biochemical process. As a result, not only cytokinin synthetase, but also cytokinin-metabolizing enzymes are required to maintain stable cytokinin levels (Frebort et al., 2011). The level of active cytokinin can be controlled by

binding to sugars (mostly glucose) or by irreversible cleavage of cytokinin oxidases, which are enzymes that degrade active cytokinin (CKXs) (Werner et al., 2006). Glucose binds to cytokinin at the purine ring's N3, N7, and N9 positions or the hydroxyl group of the pentenyl side chain, including O and N-glycosylation (Kudo et al., 2010). Glucosyltransferase catalyses O-glycosylation on the oxygen of the cytokinin side chain, which is reversed by β-glucosidase (Sakakibara, 2006). Purine ring N-glycosylation, which mainly occurs on the N7 or N9 purine ring, is considered to be irreversible (Sakakibara, 2006; Kudo et al., 2010). In bioassays, glucosyl conjugates are inactive, and the bounded cytokinins are unable to bind to histidine kinase (HK) cytokinin receptors (Spichal et al., 2004). It has been demonstrated that cytokinins bound to glucosyl conjugates do not bind to the receptors for histidine-kinase (HK) cytokinins in biological assays. CKXs are the only enzymes known to catalyse the irreversible degradation of cytokinin (Galuszka et al., 2007; Kudo et al., 2010). Flavin adenine dinucleotide (FAD) binding and substrate binding are two conserved domains in mature CKXs that have different biochemical functions and subcellular localization (Galuszka et al., 2007; Kowalska et al., 2010). By irreversibly cutting the free radicals and ribose forms of cytokinins on the N6 side chain, CKXs lower the level of active cytokinins (Werner et al., 2006). CKXs cleave both tZ and iP, but DZ, synthetic cytokinin kinetin, and 6-benzylaminopurine are resistant to cleavage (Galuszka et al., 2007). Furthermore, manipulating CKXs has a significant impact on endogenous cytokinin levels, which is discussed in great detail in the following section.

ROLE OF CYTOKININ IN IMPROVING CROP YIELD

Cytokinin oxidases/dehydrogenases (CKX) are involved in the regulation of cytokinin-dependent processes and are important in controlling endogenous cytokinin levels (Schmüllin et al., 2003). CKX is considered to act as a negative regulator of cytokinin production and may be involved in grain development (Galuszka et al., 2001). In the early stages of grain development in *Oryza sativa* (rice), cytokinin plays an important role in regulating grain filling pattern, which in turn affects grain filling percentage. Moreover, it is evident in rice endosperm, cell division is regulated by CK in the grains at the early stages of grain development (Yang et al., 2000; Yang et al., 2003). Furthermore, stay-green traits and senescence are closely linked through CK, which acts as a general coordinator between the two. *Triticum aestivum* (wheat) yields can be increased by using stay-green, as well as its resistance to heat stress during active photosynthesis (Yang et al., 2016). The improvement of leaf anatomical and biochemical traits, including tolerance to suboptimal temperature conditions, can lead to high yields by increasing photosynthetic productivity (Brestic et al., 2018). Numerous scientific studies indicate that the overexpression of the CKX gene resulted in a decrease in the endogenous cytokinin content of plants (Jones and Schreiber, 1997). The role of CKX in many agronomically important crops was elucidated using transgenic and genome-editing technology, which is discussed in detail in the following section. **Table 1** summarises the genetic and molecular approaches applied for manipulating the cytokinin gene in a variety of crop plant.

Earlier published reports in 2001, demonstrated that overexpression of the *AtCKX* gene in *N. tabacum* plants resulted in decreased endogenous cytokinin levels (Werner et al., 2001). Zeng et al. (2012) found that the *Gossypium hirsutum* L. gene *GhCKX* was suppression or overexpression in the model plant *N. tabacum*, resulting in over-production of cytokinin (e.g., more capsules and flowers) or deficiency of cytokinin (e.g., fewer or no flowers). Another group of researchers identified that bigger roots, smaller shoots, and smaller shoot apical meristems (SAMs) in *A. thaliana* when *AtCKX1* and *AtCKX2* genes were overexpressed. But overexpression of *AtCKX7* in the model plant results shorter primary roots were reported (Kollmer et al., 2014; Werner et al., 2001). In addition, *ckx3 ckx5* double mutants had more siliques and larger SAM (Bartrina et al., 2011). The findings support the critical role of cytokinin in a variety of model plants.

Zalewski et al. (2010) used hairpin RNA interference to successfully silenced the expression of *HvCKX1* and *TaCKX1* gene resulting in lower CKX activity and significant increase in grain productivity and greater root weight in *Hordeum vulgare*. Raspor et al. (2012) and team overexpressed the *AtCKX2* gene in *Solanum tuberosum* L. cv. Désirée that resulted in significantly lower levels of bioactive cytokinins when compared to the control. Furthermore, Mrìzová et al. (2013) found that overexpression of CKX induces CK-deficiency in *H. vulgare*, prevents them from flowering and resulting morphological changes such as distinctively enlarged root systems and retarded development of aerial parts. The overexpressed plants also displayed higher root-to-shoot ratios when compared to wild-type (WT). Gu et al. (2015) utilized the *An-2* gene (from *Oryza sativa*) encoding for Lonely Guy like protein 6, was cloned into *O. rufipogon* (brownbeard rice) exhibited a pleiotropic effect on root length, tiller number, grain number and awn length. Cloned *An-2* in the *O. rufipogon* background increased the endogenous cytokinin concentration, which promoter rice awn elongation. In a different study, use of RNAi mediated silencing of rice cytokinin gene showed downregulation of *OsCKX2* that led to a higher tiller number and grains per with delayed senescence (Yeh et al., 2015). He et al. (2018) overexpressed the *OsAFB6* (*Auxin-signaling F-Box 6*) which increased the cytokinin levels by supressed the *Gn1a* (*OsCKX2*) and increase the IAA concentration in the rice panicle. In the overexpressed lines, the increased cytokinin delayed heading but improved grain yield. In *Jatropha curcas*, CRISPR/Cas9 technology was used to create knockout mutants of *JcCYP735a*, which resulted in significantly lower concentrations of tZ and tZ-riboside (tZR), as well as severely stunted growth (Cai et al., 2018). In addition, Halubavaet et al. (2018) generated CRISPR mediated knock out of *HvCKX1* gene generated homozygous transgenic plants with reduce root growth and increase in grain productivity. Li et al. (2018) focused on silencing the *TaCKX2.4* gene in *Triticum aestivum*, which reduced the activity of the cytokinin oxidase in transgenic plants, resulting in cytokinin accumulation.

TABLE 1 | Summary of cytokinin oxidase/dehydrogenase (CKX) expression/activity on yield attributes in agronomically important crops.

S. No	Cultivar	Technique used	Gene	Observation	Reference
Oryza sativa (Cereal)					
1	*O. sativa* ssp. *japonica* cv. Nipponbare and Zhonghua 11	CRISPR/Cas9	*OsCKX4* and *OsCKX9*	The double mutant had a significantly greater number of tillers, compared to the wild type	Rong et al. (2021)
2	*O. sativa* ssp. *japonica* cv. Nipponbare	CRISPR/Cas9	*OsCKX11*	Genome edit lines were found to be effective in delaying leaf senescence, increasing grain number, and coordinating source and sink regulation	Zhang et al. (2021)
3	*O. sativa* ssp. *japonica* cv. Nipponbare and Wuyunjing 30	Overexpression lines	*RGG1*	Cytokinin content was found to be lower in the overexpression lines	Tao et al. (2020)
4	*O. sativa* cv. Nipponbare	RNAi and CRISPR/Cas9	*OsNAC2* (encodes a NAC TF)	The primary root length and number of crown roots both were increased in the modified plant	Mao et al. (2019)
5	*O. sativa* cv. Nipponbare	Overexpression lines	*Os6*	Identified in rice that glycosylated cytokinin *in vitro*	Li et al. (2019)
6	*O. sativa* ssp. *japonica* cv. Nipponbare and Zhonghua 11	CRISPR/Cas9, overexpression lines	*CKX9* (responds only to strigolactone)	Mutants and overexpressing transgenic plants both exhibited significant increases in tiller number while simultaneously experiencing significant decreases in panicle size and plant height	Duan et al. 2019
7	*O. sativa* cv. Zhonghua 11	Overexpression lines	*OsAFB6* (*Auxin-signaling F-Box 6*)	Transgenics lines significantly increased the number of spikelets per panicle and primary branch number all led to a 50% increase in grain yield	He et al. (2018)
8	*O. sativa* cv. TNG67	RNAi	*OsCKX2*	Transgenic crop demonstrated improved yield with increased tiller number	Yeh et al. (2015)
9	*O. sativa* cv. Koshihikari	RNAi and overexpressed lines	*OsCKX2* (*Gn1a*)	The number of reproductive organs in transgenic lines increased, resulting in increased grain yield	Ashikari et al. (2005)
Triticum aestivum (Cereal)					
10	*T. aestivum* cv. Paragon and Bobwhite	CRISPR/Cas9 and TILLING	*GW2*	Increased GS and TGW were observed in the knockout mutant	Wang et al. (2018)
11	*T. aestivum* cv. Kenong 9204	RNAi	*Ta2.2.1-3A*	Displayed increased grain yield	Li et al. (2018)
12	*T. aestivum* cv. Kontesa	RNAi	*TaCKX1*	Transgenic lines had a positive effect on plant productivity	Zalewski et al. (2010)
Hordeum vulgare (Cereal)					
13	*H. vulgare*	CRISPR/Cas9	*HvCKX1*, *HvCKX3*	The root phenotype of the knockout mutant was altered, but grain yields were not increased	Gasparis et al. (2019)
14	*H. vulgare* cv. Golden Promise	RNAi and CRISPR/Cas9	*HvCKX1*	Transgenic lines exhibited decreased root growth, but they had more tillers and grains than WT, and the total yield increased to 15%	Holubová et al. (2018)
15	*H. vulgare* cv. Golden Promise	Transgenic lines	*AtCKX1*, *AtCKX2*	Transgenic plants have larger root systems and are able to withstand long-term droughts than WT.	Ramireddy et al. (2018)
16	*H. vulgare* cv. Golden Promise	Transgenic lines	*AtCKX1*	Roots with higher drought tolerance were observed in the overexpressing line	Pospíšilová et al. (2016)
17	*H. vulgare* cv. Golden Promise	Overexpression lines	*HvCKX1*	Overexpression plant lines were unable to flower with rapid root proliferation and high root-to-shoot ratios	Mrizová et al. (2013)
18	*H. vulgare* cultivar Golden Promise	RNAi	*HvCKX1*, *HvCKX2*	Transgenic lines resulted in increased productivity which was achieved through increased seed production and grain yield	Zalewski et al., 2010; 2012
Jatropha curcas (Biofuel)					
19	*J. curcas*	CRISPR/Cas9	*IPTs*, *CYP735A* and *CKXs*	Displayed spatio-temporal expression	Cai et al. (2018)
Brassica napus (Oilseed)					
20	*B. napus* cv. Kristina	Transgenic plants	*AtCKX2*	The transgenic lines developed displayed increased number of lateral and adventitious roots. Moreover, the leaves of transgenic plants accumulated higher levels of macro- and microelements	Nehnevajova et al. (2019)
Solanum tuberosum (Tuber crop)					

(Continued on following page)

TABLE 1 | (*Continued*) Summary of cytokinin oxidase/dehydrogenase (CKX) expression/activity on yield attributes in agronomically important crops.

S. No	Cultivar	Technique used	Gene	Observation	Reference
21	*S. tuberosum* cv. Désirée	Overexpression lines	*AtCKX2*	Overexpressing lines exhibited fewer shoot and remarkably increased CKX activity. Under inducing conditions, tuberization was found to be improved in these lines	Raspor et al. (2012)

Abbreviations: RNAi, RNA interference; TF, transcription factor; GS, grain size; TGW, thousand grain weight.

Increased cytokinin accumulation in inflorescence meristems results in an increase in the number of reproductive organs, which leads to an increase in the number of grains per spike, spike length, thousand-grain weight (TGW), seed length, and seed width. In *H. vulgare*, Gasporis et al. (2019) reported CRISPR-mediated knockout of the *HvCKX1* and *HvCKX3* genes. The CKX enzyme was found to be lower in the spikes of these knockout, and the root surface, morphology, and hair were all greater in comparison to the WT. Increased cytokinin glycoside levels were observed in *A. thaliana* overexpressing the rice Os6 cytokinin glycosyltransferase gene (Li et al., 2019). In addition, Nehnevajova et al. (2019) found that overexpressing the *AtCKS2* gene in oilseed *Brassica napus* increased the root-to-shoot ratio. CRISPR-edited RGG1 (G proteins) in *O. sativa* decreased endogenous cytokinin levels and reduced grain size and plant development, as demonstrated by Tao et al. (2020). It has been shown that during leaf senescence *OsCKX11* plays an antagonistic role in both the cytokinin and abscisic acid (ABA) pathways. The downregulation of ABA biosynthesis genes and the upregulation of ABA degradation genes in CRISPR/Cas9 edited *osckx11* mutants result in a reduction of ABA content in flag leaves and, as a result, regulate leaf senescence, demonstrating the relationship between cytokinin and ABA. These results show that *OsCKX* genes serve as a link between cytokinin and other plant hormones (Zhang et al., 2021). Similarly, in 2021, Rong et al. demonstrated an efficient method of CRISPR-mediated genome editing in rice cultivars Nipponbare and Zhonghua 11 to create knock out mutants of *OsCKX4* and *OsCKX9* gene. The mutant had a higher number of tillers than the wild type in both field and pot experiments. In comparison to the control, the double mutant *osckx 4* and *osckx 9* created through genome editing techniques had a higher tiller number. These findings shed light on the functions of *CKX* genes in a variety of crop species and could serve as a foundation for future research aimed at increasing yield potential.

CRISPR/CAS9: A RAPIDLY EVOLVING BIOLOGICAL TOOL FOR EDITING PLANT CYTOKININ

CRISPR/Cas9: Mechanism

The Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease9 (CRISPR/Cas9) gene editing system was designed on the adaptive immune defence mechanism that the bacteria used to attack the entering viruses and plasmids (Pramanik et al., 2020). In a nutshell, CRISPR/Cas-mediated editing works by using short RNA sequences called guide RNAs (gRNAs) to complementarily target DNA, which is then cleaved by a Cas endonuclease. The DNA cleavage recognition motif, known as a protospacer adjacent motif (PAM), is found on the endonuclease. A few base pairs away from the PAM, the Cas protein cleaves DNA. CRISPR/Cas, like other methods of gene editing, takes advantage of two different types of DNA repair mechanisms i.e., nonhomologous end-joining (NHEJ) and homologous recombination (HR). In plant cells, NHEJ is the primary repair mechanism, and its imprecise repair results in indels (insertions or deletions) within a gene sequence, causing gene expression to be disrupted (Puchta, 2005). The knockout lines generated are often used in reverse genetics to decipher the role and function of a particular genes. But at the other hand, plant cells are less likely to repair themselves through HR because it is much less effective than NHEJ (Schmidt et al., 2019). Additionally, homology-directed repair (HDR) is a technique that involves delivering a DNA repair template along with the CRISPR/Cas components in order to insert a DNA sequence. Moreover, HDR is inefficient in plants, with only one in every 10^5–10^4 transformation events, owing primarily to the fact that NHEJ is the principal repair mechanism in plants (Horvath and Barrangou 2010).

CRISPR/Cas9: Development of the Genome Editing Tools

Discovered in 1987 by Japanese scientists, the *Escherichia coli* genome contained some previously unknown tandem repeats, but they did not investigate further for their biological role (Ishino et al., 1987). Although these sequences were given the name Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in 2002, the significance of these sequences remained unknown until recently (Jansen et al., 2002). Protospacer-adjacent motifs (PAMs) were discovered by three separate research groups in 2005, and it was hypothesised that they would guide the type II Cas9 nuclease to cut DNA (Mojica et al., 2005; Bolotin et al., 2005). Barrangou et al., in 2007, established that the CRISPR system is indeed a prokaryotic adaptive immune system, demonstrating that bacteria can change their resistance to phages by incorporating phage gene sequences (Barrangou et al., 2007). Brouns et al., in 2008 discovered that in order to perform a defensive action, the non-coding RNA transcribed from the CRISPR proto-inter-

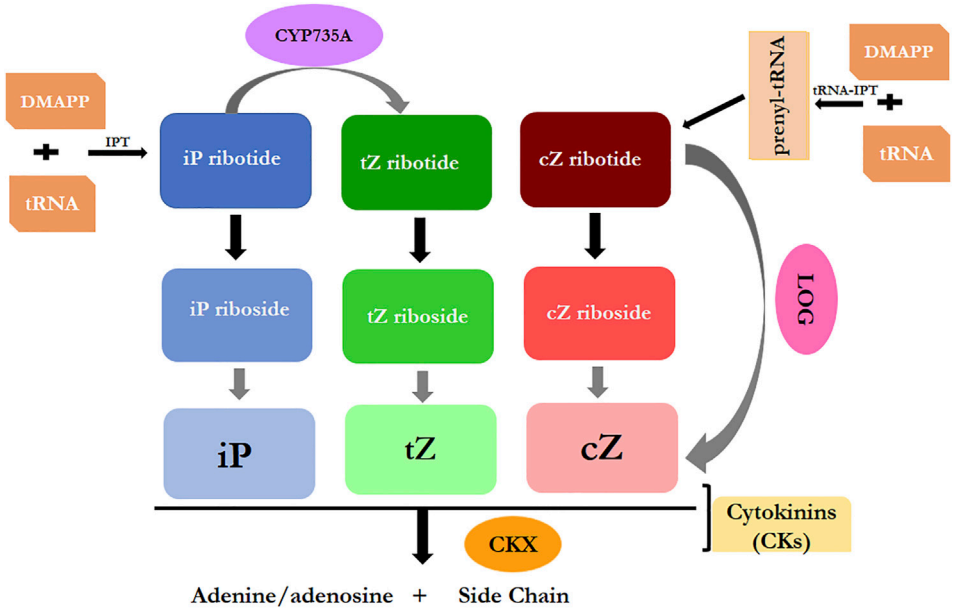

FIGURE 2 | Schematic representation for the cytokinin biosynthesis and degradation pathways.

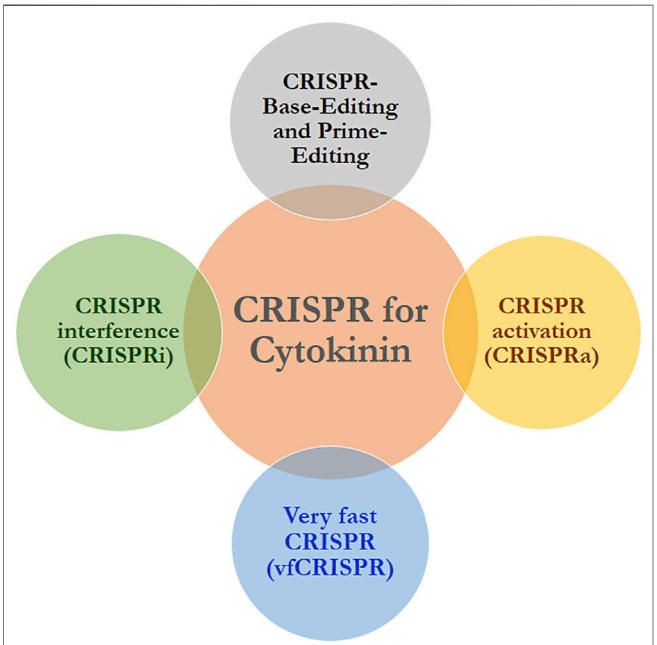

FIGURE 3 | Proposed CRISPR/Cas9 applications to target cytokinin in crop plants. CRISPR knock out (CRISPR KO) is presently successfully applied (reported in the review). We propose CRISPR-Base-Editing and Prime-Editing, CRISPRi, CRISPRa and vfCRISPR can also be used in this regard.

regional sequence guides the Cas protein to target-specific part of DNA (Brouns et al., 2008). Deltcheva et al. demonstrated in 2011 that trans-coding crRNA (tracrRNA) is involved in the pre-crRNA processing and maturation process, and their research identified new pathways for crRNA maturation (Deltcheva et al., 2011). Furthermore, *in vitro* experiments in 2012 showed that mature crRNA formed a unique double-stranded RNA structure with tracrRNA through base complementary pairing, directing Cas9 protein to cause double-stranded DNA break (Jinek et al., 2012). It has been well established that the CRISPR/Cas9 system has successfully edited numerous agronomical traits in an variety of crop species (Jaganathan et al., 2018). Additionally, the dCas9 protein mutant (endonuclease-deficient) was first created in 2013, losing its nuclease activity (Qi et al., 2013). By fusing the dCas9 protein with transcription regulators that activate or inhibit gene transcription, the CRISPR activation (CRISPRa) and interference (CRISPRi) tools were developed (**Figure 3**) (Maeder et al., 2013; Gilbert et al., 2013). To address the issue of unexpected disruptions in the CRISPR/Cas9 gene editing system, Komor et al. fused APOBEC (cytosine deaminase) with CRISPR/Cas9. Under the guidance of gRNA, this modified Cas9 performs the C→T (or G→A) conversion without causing DSB. With the help of this base editor, a variety of point mutations in the genome could be successfully corrected (Komor et al., 2016). In addition, the Adenine Bases Editor (ABE) was created to convert A-T base pairs to G-C base pairs (**Figure 3**) (Gaudelli et al., 2017). The single-base editing and CRISPR/Cas9 systems were further improved by the team, which resulted in a significant decrease in the miss rate of the single-base editor and an increase in the target range of spCas9, respectively (Doman et al., 2020; Miller et al., 2020). Gilpatrick et al., for instance, used Cas9 and adapter ligation to develop a nanopore Cas9-targeted sequence (nCATS) for third-generation nanopore sequencing by modifying the target DNA region and altering the target genome's structure, allowing for the reading of long fragments at a low cost (Gilpatrick et al., 2020). Recently, researchers developed a

caged RNA strategy that enables Cas9 to bind to DNA but does not cleave prior to light-induced activation. This method was known as very fast CRISPR (vfCRISPR) that creates double-strand breaks (DSBs) at the submicrometer and second scales (**Figure 3**). Due to the high accuracy of the vfCRISPR and the ability to edit only one allele at a time, it can be used to investigate complex genetic traits (Lino et al., 2018). Taken together, these advancements enabled the CRISPR/Cas9 transition from a blunt to a precise genome editing tool.

CRISPR/Cas9: RNP-Mediated DNA-Free Genome Editing

Plant cells and tissues are most commonly targeted by Agrobacterium or particle bombardment-mediated transformation with DNA containing CRISPR expression cassettes. This is the most common method for the delivery of CRISPR reagents, such as Cas proteins and gRNAs, into plants. Concerns and regulatory burdens arise from the random integration of CRISPR cassettes into plant genomes. There are concerns about the random integration of CRISPR cassettes into genomes which results in imposing regulatory burden. Breeding can be used to remove these transgene elements, but this process is time- and labor-intensive, making it unsuitable for species or varieties that have long juvenile growth periods or that are typically vegetatively propagated, making it impractical. It has been possible to generate transgene-free genome-edited plants by using transient expression of transgenes without selection, which has been found to be effective in preventing the integration of foreign DNA (Iaffaldano et al., 2016; Zhang et al., 2016). Despite the fact that particle bombardment can be used to deliver a wide range of cargo, it can also cause genome damage and random DNA integrations into the plant genome, making it difficult to detect and eliminate the transgenes (Banakar et al., 2019; Liu et al., 2019). When transgenes are continuously expressed, the genome is exposed to CRISPR agents, which increases the risk of off-target mutations and chimeric mutants being generated (Fu et al., 2013; Pattanayak et al., 2013; Hashimoto et al., 2016). Furthermore, efficient species-specific expression systems, such as optimal promoters and terminators, as well as codon optimization, are required for plasmid-based CRISPR genome editing to be successful. When editing multiple targets, an efficient multiplexing system is also essential, and this is especially true for polyploid plants, where more than one gRNA may be required to knock out a single gene. The integration position of transgenes can also influence their expression (Matzke and Matzke, 1998). Furthermore, Cas protein expression based on DNA is toxic in some mammalian cell types and organisms (Morgens et al., 2017; Foster et al., 2018; Jiang et al., 2021). To avoid the problems that come with DNA delivery, particle bombardment can be a alternative method to deliver mRNA encoding Cas proteins along with the gRNA(s) into the plant system (Zhang et al., 2016). It is also possible to introduce CRISPR-associated ribonucleoproteins (RNPs) into plants that already contain the Cas protein and gRNA. Due to the fact that there is no recombinant DNA involved in this process when the gRNAs are chemically synthesised, plants edited by RNPs can be considered to be transgene free. RNPs, on the other hand, are only transiently present in plant cells and are degraded by endogenous nucleases and proteases, reducing the likelihood of off-target effects and mosaicism that can result from prolonged exposure of genomic DNA to CRISPR-based technologies (Kim et al., 2014; Kim et al., 2017; Liang et al., 2017). RNPs can be used for any organism that does not have delivery barriers, and there is no need to consider promoter compatibility or multiplexing strategies when using RNPs for any organism. Furthermore, multiple gRNAs can be used concurrently to target distinct genomic regions. If there is no crosstalk between the different types of CRISPR/Cas systems and orthogonal Cas proteins, they can be used simultaneously, allowing for multiple editing outcomes (Najm et al., 2018). It is also possible to use multiple types of CRISPR/Cas systems and orthogonal Cas proteins at the same time if there is no crosstalk between them, allowing for the achievement of multiple editing outcomes. CRISPR/Cas delivered via plasmid, on the other hand, causes cell or organism toxicity, which could be avoided by using RNP-mediated genome editing (Shin et al., 2016; Foster et al., 2018; Jiang et al., 2021). *Caenorhabditis elegans*, human cells, mice and plants all have been shown to benefit from RNP-mediated genome editing (Cho et al., 2013; Kim et al., 2014; Woo et al., 2015; Zuris et al., 2015; Foster et al., 2018; Li et al., 2020). Additionally, the *in vitro* and *in vivo* use of RNPs for screening gRNAs is well-established. Even though RNP-mediated genome editing does not require transcription or translation, it can be completed as soon as cells are transfected with the RNP construct (Kim et al., 2014). There is evidence of RNP-mediated genetic engineering in plant protoplasts, as well as RNP-edited plants (Jiang et al., 2021). Therefore, RNP-based CRISPR technology has the potential to generate transgene-free and improved germplasm that can be more easily commercialised, it still faces numerous technological constraints and challenges.

CONCLUSION

It is well established that cytokinins play an important role in many aspects of plant growth and development, and their positive impact on grain yield is well documented. Significant efforts have been made over the last decade to improve yields by utilizing endogenous cytokinins. Reduced CKX expression has increased yield regardless of whether the crop is monocot or dicot. Likewise, according to Bartrina et al. (2011), the function of CKX genes in yield determination has been evolutionarily conserved and is of functional significance for all or most of the agronomically important crops (Bartrina et al., 2011). To achieve fine control, it appears necessary to identify the spatio-temporal expression patterns of CKX gene family members expressed in the shoot apical meristem (SAM) and/or during seed development. This information will aid in the identification of gene-specific, functionally associated

markers for marker assisted selection (MAS) methods, as well as in the induction and/or detection of beneficial mutations using a targeting induced local lesions in genomes (TILLING) strategy. In fact, even the CRISPR technique will also require such in-depth understanding.

Despite their multifaceted role, manipulating cytokinins is challenging, due to their pleiotropic nature as well as their multigene family composition, which allows for spatio-temporal expression patterns (Jameson and Song, 2016). However, current genetic approaches (RNAi, CRISPR) are now been developed to specifically target members of the CKX gene family, which may result in increased seed yield, without any detrimental effect on the plant phenotype (**Table 1**). By targeting cytokinin metabolism at the genetic level, modern genome-editing tool, CRISPR open up new avenues for crop improvement. Moreover, the use of novel GE tools, allows for the simultaneous targeting of multiple genes by a single construct which vastly enhancing the scope of genome editing (Gasparis et al., 2018; Ogonowska et al., 2019). Combining rapidly evolving advancements in CRISPR/Cas tool with knowledge of IPT or CKX gene function could speed up the development of new, higher-yielding cultivars that could shape future agricultural. Whether IPT or CKX is targeted, and how the cytokinin level is altered, may be dependent on whether the crop is considered to be in a sink- or a source-limited environment. In addition, cytokinins have also been implicated in the response to both abiotic and biotic stress responses (Cortleven et al., 2019). As a result, targeting CKs will significantly enhance crop yields while also increasing tolerance to various environmental stress in a wide range of agronomically important plants.

AUTHOR CONTRIBUTIONS

SM: Primary draft, MG: primary draft, tables, revision, UA: conceptualization and revision, DR: primary draft, data curing, NK: data curing, TM: conceptualization and revision, AM: Guidance, data curing, NJ: figures, tables, ML: figures, tables, revision, RT: figures, tables, revision, MK: conceptualization and revision, Radha: conceptualization and revision, AG: guidance, data curing, RB: revision and response, JP: figures, tables, revision, conceptualization, project administration and funding acquisition, AD: revision, conceptualization, overall guidance.

FUNDING

The APC is financed by Wrocław University of Environmental and Life Sciences.

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frontiers | Frontiers in Genetics

SYSTEMATIC REVIEW
published: 20 May 2022
doi: 10.3389/fgene.2022.900324

Chickpea Biofortification for Cytokinin Dehydrogenase *via* Genome Editing to Enhance Abiotic-Biotic Stress Tolerance and Food Security

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Globally more than two billion people suffer from micronutrient malnutrition (also known as "hidden hunger"). Further, the pregnant women and children in developing nations are mainly affected by micronutrient deficiencies. One of the most important factors is food insecurity which can be mitigated by improving the nutritional values through biofortification using selective breeding and genetic enhancement techniques. Chickpea is the second most important legume with numerous economic and nutraceutical properties. Therefore, chickpea production needs to be increased from the current level. However, various kind of biotic and abiotic stresses hamper global chickpea production. The emerging popular targets for biofortification in agronomic crops include targeting cytokinin dehydrogenase (*CKX*). The *CKXs* play essential roles in both physiological and developmental processes and directly impact several agronomic parameters i.e., growth, development, and yield. Manipulation of *CKX* genes using genome editing tools in several crop plants reveal that *CKXs* are involved in regulation yield, shoot and root growth, and minerals nutrition. Therefore, *CKXs* have become popular targets for yield improvement, their overexpression and mutants can be directly correlated with the increased yield and tolerance to various stresses. Here, we provide detailed information on the different roles of *CKX* genes in chickpea. In the end, we discuss the utilization of genome editing tool clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) to engineer *CKX* genes that can facilitate trait improvement. Overall, recent advancements in *CKX* and their role in plant growth, stresses and nutrient accumulation are highlighted, which could be used for chickpea improvement.

Keywords: biofortification, chickpea, cytokinindehydrogenase *(CKX)*, genome-editing, stress

OPEN ACCESS

Edited by:
Santosh Kumar Gupta, National Institute of Plant Genome Research (NIPGR), India

Reviewed by:
Surendra Pratap Singh, Chhatrapati Shahu Ji Maharaj University, India
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *20 March 2022*
Accepted: *22 April 2022*
Published: *20 May 2022*

Citation:
Mahto RK, Ambika, Singh C, Chandana BS, Singh RK, Verma S, Gahlaut V, Manohar M, Yadav N and Kumar R (2022) Chickpea Biofortification for Cytokinin Dehydrogenase via Genome Editing to Enhance Abiotic-Biotic Stress Tolerance and Food Security. Front. Genet. 13:900324. doi: 10.3389/fgene.2022.900324

INTRODUCTION

Micronutrient malnutrition, often known as "hidden hunger" affects more than half of the world's population, with pregnant women and children in developing nations bearing the brunt of the burden. According to the World Health Organisation (WHO), more than 2 billion people are suffering from hidden hunger and one of the most important responsible factors for malnutrition is food insecurity which affects almost a billion people worldwide (FAO, 2013). Food security is defined as when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for active and healthy life (FAO, 2016). This is particularly an issue in developing countries where families cannot afford or get access to sufficient and nutritious food. Each year twenty million infants are born with low body weight. As per the malnutrition report around 150.8 million stunted, 50.5 million wasted, and 38.3 million overweight children under five years of age are found (WHO, 2018; Kumar and Pandey, 2020; Ahmed et al., 2022). The reason for malnutrition is due to the insufficient or poor-quality supply or uptake of nutrition. The consumption of legumes in daily diets, especially in developing countries (Afro-Asian countries), could majorly eradicate protein malnutrition. Legumes could act as a base for the development of many functional foods to promote health benefits in humans (Maphosa and Jideani, 2017; Kumar and Pandey, 2020; Ahmed et al., 2022).

Chickpea (*Cicer arietinum* L.) is the world's second largest, cool season food legume. It is in high demand owing to its high nutritional value. Chickpea is considered invaluable because it provides food for human consumption and feed to livestock. Owing to these astounding properties of chickpea, its production needs to be enhanced to feed and ensure nutritional health and well-being of world's population. It is also essential to involve the screening techniques, which is useful to promote the breeding program for increasing the growth of chickpea (Talip et al., 2018). However, various factors hamper the yield of this crop. Climate variability has altered plant physiology in a variety of ways. Multiple stressors on plants are increased as a result of environmental extremes and climate unpredictability (Thornton et al., 2014). Heat stress reduces grain yield and productivity, cold stress causes sterility, and drought stress has a deleterious impact on plant morpho-physiology (Barlow et al., 2015). The generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($.O_2$), and hydroxyl radicals ($.OH^-$) are accelerated in plant tissues due to harsh circumstances. In addition to other stress hormones like abscisic acid (ABA), jasmonate, and salicylic acid, cytokinins (CKs) also play a significant role in increasing plant stress tolerance and regulate the action of plants defensive mechanisms. Thus, understanding the roles of cytokinin in plant responses to abiotic stressors is very critical imperative.

The cytokinins have been largely involved in the regulation of plant yield, particularly by influencing the grain related traits i.e., number, size and growth of the root (Yamburenko et al., 2017). Moreover, they are found to be involved in stress regulations (Cortleven et al., 2019) and plant mineral concentration status (Gao et al., 2019). It has also been reported that cytokines negatively affect micronutrient uptake regulations (Gao et al., 2019). Cytokinin dehydrogenase (*CKXs*) is a key enzyme that regulates the cytokinin hormone level in plants (Sakakibara, 2006). It is a small gene family, for instance in Arabidopsis only seven *CKX* genes were reported (Werner et al., 2003). Several studies in Arabidopsis showed that *CKX* genes regulate plant growth and developmental processes including shoot and root development, reproductive meristem activity (Werner et al., 2006; Bartrina et al., 2011), and mineral [phosphorus (P), calcium (Ca), sulfur (S) and microelements like copper (Cu), manganese (Mn), iron (Fe), and zinc (Zn)] accumulations (Werner et al., 2010; Bartrina et al., 2011; Chang et al., 2015). However, related information and functional studies about *CKX* genes in chickpea and their utilization to improve agronomic traits are lacking. Various techniques are being employed to develop a sustainable agriculture system to decrease food insecurity. One such method is Genome editing (GE) for crop improvement that has the potential to create a climate-resilient agriculture system on a global scale (Liu et al., 2013). GE technologies have had a significant impact on plant breeding techniques including new strategies for making rapid and precise changes in crop genomes to protect plants from various challenges and enhance crop outputs (Taranto et al., 2018). In genome editing methods, site-specific endonucleases such as zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and CRISPR-Cas9 are used (Zhu et al., 2017). Unlike the ZENs and TALENs genome editing tools, the CRISPR/Cas9 system is proving to be the most effective GE technology since it is cost-efficient, rapid, accurate, and allows for various site-specific genome editing (Abdelrahman et al., 2018a). In comparison to other genome editing methods like TALENs/ZFNs, CRISPR-based techniques have been intensively investigated in plant genomes. It also offers a lot of potential for assisting crop breeders in developing high-yielding, stress-resistant varieties (Abdelrahman et al., 2018b). Most importantly, CRISPR/Cas9 is coming up as a straight forward, environmentally benign strategy for making genome-edited non-transgenic plants to combat environmental extremes and maintain food security (Haque et al., 2018).

Apart from genome editing tools like CRISPR-Cas9 which are being increasingly used nowadays to develop stress tolerant varieties in different crops, there are some alternative strategies as well. Selection from landraces, hybridization to develop novel variety followed by pedigree selection, mutation breeding, and exploitation of hybrid vigour are examples of traditional breeding procedures that have resulted in considerable improvements in stress tolerance and nutritional quality. Applications of PGPRs are being used to alleviate abiotic stresses and increase productivity in economically important crops including rice, soybean, lettuce, tomato, maize and wheat. Nano-biotechnology has also proven to be a promising tool for sustainable agriculture, seed treatment and germination, plant growth and development, disease diagnostics, and detection of harmful agrochemicals (Nuruzzaman et al., 2016).

In this comprehensive review article, we emphasize the importance of legumes with reference to chickpea,

malnutrition and food security, biotic and abiotic stresses, and the role of cytokinin. We also discuss how recently developed genome editing technologies such as CRISPR/Cas9 are being utilized to engineer *CKX* genes to improve agricultural traits and biofortification in chickpea.

LEGUMES AS BOON TO MANKIND

Legumes are plants of the Leguminosae/Fabaceae family that bear seeds in pods (Staniak et al., 2014; Kouris-Blazos and Belski, 2016) and as a distinguishing feature fix atmospheric N_2 in symbiosis with suitable rhizobia. Agriculturally significant legumes fix 40–60 million metric tonnes N_2, along with an additional 3–5 million tonnes by wild legumes, annually (Smil, 1999). The principal edible legumes are bean, broad bean, chickpea, cowpea, pea, pigeon peaand lentil (National Academy of Science, 1994). However, peas, broad beans, lentils, soybeans, lupins, sprouts, mung bean, green beans, and peanuts are common legumes utilized for human consumption and are known as grain or grain food legumes (Yorgancilar and Bilgicli, 2014). Food legumes are classified into two types: oilseeds and pulses. The oilseeds are high-oil-content legumes such as soybeans and peanuts, whereas the pulses are all dry seeds of cultivated legumes eaten as traditional food. These seeds are recognized globally as a low-cost meat substitute and regarded as the second most important dietary source after cereals (Kouris-Blazos and Belski, 2016). Legumes are high inprotein, essential amino acids, complex carbohydrates, dietary fibre, unsaturated fats, vitamins, and critical minerals, all of which are important in the human diet (Bouchenak and Lamri-Senhadji, 2013; Clark et al., 2014; Rebello et al., 2014). Due to the abundance of useful bioactive chemicals, legumes also have been assigned economic, cultural, physiological, and therapeutic functions in addition to their nutritional excellence.

Legumes are an excellent source of high-quality protein, including 20–45% protein and particularly high in the important amino acid lysine (Philips, 1993). Peas and beans contain 17–20% protein, whilst lupins and soybeans contain 38–45% protein (Mlyneková et al., 2014; Kouris-Blazos and Belski, 2016). Legumes contain twice the protein level of cereals and are richer in protein than most of the other plant diets (Leonard, 2012; FAO, 2016; Kouris-Blazos and Belski, 2016). Leguminous proteins, with the exception of soybean protein, are poor in the important sulphur-containing amino acids namely methionine, cysteine, and cysteine, as well as tryptophan, and are thus regarded as an inadequate source of protein (Kouris-Blazos and Belski, 2016). The primary components of leguminous protein are albumins and globulins, which are further subdivided into vialin and legumin. Vialin is the primary protein group in most of the legumes and is defined by a low quantity of sulphur-containing amino acids, which reflects those legumes have small amounts of sulphur-containing amino acids (FAO, 2016). In terms of protein, legumes and cereals complement each other because cereals are high in sulphur-containing amino acids (poor in legumes) and low in lysine (high in legumes) (Staniak et al., 2014). As a result, when beans are combined with grains, the protein quality improves dramatically (FAO, 2016).

Legumes contain up to 60% carbohydrates by dry weight and are source of complex energy-giving carbohydrates (Leonard, 2012). Leguminous starch digests more slowly than cereal and tuber starch. As a result, beans have a low glycemic index (GI) rating for blood glucose control (Philips, 1993; Khalid and Elharadallou, 2013) making them ideal for diabetic patients and those at greater risk of acquiring diabetes. In general, legumes are beneficial for people who want to live a healthy, disease-free lifestyle (Bouchenak and Lamri-Senhadji, 2013). Legumes are also a rich source of dietary fiber (5–37%), with large levels of both soluble and insoluble fibers (Philips, 1993; Leonard, 2012; Kouris-Blazos and Belski, 2016). Diets high in dietary fiber have been linked to plenty of health advantages. Constipation, obesity, diabetes, heart issues, piles, and various malignancies are among the various diseases and ailments that can be prevented and treated (Maphosa and Jideani, 2016; Tamang et al., 2016).

Except for peanuts (45%) and soybeans (47%), legumes have no cholesterol and are generally low in fat, with 5% calories from fat (Messina, 2016). Legumes have a high concentration of mono- and polyunsaturated fatty acids (PUFA) and almost no saturated fatty acids (Kouris-Blazos and Belski, 2016). Kidney beans and chickpeas have the highest levels of PUFA (71.1%) and MUFA (34%), respectively (Kouris-Blazos and Belski, 2016). Because the human body cannot synthesize these PUFAs, they must be consumed through the diet (FAO, 2016). Legumes are high in B-complex vitamins like foliate, thiamin, and riboflavin, but low in fat-soluble vitamins and vitamin C. (Kouris-Blazos and Belski, 2016). Folate is an important nutrient shown to minimize the likelihood of neural tube abnormalities such as spina bifida in newborns (FAO, 2016; Messina, 2016). Zinc, iron, calcium, selenium, phosphorus, copper, potassium, magnesium, and chromium are also found in legumes (Brigide et al., 2014; Kouris-Blazos and Belski, 2016). These micronutrients serve critical physiological functions in bone health (calcium), enzyme activity and iron metabolism (copper), carbohydrate and lipid metabolism (chromium, zinc), hemoglobin production (iron), antioxidative action, protein synthesis, and plasma membrane stability (zinc) (Mogobe et al., 2015). Legumes are often low in sodium, which is ideal given current developments advocating sodium reduction (Leonard, 2012).

CHICKPEA AS A WONDER LEGUME

Chickpea has been identified as the second most important legume and it has numerous economic facilities. According to Chen et al. (2020), India has been identified as the largest chickpea producer with nearly 65% of total global chickpea production. It is being cultivated on about 12 million hectares and its annual production rate is 9 million tonnes. Chickpea contains high protein content, and is also rich in dietary fibres, calcium, zinc, phosphorus and magnesium. Due to a heavy breeding programme, the production rate of chickpea is gradually increasing in the last thirty years. Determination of

the effectiveness and importance of yield components have been identified as the main target. According to Coyne et al. (2020), it has been reported that there is a positive relationship between plant height, seed mass, number of pods per plant and number of branches. The grain yield of chickpea is dependent on different quantitative characteristics, such as environmental location and genetic factors.

Chickpea is a true self-pollinated diploid (2 n = 2 x = 16) with an estimated genome size of 738 Mb having 28,269 genes (Varshney et al., 2013). It is the second most economically important pulse crop with the production of 14.24 million metric tons (FAOSTAT, 2019). Chickpea is valuable because it provides both human food and livestock feed and there is a growing demand for chickpea due to its nutritional value. It is an effective source of protein, carbohydrates, minerals and vitamins, dietary fiber, folate, β-carotene, anti-oxidants, micronutrients (phosphorus, calcium, magnesium, iron and zinc) and health-promoting fatty acids (Sharma et al., 2013a; 2013b). It is rich in carbohydrates and proteins, which altogether account for 80% of the total mass of dry seed (Chibbar et al., 2012). Chickpea's protein content trends vary considerably by17–22% as a % of the total dry seed mass before dehulling and 25.3–28.9% after dehulling (Hulse, 1989; Misra et al., 2016) and is about 2-3 folds more than cereals. The composition of amino acid in chickpea is well balanced having minimal amino acid containing sulphur i.e., methionine, cysteine and a considerable amount of lysine making it an excellent combination with cereals, which are good source of sulfur-containing amino acids. It is rich in carotenoids responsible for the yellow color of the cotyledon. The prominent and widely distributed carotenoid in chickpea is β-carotene and is more efficiently transformed to vitamin A than any other carotenoids. Chickpea has a higher amount of β-carotene on a dry seed weight basis than "golden rice" endosperm or red wheat. It has a higher dietary fiber content (~18–22 g), particularly in comparison to wheat (~12.7 g) and a higher fat content, especially in comparison to other pulses or cereals and two polyunsaturated fatty acids (PUFAs) namely, linoleic and oleic acids that constitute approximately ~50–60% of chickpea fat, therefore works as cholesterol reducer food.

ABIOTIC AND BIOTIC STRESSES IN CHICKPEA

External factors that negatively affect plant growth, development, or productivity are referred to as stress in plants (Verma et al., 2013). Plants face abiotic and biotic the two main types of stresses and as sessile organisms are continually confronted with a variety of biotic and abiotic stressors. They require continual alterations at the molecular level in order to adapt to changing conditions. Epigenetic regulators provide efficient and effective controls to promote plant survival by increasing their tolerance to stresses (Richards, 2006; Hirayama and Shinozaki, 2010). Different chemical alterations at the molecular level that regulate gene expression are involved in epigenetic control. Today, epigenetics refers primarily to alterations that are related to chemical modifications not to changes in DNA sequence and can be passed on through generations (Feng et al., 2010; Fujimoto et al., 2012). Plants use three types of epigenetic regulatory systems to resist severe conditions caused due to stress conditions: DNA methylation, histone modification, and RNA interference (RNAi). Plants respond to stresses in a variety of ways, including changes in gene expression, cellular metabolism, growth rates, crop yields, and so on. Severe stresses cause crop plants to die by inhibiting flowering, seed development, and inducing senescence (Verma et al., 2013). Abiotic stress is the adverse effect on biological organisms in a particular environment caused by non-living elements. Toxic abiotic stressors, such as hyper drought and salinity, low or high temperatures, depleted or surplus water, high salt levels, heavy metals, and UV radiation are examples of abiotic stress which pose a threat to plant development and growth, resulting in a significant agricultural production penalty throughout the globe. These stresses also adversely affect plant nutrition, for instance when water availability is limited due to drought, total nutrient intake is lowered, and mineral nutrient concentrations in agricultural plants are frequently reduced. Water shortages have the most significant impact on nutrient transport to the root, root growth and extension. The interference of nutrient uptake and unloading mechanisms as well as lower transpiration flow results in reduced nutritional absorption (Marschner 1995; Baligar et al., 2001). Due to lower tissue nutrient concentrations, root development is inhibited when the soil temperature is too low. Nutrient insufficiency may lead to stunted or dying of plant tissue as well as yellowing of leaves. A reduction in crop output or a decline in plant quality growth might be the outcome of nutrients shortage. Traditional and contemporary methods of plant breeding that aim to improve stress tolerance would benefit from a better knowledge of how plants respond to abiotic stresses.

Apart from the abiotic stress, there are many other biotic stresses, such as insect pests, plant-parasitic nematodes and chickpea diseases. Identification of these diseases is accountable for reducing the risk of chickpea cultivation. According to Kumar and Naik (2020), the major disease of chickpeas, such as ascochyta blight (*Ascochyta rabie*), phytophthora root rot (*Phytophthora medicaginis*) and *Botrytis cinerea* is accountable for decreasing the growth of chickpea. There are some major insect pests, such as *Helicoverpa punctigera* and *Helicoverpa armigera,* which are accountable for reducing the nutritive value of chickpea (Abdelrahman et al., 2018a). Moreover, the plant-parasitic nematodes, such as root-lesion nematodes and Crystormin nematodes are accountable for decreasing the 14% growth of chickpea.

The pod borer (*Helicoverpa armigera*) is the most dangerous, followed by the pod fly. Nematodes, which have been efficiently managed by bio-agents, are another key pest impacting chickpea. Wilt and root-knot nematodes are crucial in terms of distribution and chickpea yield damage. Chickpeas are typically grown as rainfed crops since they require less irrigation than competitive crops such as cereal. Post-harvest losses are responsible for 9.5% of total chickpea production. Among post-harvest processes, storage accounts for the greatest amount of loss (7.5%). Processing, threshing, and transportation all result in 1%,

0.50%, and 0.50% losses, respectively. Chickpeas are likewise the most vulnerable to insect damage (5%) among storage losses, compared to wheat (2.5%), rice (2%), and maize (3.5%) (Deshpande and Singh, 2001). Unfortunately, improvements in legumes yield have lagged as those of cereals.

Worldwide, plant productivity is hampered by a lack of water and high salinity. Plants have evolved sophisticated and sensitive defense systems that allow them to signal immediately, respond to, and adapt to a variety of challenges, including drought and excessive salt (Yamaguchi-Shinozaki and Shinozaki, 2006; Tran L. S. P. et al., 2007; Tran L.-S. P. et al., 2007). Plant's defensive responses to abiotic and biotic stress factors are regulated by various phytohormones.

ROLE OF CYTOKININ IN PLANT GROWTH, STRESSES AND BIOFORTIFICATION IN CHICKPEA

Cytokinins appear to be implicated in stress reactions, according to growing evidence (Tran L.-S. P. et al., 2007; Argueso et al., 2009) and regulate various aspects of root growth, architecture, and function and plays a crucial regulatory role in a variety of developmental and physiological plant processes (Werner and Schmulling, 2009; Hwang et al., 2012). Cytokinins have been identified as a key signal that passes from roots to shoots (Letham, 1994). According to recent research, the abscisic acid (ABA) and cytokinin ratios in xylem sap are critical for stress signaling (Alvarez et al., 2008; Schachtman and Goodger, 2008). Drought, for example, reduces the generation and distribution of cytokinins from roots. The major enzymes involved in cytokinin metabolism in plants such as *Arabidopsis thaliana* are adenosine phosphate-isopentenyltransferases (IPTs) and cytokinin oxidases/dehydrogenases (CKX) (Hirose et al., 2008; Werner and Schmulling, 2009). Cytokinin oxidases/dehydrogenases accelerate irreversible cytokinin breakdown by selectively cleaving unsaturated isoprenoid side chains, culminating in the synthesis of adenine/adenosine and the associated side-chain aldehyde (Sakakibara, 2006; Werner et al., 2006).

Plant cytokinin levels have been altered in genetic experiments assuming usually a negative participant in stress response (Nishiyama et al., 2011). For example, in transgenic tobacco plants, overexpression of the cytokinin degrading enzyme cytokininoxidase/dehydrogenase improved drought and heat stress tolerance (Macková et al., 2013). However, recent research suggests that cytokinins (CK) are N6-substituted adenine derivatives that were first identified as a major regulator in plant developmental processes such as organ formation, apical dominance, leaf senescence (El-Showk et al., 2013) and may play a significant role in drought stress adaption as a positive regulator (Hai et al., 2020). For example, in transgenic cotton (Kuppu et al., 2013), creeping bentgrass (Xu et al., 2016), eggplant (Xiao et al., 2017), and tropical maize (Leta et al., 2016), ectopic expression of the isopentenyltransferase gene (IPT), which encodes a rate-limiting enzyme in cytokinin biosynthesis, increases endogenous cytokinin levels. According to a new rice cytokinin-responsive transcriptome analysis, a substantial number of genes are implicated in both biotic and abiotic stressors (Raines et al., 2016). Temperature, drought, osmotic stress, salinity, nutritional stress, plant diseases, and herbivores are among the environmental conditions where cytokinin is said to be essential for responses (Raines et al., 2016; Cortleven et al., 2019).

Accordingly, multiple functional studies were undertaken to determine the *CKX*-mutant derived tolerance mechanisms. For example, partial root-zone drying resulted in lower cytokinin concentrations in leaves, buds, and shoot tips. This increased apical dominance and aided in overcoming drought stress, particularly when combined with ABA modulation of stomatal apertures. In plants exposed to drought stress, tolerance responses may be induced by manipulating endogenous cytokinin levels, either by deletion of the biosynthesis genes isopentyltransferase or by overexpression of cytokinin oxidase (*CKK*)-encoding degradation genes. Meanwhile, heat stress is known to lower cytokinin levels, and thus exogenous cytokinin application has generally been shown to improve plant heat stress responses, combating the negative effects of heat stress on photosynthesis and chloroplast growth. Additionally, N6-(D2-isopentenyl) adenine (iP) and trans-zeatin (tZ), the biologically active free-base forms of cytokinins, were found to play a key role in tolerance mechanisms, thought to be *via* yielding higher relative abundances and affinities for cytokinin receptors. Thus, *CKX* enzymes play a key role in controlling cytokinin concentrations, which influences plant growth and development. Plants evolve through structural and metabolic adaptations to cope with stress, such as increased root area and leaf curling when subjected to dryness, and increased production of antioxidant chemicals like carotenoids, proline, and ascorbic acid. Plants with bigger root systems have a higher chance of competing for nutrients and surviving in low-nutrient environments (Passioura, 1981; Brown et al., 1989; Saxena et al., 1993; Morita and Nemoto, 1995; Kondo et al., 1999; Steele et al., 2006; Henry, 2013). Root biomass and the availability of soil resources like water and minerals have a big impact on seed output and quality. W31:CaCKX6 expressions in chickpea roots have shown to boost root biomass, shoot biomass and yield (Khandal et al., 2020). The broader root network, obtaining more nutrients from the soil and enhancing the plant's lifetime, are ascribed to the increased vegetative and reproductive growth of shoots in chickpea lines with W31: CaCKX6.

Chickpea lines expressing W31:CaCKX6 had higher relative water content (RWC) in their leaves, indicating that they were more drought tolerant. Better leaf RWC mixed with lower ABA levels may have contributed to higher carbon assimilation under long-term drought conditions. Chickpea plant introgressed with W31:CaCKX6 in chickpea root produced the seeds having better concentrations of zinc, iron, copper, phosphorus, magnesium, and potassium (Khandal et al., 2020). Thus, increasing the root network through local biofortification of cytokinin and lowering the ABA content employing genome editing in combination with classical breeding may be an effective approach for maintaining the balance for enhanced yield, grain quality and stress tolerance.

Potentials of Cytokinin Dehydrogenase

A search of the annotated *Medicago truncatula* genome assembly turned up nine CKX-encoding genes (Young et al., 2011). A comparable search of publicly available annotated chickpea genome and transcriptome sequences (Garg et al., 2011; Varshney et al., 2013) revealed the presence of 10 non-redundant genes that encode proteins with sequence similarities to seven Arabidopsis CKX proteins (Schmulling et al., 2003). They were annotated as *C. arietinum* cytokinin oxidases/dehydrogenases (*CaCKX*) and as a result, the chickpea genome has ten *CaCKX* genes. Climate change and population growth have put pressure on the agriculture industry to enhance productivity, resulting in the development of new, improved technologies aimed at improving crop's ability to remain productive in conditions such as high temperatures and low moisture availability.

Current Status of *CKX* in Chickpea

The current review highlights the effectiveness of the spatio-temporal regulation of cytokinin, which is significant for nodule development. Investigating the root manipulation for cytokinin is essential for managing the growth of chickpea. Promoter-driven CaWRKY31 in chickpeas, CaCKX6 expression resulted in a larger root system, increased CKX activity in the root, and increased seed yield. With the help of W31:CaCKX6 construct and the chickpea cultivar Pusa 362, T4 transgenic chickpea plants were created (IC296139). Root nodulation and nitrogen fixation were not affected while increasing the CKX activity. When grown in soil rite pots in a controlled growth environment, chickpea transgenic plants showed up to 1.8-fold increase in root length and lateral root numbers in the 10 days post germination (dpg) stage. In soil-grown 30 dpg plants, CKX activity was measured, and only the root showed a 2.1–3.7 fold increase over the untransformed plant, whereas CKX activity in the shoot tissue remained unchanged. The total length of the roots rose by 1.5–1.85 times. The average amount of biomass in the shoots increased by up to 20%. In transgenic chickpea lines, the root-to-shoot biomass ratio was raised by up to 1.7 times. According to two-year growth statistics, average seed number per plant increased by 20%–25%, with no significant variance in 100 seed weight. Statistical significance was often poor due to variance in seed counts between individual plants of a line. CaCKX6 expression in the roots also increased mineral content in seeds like the concentrations of Zn (27 %–62%), Cu (26 %–61%), Fe (22 %–48%) etc. were all greater in transgenic lines' seeds (Khandal et al., 2020).

Due to the presence of effective nutrients, the global economic demand and importance of chickpea are gradually increasing. It has been detected that chickpea is one of the good sources of multivitamins, such as niacin, riboflavin, thiamin, vitamin A (β carotene) and folate for the fulfilment of nutritional requirements. There are three primary components, such as inadequate supply, food accessibility and inappropriate food, which are accountable for food insecurity. Maintenance of sustainability in the cultivation of chickpea to fulfil nutritional requirements and increase economic values has enhanced food security. However, it has been identified that the production of chickpea is dependent on several challenging situations, different abiotic stresses, such as high and low temperature and drought.

BIOFORTIFICATION STRATEGIES

Agronomic practices and plant breeding is accountable for providing sufficient nutrients to people. However, one approach towards achieving greater food security is through improving the nutrient value of the food that is consumed. This may be achieved through biofortification approaches *via* breeding for enhanced concentrations of bioavailable nutrients within staple food crops. Several efficacy studies have demonstrated that the biofortification of staple crops can effectively alleviate micronutrient malnutrition or "hidden hunger" among vulnerable populations across the world. Biofortification is one of the innovative techniques, which is used to increase the level of nutrients such as minerals, vitamins and minerals for the enhancement of product's demand. The nutritional value of legumes including other crops can be increased with the help of various methods such as traditional breeding, molecular technologies, transgenic approaches or genome editing approaches, thereby preventing malnutrition. The former is a well-established, albeit is a labor-intensive and long-term operation.

Molecular Technology-Assisted Biofortification

The efforts have mainly focused on cereal grain staple species, whereas the application of this approach to grain legume/pulse crops has been largely overlooked. The process of biofortification in agronomic crops includes targeting the cytokinin gene family. The cytokinins are one of the phyto hormones that play essential roles in both physiological and developmental processes and directly impact several agronomic parameters, including growth, development and yield, including root extension and branching during post-embryonic advancement. The root-specific degradation of cytokinin was used to engineer maize genetically (*Zea mays* L.) plants to have a larger root system. Root-specific expression of a cytokinin oxidase (CKK)/dehydrogenase (CKX) gene of *Arabidopsis* caused the formation of up to 46% more root dry weight while shoot growth of the same transgenic lines was similar to the control plants. Meanwhile, the concentrations of K, P, Mo and Zn were significantly increased in the leaves of the transgenic plants. Subsequently, fine-tuning of cytokinin metabolism by root-specific expression of a cytokinin degradation enzyme was undertaken to improve both Zn nutrient level and yield traits.

Biofortification in Chickpea

Chickpea has been identified as one of the effective nutritious crops, for decreasing the negative impact of nutritional deficiencies. According to Yadav et al. (2019), there are nearly 40–60% of low digestible carbohydrates, 4–8% of essential fats, 15–22% of proteins and a sufficient range of vitamins and minerals (Wang et al., 2020). The presence of these nutrients

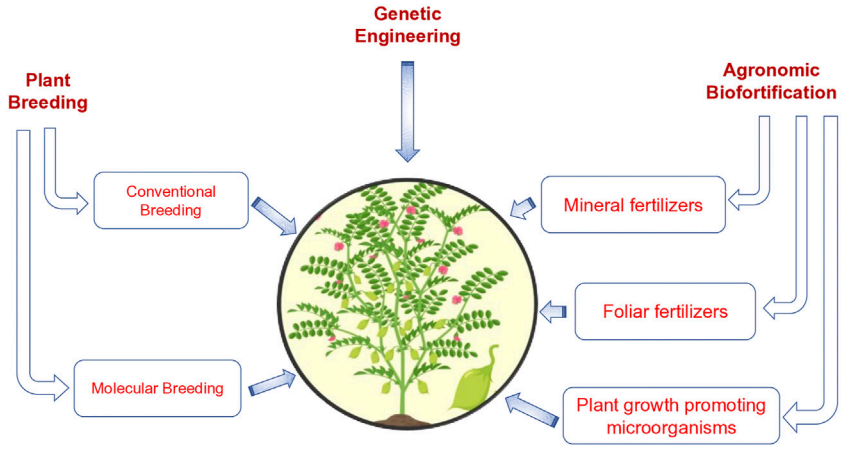

FIGURE 1 | Diagram showing the various approaches to biofortified chickpea.

is essential for increasing the nutritional value of chickpeas. Henceforth, the fatty acid composition is accountable for increasing the value of the seed. It has been identified that fat is essential for governing the texture, flavour, shelf life, nutritional composition and aroma. Therefore, the involvement of the biofortification of essential fatty acids is significant for the fulfilment of the nutritive value of crops including chickpea (Abdelrahman et al., 2018b). Some potential area for biofortification in chickpea is given in **Figure 1**.

Malnutrition has been considered as the cause of global calamity in Asia and Africa. According to Ninan et al. (2019), the current biofortification efforts focus on the enrichment of significant micronutrients and decreasing the anti-nutrient factors. Implementation of the Agronomic approaches, such as fertilizer application is essential for the enrichment of different minerals, such as Zn, Se and Fe. The combined application of Zn, Fe and urea is accountable for increasing the Zn and Fe concentration in the chickpea. It has been detected that the implementation of the transgenic approaches is one of the most efficient for iron biofortification in chickpea. According to Lastochkina, (2019), over expression of the nicotinamide synthesis, such as ferritin (GmFER) and 2 ($CaNAS_2$) is essential for increasing the Fe concentration rate in chickpea (Toğay et al., 2019). The biofortification process focused on the macro nutritional traits. Linoleic acid (LA; ω-6) has been identified as the essential fatty acid to facilitate human health. Whereas (α-linolenic acid) ALA is the other most essential fatty acid for managing human health benefits (Talip et al., 2018). It has been identified that there are about 3.8–10.2% of general facts in chickpeas. Enhancement of the nutritional values in chickpea is essential for managing the growth of chickpeas, simultaneously its quality and economic value.

Conversely, developments in molecular technologies based biofortification and the availability of improved species-specific genomic resources have led to the evolution of gene editing methods with targeted precision and validated outcomes within a relatively short time frame. Emerging popular genomic targets for the focus of biofortification efforts in food crop species are members of the cytokinin gene family expression pathway, phytohormones essential for many varied physiological and developmental processes.

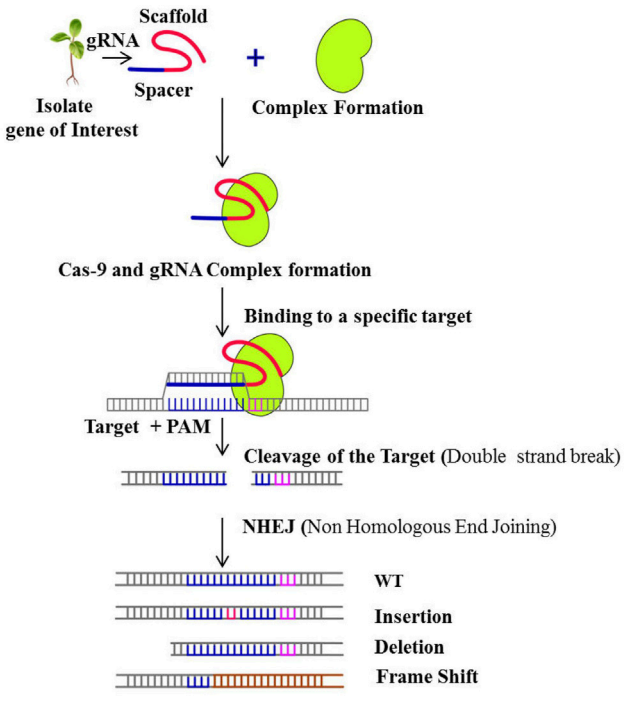

FIGURE 2 | Schematic presentation of mechanism of CRISPR/Cas-9 gene editing technology.

GENETIC ENGINEERING OF *CKX* GENES

Genomic editing or genetic engineering is an important aspect of today's world, in which the DNA is inserted, modified or deleted. First genome editing technologies were developed in the 1900s

(Kiran and Chimmad, 2018). These technologies act like scissors and cut the DNA at specific sites. The most efficient tool for genome editing is the CRISPR/cas9 system. This system is mainly used in the production of genetically modified organisms (GMOs) and genomic engineering. CRISPR/cas9 has extended the scope of agricultural research allowing for new potentials to generate novel plant types with undesirable features removed or significant characters added such as acrylamide-free potatoes (Halterman et al., 2015); non-browning apples, mushrooms and potatoes; low phytic acid maize (Liang et al., 2014); blast disease resistant rice (Wang et al., 2016) and powdery mildew resistant wheat (Wang et al., 2014). CRISPR technology is continually improving, allowing for more genetic manipulations such as creating knockouts, precise changes, multiplex genome engineering, and target gene activation and repression. With more precision and simplicity, CRISPR targets endogenous genes that are unable to target specifically using RNAi technology the mechanism of which can beseen in **Figure 2**. CRISPR/Cas9 uses a 100 nucleotide (nt) guide RNA (gRNA) sequence to target specific genomic loci. Using Watson and Crick base pairing through 17–20 nt at the gRNA 5′-end, sgRNA binds to the protospacer adjacent motif (PAM) on targeted DNA and guides Cas9 for selective cleavage. Cas9 accelerates DNA repair by causing DSBs (Double Stranded Breaks) in the target DNA. To induce genomic changes, gene knockouts, and gene insertions, the repair mechanism uses error-prone non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ makes random insertions or deletions in the coding area, resulting in frame shift mutations and gene knockouts. Thus, loss-of-function, gain-of-function, and gene expression analysis are possible with CRISPR technology enabling it as one of the effective plant breeding tool, which focuses on different gene action by acquiring knowledge on gene family members (GFMs) and is desperately needed.

According to Kumar et al. (2017),CRISPR/Cas9is known as one of the effective gene-editing tools, which is accountable for manipulating cytokinin dehydrogenase (Kumar et al., 2017; Jha et al., 2018). The GFMs expression is essential for cytokine biosynthesis and destruction for managing the gene factors. According to Ninan et al. (2019), cytokines have been identified as the enhancement of sink activities in chickpea leaves. The primary steps in cytokine biosynthesis can be controlled by isopentenyltransferase (IPT). Henceforth, the cytokinin dehydrogenase or oxidase is accountable to control the process of cytokinin degradation. Involvement of the DNA sequencing technology is essential for gathering delta knowledge in the gene concept. Cytokinin is identified as one of the effective plant hormones, which is accountable for regulating plant development. The *PsCKX7* (*Pisum sativum* cytokinin dehydrogenase) gene when down-regulated, cytokinin levels increased in roots, shoots and leaves also involves delaying of senescence. It is noteworthy that *PsCKX5* and *PsCKX7* express in the sink and mature leaves respectively (Ninan et al., 2019). In rice, Zhang et al. (2021) performed CRISPR/Cas9 editing to target serval *CKX* genes. They found that *OsCKX11* (*Oryza sativa* cytokinin dehydrogenase) have simultaneously regulates cytokinin-mediated leaf senescence and grain number (**Figure 3**).

The cytokine dehydrogenase (*CKXs*) is known as an essential protein for an irreversible breakdown of cytokinin's. It is

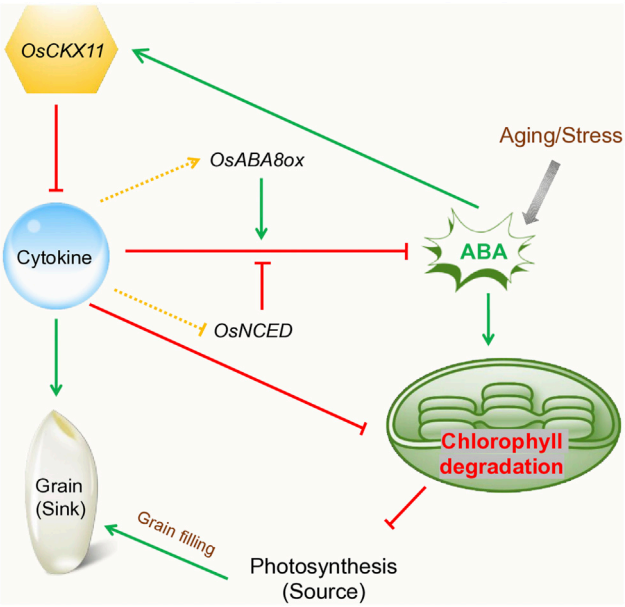

FIGURE 3 | Schematic presentation showing the role of *OsCKX11* in cytokinin-mediated leaf senescence and grain number in rice (Modified from Zhang et al., 2021)

significant for the molecular evolution for the determination of the homologous protein. Uses of these gene-editing tools are essential for detecting the presence of *CKX* in prokaryotic and eukaryotic. Apart from this, it has been identified that *CKX* plays a significant role in the improvement of plant life. Controlling the plant development process is beneficial for managing the abiotic and biotic stress for influencing the nutritive value of chickpea (Abdelrahman et al., 2018a). Cytokinin dehydrogenase is an essential plant hormone for promoting cell division. Promotion of primary cell growth and differentiation is essential for increasing the growth of this hormone. The involvement of the gene-editing tools helped in gene formation for improving the growth of its products. Apart from cytokinin, ethylene exhibition is equally important for managing the growth of plants. Involvement of the photosynthetic machinery process is essential for stimulating the growth of chickpea (Jyothi et al., 2018). Cytokinin is accountable for increasing the grain size and grain numbers for yielding the components of this plant.

In recent years, the Clustered Regularly Interspaced Short Palindromic Repeats Cas9 (CRISPR/Cas9) genome editing method has revolutionized targeted gene editing in plants (Woo et al., 2015; Baek et al., 2016; Malnoy et al., 2016; Liang et al., 2017; Kim et al., 2018; Lin et al., 2018; Murovec et al., 2018; Osakabe et al., 2018; Johansen et al., 2019; Petersen et al., 2019). CRISPR/Cas9 genome editing has a wide range of applications in agricultural improvement, including the development of designer genetically modified non-GM crops. The application of this strategy to plant breeding for the production of new crop varieties with greater tolerance to environmental challenges is a major focus of agricultural scientists (Khatodia et al., 2016;

Noman et al., 2016). CRISPR/Cas9 gene-editing tools have been utilized for gene activation, repression, knockout, knockdown, repression, and for altering epigenetic modifications in several plants crops such as Arabidopsis (Feng et al., 2014), apple (Osakabe et al., 2018), citrus, carrot (Klimek-Chodacka et al., 2018), grape (Nakajima et al., 2017), tomato (Wang et al., 2019), rice (Zhang et al., 2014), sorghum (Liu et al., 2019), maize and soybean (Chilcoat et al., 2017), and wheat (Zhang et al., 2016). CRISPR/Cas9 genome editing was utilized to discover abiotic stress response in Arabidopsis plants; the findings revealed that *OST2* (proton pump), a mutant allele produced through editing, changed stomatal closure under environmental stress (Osakabe et al., 2018). Another recent maize work employed the CRISPR/Cas9 method to create unique allelic variants that could be exploited to engineer drought-tolerant crops. This system genetically modified *ARGOS8*, whose over expression can result in lower ethylene sensitivity. Field investigations demonstrated that *ARGOS8* variants had higher grain yield under drought stress; further, no yield loss was documented under well-watered conditions (Shi et al., 2017).

CONCLUSION AND FUTURE PROSPECTS

This present review highlights the role of *CKX* genes in chickpea growth and development traits, biotic and abiotic stress regulation, and biofortification. Chickpea is the most economically important product all over the world. There are various types of stress like heat, cold, drought and so on those are faced by the crop plant. Due to global warming, a temperature rise is a frequent event in today's world that causes the drought condition. Heat stress causes severe damage to the leaves and also ruptures the membrane. All these factors adversely affect the agronomic traits of chickpea. CKs play many crucial roles in plants when they experience any kind of stress. Phytohormones in the cytokinin family control root length and branching in the post-embryonic stages. Cytokinin oxidases or dehydrogenases (CKXs) are enzymes that degrade cytokinin in order to study its biological functions and engineer root development. A chickpea root-specific promoter of CaWRKY31 may be used to explore how cytokinin depletion affects root development and drought tolerance in *Arabidopsis thaliana* and chickpea with definite and indeterminate growth patterns, respectively. In *Arabidopsis* and chickpea, root specific expressions of CaCKX6 increased lateral root number and plant biomass without affecting shoot vegetative and reproductive development. Root CKX activity was elevated in transgenic chickpea lines. The root-to-shoot biomass ratio was greater in soil-grown advanced chickpea transgenic lines, and the plants had improved long-term drought resistance. Nutrient fixation in the roots and leaves of these chickpea varieties was unaffected. In certain transgenic lines, the seed output was up to 25% greater with enhanced concentrations of zinc, iron, potassium, and copper without corresponding decrease in protein content. Apart from this other phytohormones also play an important role in alleviating stress condition in chickpea. ABA plays an important role to reduce oxidative damage in chickpeas. It interacts with the various types of antioxidants to reduce stress and reduces the ROS production in the plant body which harms the plants. It has also introduced some heat shock protein to provide tolerance against the heat. Salicylic acid also plays an important role against abiotic and biotic stress as well as against pathogens and herbivores. However, in chickpea, functional characterization studies of *CKX* genes have just started. Gene editing tools such as TALENs or CRISPR/Cas9 can play crucial role in this context. Still, less functional studies exist in the case of stress regulation and biofortification. This is a potential area for research to unravel the CK signaling networks and their cross talk elucidating its biochemical pathways which will draw a detailed picture and pave the road towards developing tolerant crops, and in the long-term, more sustainable agriculture. Similarly, many more such genes are hidden in the plant genome, which are required to be explored and investigated to harness and develop cultivars with a higher yield, better abiotic stress resistance and biofortification.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RK conceptualized and supervised the manuscript writing. RM, Ambika, CS, BC, RS and SV collected the related literature and contributed to the original writing. RK, VG, MM and NY extended their assistance in inference, review, and editing of the manuscript. All authors went through the final manuscript draft and approved.

FUNDING

The study was supported by ICAR-Indian Agricultural Research Institute, New Delhi.

ACKNOWLEDGMENTS

The 1st and lead author gratefully acknowledges the DST-Science and Engineering Research Board for providing the financial support to carry out the manuscript preparation. All authors acknowledge the ICAR-Indian Agricultural Research Institute, New Delhi; UAS-GKVK, Bangalore, India; GGS Indraprastha University, Delhi, India; CSIR-IHBT, Palampur, India; CFT, University of Allahabad, India for providing necessary support for the study along with Prof Rebecca Ford, Griffith University, Queensland, Australia and Prof Syed S H Rizvi, Cornell University for reviewing and meticulous scientific editing of the 1st draft of the manuscript.

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frontiers | Frontiers in Genetics

ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fgene.2022.884106

Transcriptome Analysis of *Pennisetum glaucum* (L.) R. Br. Provides Insight Into Heat Stress Responses

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: 25 February 2022
Accepted: 19 April 2022
Published: 02 June 2022

Citation:
Maibam A, Lone SA, Ningombam S, Gaikwad K, Amitha Mithra SV, Singh MP, Singh SP, Dalal M and Padaria JC (2022) Transcriptome Analysis of Pennisetum glaucum (L.) R. Br. Provides Insight Into Heat Stress Responses. Front. Genet. 13:884106. doi: 10.3389/fgene.2022.884106

Pennisetum glaucum (L.) R. Br., being widely grown in dry and hot weather, frequently encounters heat stress at various stages of growth. The crop, due to its inherent capacity, efficiently overcomes such stress during vegetative stages. However, the same is not always the case with the terminal (flowering through grain filling) stages of growth, where recovery from stress is more challenging. However, certain pearl millet genotypes such as 841-B are known to overcome heat stress even at the terminal growth stages. Therefore, we performed RNA sequencing of two contrasting genotypes of pearl millet (841-B and PPMI-69) subjected to heat stress (42°C for 6 h) at flowering stages. Over 274 million high quality reads with an average length of 150 nt were generated, which were assembled into 47,310 unigenes having an average length of 1,254 nucleotides, N50 length of 1853 nucleotides, and GC content of 53.11%. Blastx resulted in the annotation of 35,628 unigenes, and functional classification showed 15,950 unigenes designated to 51 Gene Ontology terms. A total of 13,786 unigenes were allocated to 23 Clusters of Orthologous Groups, and 4,255 unigenes were distributed to 132 functional Kyoto Encyclopedia of Genes and Genomes database pathways. A total of 12,976 simple sequence repeats and 305,759 SNPs were identified in the transcriptome data. Out of 2,301 differentially expressed genes, 10 potential candidate genes were selected based on log2 fold change and adjusted *p* value parameters for their differential gene expression by qRT-PCR. We were able to identify differentially expressed genes unique to either of the two genotypes, and also, some DEGs common to both the genotypes were enriched. The differential expression patterns suggested that 841-B 6 h has better ability to maintain homeostasis during heat stress as compared to PPMI-69 6 h. The sequencing data generated in this study, like the SSRs and SNPs, shall serve as an important resource for

Abbreviations: nt, nucleotides; h, hours; BLAST, Basic Local Alignment Search Tool; SSRs, simple sequence repeats; mm, milli metre; ESTs, expressed sequence tags.

the development of genetic markers, and the differentially expressed heat responsive genes shall be used for the development of transgenic crops.

Keywords: *Pennisetum glaucum* (L.) R. Br., heat stress (HS), flag leaf, RNA sequencing (RNAseq), SSRs (simple sequence repeats), SNPs (single-nucleotide polymorphisms)

INTRODUCTION

Temperature is a key physical parameter which affects the growth and development of a plant. According to the Intergovernmental Panel on Climate Change (IPCC), there has been an average increase of 4°C in global atmospheric temperature since the late 20th century (Porter et al., 2014). A plant undergoes a number of morphological, physiological, biochemical, and molecular changes during heat stress to ensure its survival (Wahid et al., 2007). These changes include reduction in chlorophyll content, changes in membrane fluidity and protein stability, production of reactive oxygen species (ROS), secondary signaling, and transcriptional changes. In some crops, the occurrence of heat stress during the flowering period leads to poor grain setting (Iqbal et al., 2009; Moriondo et al., 2011). Increased water stress due to heat throughout the growing cycle can reduce the crop yield (Lobell et al., 2013). Despite the impact of high temperature on plant growth and crop yield being quite profound, the underlying heat tolerance mechanisms are not clearly understood in many crops.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.], also known as *Cenchrus americanus* (L.) Morrone, is widely grown in the African and Indian subcontinents, since prehistoric times. The crop's main center of diversity is known to be the Sahel zone of West Africa. Pearl millet is a C4 species having diploid number 2n = 14 and genome size around 1.79 Gb (Varshney et al., 2017) and is mostly grown under drought-prone semi-arid and arid tropics in the regions with 200–800 mm of annual rainfall. Pearl millet is grown in an area of about 31 million hectares worldwide in more than 30 countries with more than 90 million poor people depending on it for food and income (http://exploreit.icrisat.org/profile/Pearl%20Millet/178 accessed on 6 February 2022). Optimum temperature required for the growth of pearl millet is about 30–35°C (Maibam and Padaria, 2015). It is well known for its tolerance to extreme environmental conditions. Limited genomic resources are available as compared to other crop species.

Next-generation sequencing (NGS) based technology for the analysis of transcriptome is more powerful and accurate compared to the Sanger based EST sequencing, suppression subtractive hybridization, and hybridization based microarrays (Marioni et al., 2008; Agarwal et al., 2010). Moreover, over the last decade, several NGS platforms including the illumina are becoming more affordable and efficient for transcriptome sequencing. Additionally, transcriptome sequencing has emerged as an alternative core technology for the discovery and understanding of genes associated with desired traits, where full genome sequencing is not economically feasible especially in case of nonmodel plants. RNA-sequencing (RNA-Seq) has become a benchmark tool for whole transcriptome gene expression quantification and identification of differentially expressed genes (DEGs). It provides scope for the identification of probable candidate genes involved in abiotic and biotic stress tolerance and further for the development of molecular markers (Wang et al., 2009; Garber et al., 2011; Strickler et al., 2012). Third generation sequencing technology such as PacBio provides long/full length transcripts but still having high single error base rate (Sun et al., 2020).

Enormous amounts of ESTs generated from various transcriptomic studies and other genomic sequences are available in public databases for many model plant species (James et al., 2015). However, limited research emphasis has been given to the nonmodel crops including pearl millet, as evidenced by the presence of only 75,499 ESTs (https://www.ncbi.nlm.nih.gov/nuccore/?term=pearl+millet+ESTs, Accessed on 12th April 2022) of this crop in GenBank. It is now possible to assemble transcripts without the reference genome *via de novo* assembly using trinity (Haas et al., 2014) and/or one of the several other available software tools. Recently, genome wide expression profiling in various nonmodel plant species growing under abiotic or biotic stresses, for various biosynthetic pathways or developmental stages, has been carried out using RNA-sequencing. These plants include *Raphanus sativus* L. (Nie et al., 2016), *Scrophularia ningpoensis* Hemsl. (Pan et al., 2017), *Camellia sinensis* (L.) Kuntze (Guo et al., 2017), *Lilium* genotypes (Hu et al., 2017), *Brassica rapa* L. (Greenham et al., 2017), *Sesamum indicum* L. (Dossa et al., 2017), *Asparagus officinalis* L. (Li et al., 2017), and *Agrostis stolonifera* L. (Ma et al., 2017). Transcriptome studies performed for pearl millet have mostly covered drought and/or salinity stresses (Dudhate et al., 2018; Jaiswal et al., 2018; Shinde et al., 2018; Shivhare et al., 2020) and only two studies (Sun et al., 2020, 2021) have covered transcriptome of pearl millet under heat stress. Wherein they used only one variety to study the transcriptome, different parts of the plant viz. roots, leaves, and whole plant were used for RNA extraction. Given the limited number of studies available on transcriptome under heat stress in pearl millet, it would be appropriate to study the transcriptome of this crop under heat stress to gain novel insights into the underlying mechanism. For the said purpose, we chose two varieties having contrasting feature visa-a-vis their heat stress tolerance (heat tolerant genotype-841-B and heat sensitive genotype PPMI-69).

Flag leaf acts as an immediate source for panicle development during the reproductive stage in plants (Wang et al., 2011), RNA-sequencing of flag leaf subjected to heat stress during flowering stage was carried out using Illumina sequencing platform. *De novo* assembly resulted in 47,310 unigenes and further, functional annotation (gene ontology, corresponding metabolic pathways) was carried out. The aim of the present study was to unravel the

gene pool responsible for conferring heat tolerance to pearl millet. Being one among a few transcriptome reports of pearl millet in response to heat stress, the data presented here will be a primary source of information for the research on genomics and functional genomics in this orphan crop.

MATERIALS AND METHODS

Plant Materials, Heat Stress Treatment, RNA Isolation, and cDNA Library Construction

Seeds of two contrasting genotypes, 841-B and PPMI-69, were collected from Division of Genetics, Indian Agricultural Research Institute (ICAR-IARI), New Delhi, India. The genotype 841-B has higher tolerance to heat stress compared to PPMI-69 (Sankar et al., 2013, 2014). The tolerance of the genotypes was evaluated by membrane stability index (MSI) and Malondialdehyde assay (Heath and Packer, 1968). For heat stress treatment, seeds of both the genotypes were surface-sterilized and sown in plastic pots (10 inches) filled with vermiculite and grown under glasshouse (temperature 32 ± 2°C, relative humidity 70–80%, under day length of 12 h), at National Phytotron facility, IARI, New Delhi. 10 seeds per genotype were grown with one seedling per pot. At flowering stage (55 days after sowing), heat stress was applied in a growth chamber at a temperature of 42°C, relative humidity of 70–80%, and normal light conditions for different time intervals (30 min and 6 h). Plants grown at 32–34°C under normal light conditions in the glasshouse served as control. Different plant samples used in the study were given independent identity numbers (**Supplementary Table S1**). For RNA extraction, one flag leaf per plant was collected from each plant sample (both untreated and treated) respectively in biological triplicates and was immediately frozen in liquid nitrogen before storing at −80°C. RNA isolation was carried out in three biological replicates using TRIzol reagent (ThermoFisher Scientific, United States) and purified using NucleoTrap mRNA mini kit (Macherey-Nagel, Germany). DNA contamination was removed using TURBO DNase (Ambion, United States) according to the manufacturer's instructions. The RNA quality was assessed using the 2,100 Bioanalyzer (Agilent Technology, United States). One μg of the total RNA from each sample was used to purify poly-A containing mRNA molecules using Oligotex mRNA mini kit (Qiagen, Germany) as described by the manufacturer. Four independent RNA-seq libraries were constructed using TruSeq® Stranded mRNA Library Prep Kit (Illumina, United States) according to the manufacturer's instructions (**Supplementary Table S1**). The RNA libraries thus constructed were sequenced using Illumina Hiseq platform.

Determination of Physio-Biochemical Characteristics of Plants

Malondialdehyde (MDA) content of both the genotypes was estimated as described previously (Heath and Packer, 1968). Briefly, 0.5 g of leaf tissue from each genotype was taken and homogenized in 10% trichloroacetic acid (TCA) and 0.65% thiobarbituric acid (TBA). The homogenate was incubated at 95°C for 30 min and allowed to cool to room temperature (~25°C) followed by centrifugation at 10,000 × *g* for 10 min. The supernatant was collected and its absorbance was measured spectrophotometrically (Shimadzu UV–vis Spectrophotometer UV-1800, Japan) at 532 and 600 nm. The MDA equivalent was calculated in nMg^{-1} fresh weight as MDA = $[(A_{532}-A_{600})]/155] \times 100$. For the estimation of membrane stability index (MSI), the leaf samples were washed with double distilled water (DDW) to remove surface contamination and 10 leaf discs were taken in sealed vials containing 10 ml of DDW separately, followed by incubation at 4°C for 24 h. The electrical conductivity (EC1) was recorded by using a digital conductivity meter (Blum and Ebercon, 1981). For the electrical conductivity (EC2), the samples were autoclaved at 121°C for 20 min, and allowed to cool down to room temperature. The membrane stability index was calculated as per the equation: MSI (%) = 1-(EC1/EC2) × 100.

De Novo Assembly of Flag Leaf Transcriptome

The next-generation sequencing run for whole transcriptome was performed using Paired end (PE) 2 bp × 150 bp library on Illumina HiSeq 2,500. Using FastQC tools (Andrews, 2014), quality check was performed for the raw data shown in **Supplementary Table S2**. Trimmomatic was used for preprocessing the raw reads to eliminate adapter sequences and poor quality reads. Trinity program was used for *de novo* assembly with the default parameters (Grabherr et al., 2011; Haas et al., 2014). The Cluster Database at High Identity with Tolerance (CD-HIT) program (Li and Godzik, 2006) was run to remove the similar short sequences based on 90% alignment coverage to longer sequence and produces a set of 'nonredundant' (nr) representative sequences and eliminating short redundant sequences. The sequences were clustered using TGICL tools (Pertea et al., 2003) with the default parameters to produce longer, more complete consensus sequences. Gene construction was carried out using EvidentialGene tools with the default parameters to retain the biologically significant transcripts.

Annotation of Transcriptome and Identification of Simple Sequence Repeats and Single-Nucleotide Polymorphisms

The transcriptome structural annotation was performed using TransDecoder (https://github.com/TransDecoder). The functional annotation was performed using BLAST + tools, with BLASTx using a translated nucleotide query (unigenes). Gene Ontology mapping was performed using Blast2GO (Conesa and Gotz, 2008), to specify all the annotated unigenes to various categories such as biological processes, molecular functions, and cellular components. Pathway mapping of unigenes was performed using KEGG database (Ogata et al., 1999). The unigene sequences were aligned to the Clusters of Orthologous

TABLE 1 | Summary of annotations against publicly available databases.

	Number of unigenes	Percentage (%)
Total unigenes	47,310	100
NR	35,628	75.31
GO	15,950	22.77
COG	13,786	29.14
KEGG	4,263	11.97
TF	3,841	8.119

Groups (COGs database) (Tatusov et al., 2000) to predict and classify proteins. PlantTFcat online tool (http://plantgrn.noble.org/PlantTFcat/) was used to identify the transcription factor in the generated data. SSRs were identified using MIcroSAtellite (MISA) identification tool. The Microsatellite search module (MISA) is available online for the public (http://pgrc.ipk-gatersleben.de/misa/). The SNPs were identified by using GATK best practice pipeline Version 4.1.2.0 (https://software.broadinstitute.org/gatk/best-practices/), the cleaned reads were mapped against the Transfuse. fasta file using BWA aligner (http://bio-bwa.sourceforge.net/). The alignment was performed in default mode. Picard tool was used to co-ordinate, sort, and remove duplicates from the aligned bam files. The GATK tool was used for processing the alignments and variant calling. SplitNCigarReads and HaplotypeCaller from GATK tools were used for reassigning mapping qualities and variant calling, respectively.

Expression Analysis

Fragments per kilobase of transcript per million mapped reads (FPKM) unit was used to calculate the expression level of unigenes. Read count for each unigenes were calculated and then converted to FPKM using the formula: (Read count X 10^9)/(Sum of read count × Length). Differential gene expression was determined using DEGseq (Wang et al., 2010), in R package. The significant DEGs were filtered based on the adjusted *p*-value <0.005 and log ratio >1 and −1 between the samples. Heatmaps of the significant genes were generated with the heatmap package (Perry, 2018), in R package. Using R package pvclust (Suzuki and Shimodaira 2006), hierarchical clustering was performed with 1,000 bootstrap replications.

Quantitative RT-PCR

Total RNA was extracted (as described in **Section 2.1**) from the treated and control flag leaves to study the differential expression patterns under heat stress (42°C for 30 min and 6 h) of the few selected genes. A total of 10 genes (*PgDnaJ, PgGST, PgNAC67, PgTIL, PgEXP, PgHd1, PgLTP, PgUCP1, PgUCP2,* and *PgUCP3*) involved in heat stress response were selected for differential expression analysis, from the generated pearl millet transcriptome data based on the log2 fold change ≥2 (for upregulated transcripts) and adjusted *p*-values. cDNA was synthesized for studying the expression of selected DEGs with the samples of flag leaves originally used for RNA-seq, using SuperScript® III First-Strand Synthesis System (Invitrogen, United States). qRT-PCR was performed using

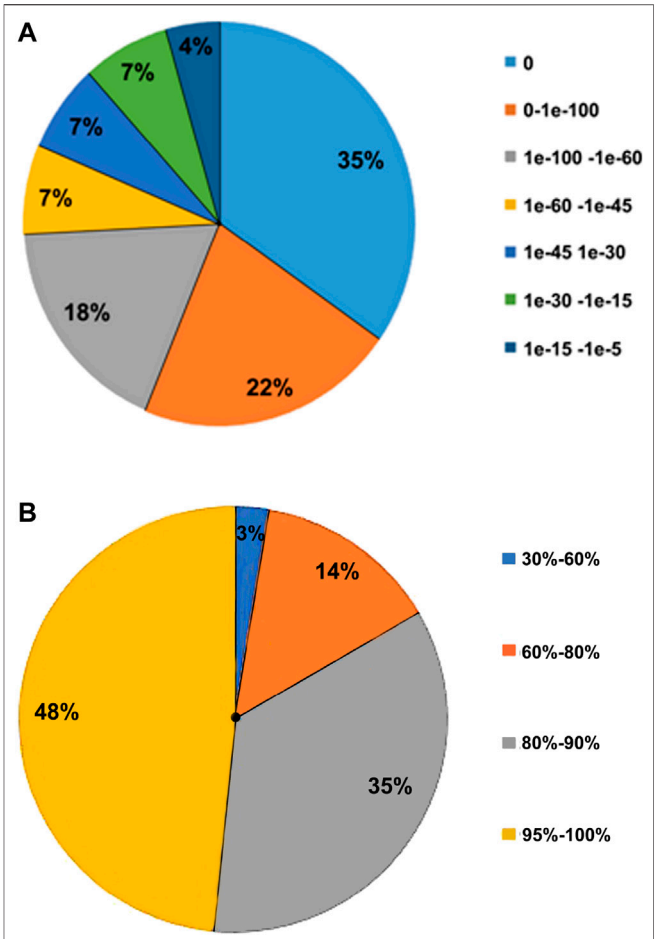

FIGURE 1 | Characteristics of sequence homology of unigenes against the NR database. **(A)**- E-value distribution of BLAST hits for each unigene. **(B)** Similarity distribution of BLAST hits of each unigene.

LightCycler® 480 System (Roche, Switzerland) and KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, United States) was used as reaction components. Gene specific primers were designed using PrimerQuest Tool (Integrated DNA Technologies (IDT), United States) (**Supplementary Table S3**). PCR program was set as: 94°C for 5 min once followed by 40 cycles at 94°C for 10 s, 60°C for 10 s, and 72°C for 10 s. *PgActin* was used as an endogenous control to normalize all the data (Sankar et al., 2021). $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change (Livak and Schmittgen 2001) among the treatments. The significance levels were calculated using two-tailed unpaired *t*-test.

RESULTS AND DISCUSSION

Determination of Physio-Biochemical Properties of *P. glaucum* Genotypes

The detection of higher content of Malondialdehyde and lower membrane stability index in genotype PPMI-69 compared to

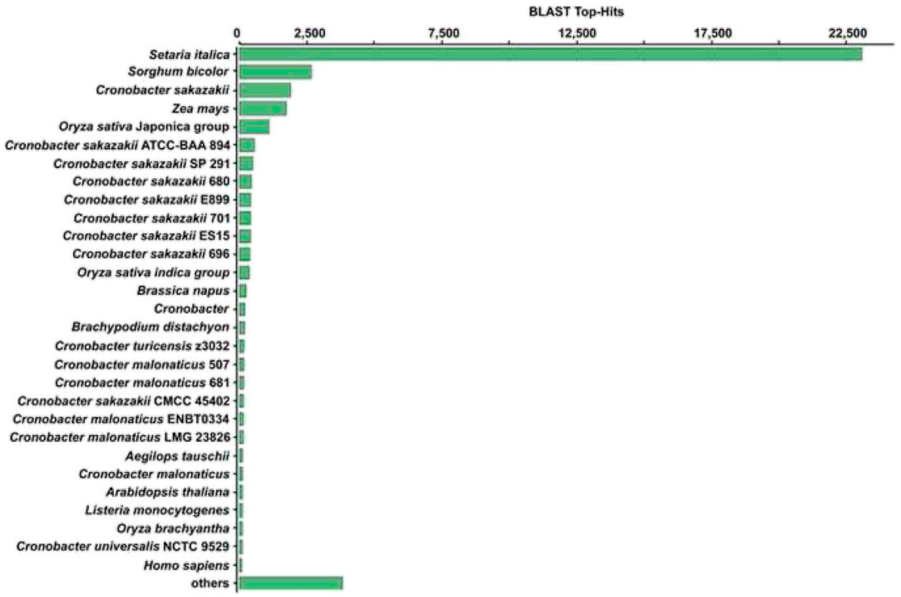

FIGURE 2 | Distribution of the top BLAST hits in different species.

that of 841-B indicated that PPMI-69 genotype is susceptible to heat stress compared to 841-B (**Supplementary Figures S1,S2**). The heat susceptibility of PPMI-69 genotype compared to that of 841-B has been previously reported (Sankar et al., 2013, 2014).

Illumina Sequencing and Raw Data Pre-Processing

Pear millet, despite being an important source of food and fodder in both arid and semi-arid regions of the world, has not been widely explored as a reservoir of heat tolerant genes. There are some genomic and very few transcriptomic studies exploring the genetic potential of pearl millet as a source of heat stress responsive genes. In the year 2016, Berthouly-Salazar et al. (2016) performed RNA sequencing of different pearl millet populations to explore their climatic adaptability. Varshney et al. (2017) reported whole genome sequencing of pearl millet using shortgun and BAC cloning approaches. Sun et al. (2020) performed Pacbio full-length RNA sequencing of pearl millet under heat and drought stress. Sun et al. (2021) reported root transcriptome of pearl millet under heat stress. Huang et al. (2021) studied the transcriptional changes in pearl millet leaves under heat stress. In this study, the whole transcriptome sequencing was performed using Paired end (PE) 2 bp × 150 bp library on Illumina HiSeq 2,500. The sequencing run produced a total raw data of 288, 876, 956 reads, details are given in **Supplementary Table S2**. After the removal of low quality sequences, ambiguous bases and adapter sequences by Trimmomatic tool (Bolger et al., 2014), a total of 274, 721, 009 high quality clean reads, containing 39, 782, 593,275 nucleotides (nts) were generated having an average length of 150 nt and GC content of 57.17%. The sequencing data has been deposited to NCBI (National Centre for Biotechnology Information), Sequence Read Archive (SRA) database under the accession number SRP151237.

De Novo Assembly of Pearl Millet Flag Leaf Transcriptome

Using the Trinity program based on the de Bruijn graph algorithm, we performed *de novo* transcriptome assemblies using their default K-mer sizes. The analysis generated 147,934 contigs having the mean length of 1,059 nt and N50 length of 1,526 nt (**Supplementary Table S4**), which is significantly higher than the average length (725 bp, N50 1,014 bp) reported by Varshney et al. (2017) and lesser than average length (3,102 bp, N50 3,302 bp) reported by Sun et al. (2020). In order to reduce the assembled contig numbers, CD-HIT software was used for grouping and estimating similarity and dissimilarity of nucleotide sequences, which resulted in the number of contigs being reduced from 147,934 to 129,893 due to the removal of redundant sequences. TGICL tools were further used to retrieve longer and complete contigs, as a result, 129,893 contigs were processed into 109,001 contigs, with N50 length of 1,649 nt. In order to retain the biologically significant contigs, EvidentialGene tools was used and these contigs were assembled in a nonredundant manner. 47,310 high quality unigenes were generated, with a total length of 59, 323, 119 nt, a mean length 1,254 nt, N50 length of 1,853 nt, and GC content 53.11% (**Supplementary Table S4**). The use of assembly tools (CD-HIT, TGICL, and EvidentialGene) led to the improvement of N50 values, compared to raw assembly. To find the read usage in the assembly, we aligned all the 274.721 million reads to 47,310 unigenes using Bowtie two software tool (Langmead and Salzberg, 2012), 72.62% of reads were

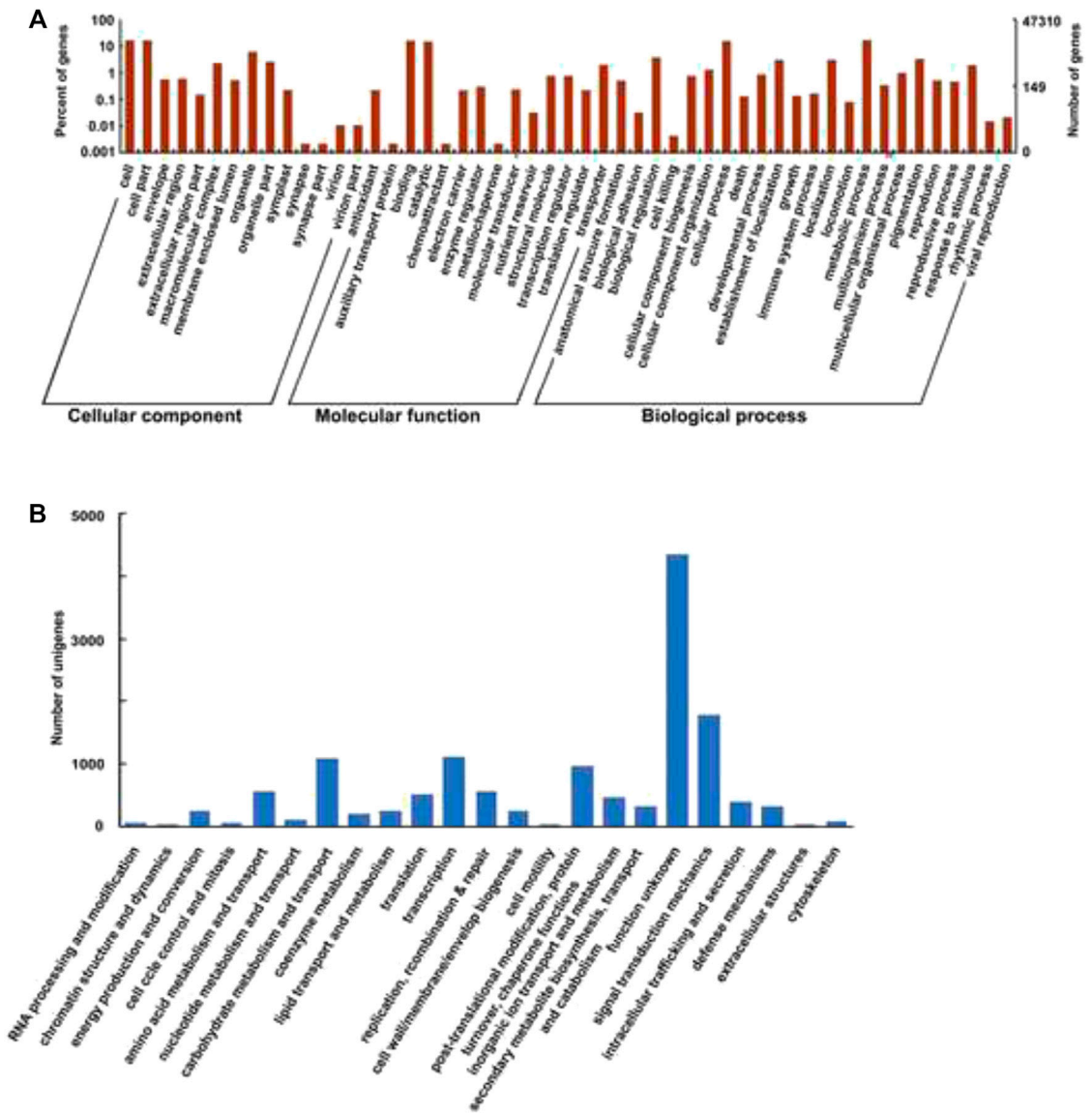

FIGURE 3 | (A)- GO (Gene Ontology) classification of the transcriptome. **(B)**- COGs (Clusters of Orthologous Groups) classification.

aligned to the assembled transcripts (**Supplementary Table S5**).

Structural and Functional Annotation and Classification of *P. glaucum* Transcriptome

The transcriptome structural annotation analysis was performed using TransDecoder tool (https://github.com/TransDecoder/TransDecoder/wiki-Date of access 17th January 2020). Out of 47,310 unigenes analyzed, 29,919 (63.24%) were found to be coding sequences, in which 11,893 unigenes (25.13%) were detected with ORFs (Open Reading Frame) (**Supplementary Table S6**). Functional annotation of all the assembled unigenes were compared to the NCBI nonredundant (nr) protein database (Blastx program) (Altschul et al., 1990) with a cut-off value of 1.0 E-06. A total of 35,628 unigenes (75.31% of all unigenes) were annotated against the nr protein database while the remaining 11,682 (24.69%) were not annotated (**Table 1**). Based on the E-value distribution of Blastx results, 81.34% of the unigenes showed E-value < 1.0E-45 while 18.66% of unigenes had E-value in the range of 1.0E-0.6 to 1.0E-45 (**Figure 1A**). 83.35% of the aligned unigenes showed more than 80% of similarity distribution (**Figure 1B**). Blast top hits analysis showed that 57.07% of the annotated sequences correspond to *Setaria italica* (L.) *P. Beauvois*, followed by *Sorghum bicolor* (L.) Moench (6.61%), *Cronobacter sakazakii* (4.72%), *Zea mays* L (4.33%), and *Oryza sativa japonica* (2.74%) (**Figure 2**). A similar trend in the distribution of BLAST top hits was observed in pearl millet subjected to heat and drought stress previously (Sun et al., 2020). The 30 top-hit species based on nr annotation are shown in **Figure 2**. Based on Gene

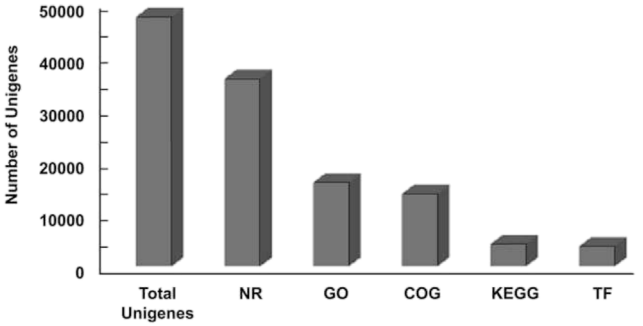

FIGURE 4 | Summary of annotation of all unigenes.

Ontology (GO), 15,950 unigenes were designated into three GO classes i.e., 23 biological processes, 14 cellular components, and 14 molecular functions as shown in **Figure 3A**. Transcriptional sequences for cellular process, biological regulation, establishment of localization, localization, pigmentation, response to stimulus, and metabolic process, among others were significantly enriched under the biological process. Within the cellular component, unigene sequences for the cell, cell part, organelle, organelle part, and macromolecular complex were identified as highly enriched categories. The major proportion of unigenes was assigned to binding, catalytic activity, and transporter categories under molecular function. Moreover, Clusters of Orthologous Groups (COGs) analysis showed that 13,786 unigenes (29.14% of all unigenes) were allocated to 23 COGs categories (**Table 1**; **Figure 3B**). Of these, maximum unigenes fall under the category of unknown functions (4,353), followed by a large number of unigenes falling under the categories of signal transduction mechanisms (1,776), transcription (1,105), carbohydrate metabolism and transport (1,095), and post-translational modification, protein turnover, chaperone functions (961). Minimum unigenes were observed to fall under the categories of extracellular structures (9) and cell motility (3). The assigned function of unigenes showed GO (44.77%) and COGs (29.14%) classifications, representing a broad range of cellular transcripts in pearl millet. We used PlantTFcat tool (http://plantgrn.noble.org/PlantTFcat/) and identified 3,841 unigenes associated with the plant transcription factors (**Figure 4**).

Identification of Heat-Responsive Genes Involved in Biological Pathways During Flowering

To identify the potential heat responsive genes and understand their role in various biological pathways, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed with a cut-off E-value of 1.0E-05. In total, 4,255 unigenes (11.94% of total unigenes) were categorized into 132 KEGG pathways (**Table 1**; **Figure 4**). The most represented pathways were the ones related to "biosynthesis of antibiotics" (10.86%), "purine metabolism" (6.46%), "starch and sucrose metabolism" (3.45%), "pyrimidine metabolism" (3.2%), "phenylpropanoid biosynthesis" (2.70%), and "glycolysis/gluconeogenesis" (2.63%). A total of 147 transcripts of starch and sucrose metabolism (3.45%) and 112 transcripts of "glycolysis/gluconeogenesis" (2.63%) were identified. Our study will provide better understanding of molecular mechanisms that are prevailing during the flowering stage of pearl millet under heat stress. Interestingly, this analysis shall help in specifying pathways related to synthesis and turnover of compounds, which have favorable effects in grain filling and yield.

TABLE 2 | Statistics related to SSRs obtained from the transcriptome of *Pennisetum glaucum*.

Total number of unigenes examined	**47,310**
Total size of examined sequences (bp)	5,93,23,119
Total number of identified SSRs	12,976
Number of unigenes containing SSRs	10,294
Number of unigenes containing more than 1 SSR	2,116
Number of SSRs present in compound formation	986

Identification of Simple Sequence Repeats and Single-Nucleotide Polymorphisms

Assembled transcriptome of *P. glaucum* was used for the identification of SSRs. Based on the criteria with a minimum of (5–10) repetitions of mono to hexa-nucleotide motifs, MISA software was used to search the SSR markers in all unigenes (Thiel et al., 2003). A total of 12,976 SSRs from 10,294 unigenes were detected, of which 2,116 unigenes had more than one SSR (**Table 2**). Among all the identified SSRs, 50.88% fall under tri-nucleotide repeats, followed by mono-nucleotides (31.88%) and di-nucleotides (13.7%), but tetra-nucleotide, penta-nucleotide, and hexa-nucleotide were represented only as a small fraction (**Figure 5**).

SSRs are simple motifs of nucleotides (1–10 nucleotides), which may occur as tandem or interspersed repeats and are abundant within the genome of prokaryotes and eukaryotes (Vieira et al., 2016). Genetic variability for heat stress tolerance in *P. glaucum* is still unexplored. Therefore, mining the SSR markers from *P. glaucum* would be utilized by breeders to develop heat stress tolerant crops. Several studies show that SSRs are not distributed randomly along the genome. For example, in case of *Arabidopsis thaliana* (L.) Heynh. rice (Lawson and Zhang, 2006) and *Gossypium raimondii* Ulbr. (Zou et al., 2012), it has been reported that the occurrence of GC-rich trinucleotides SSRs were frequent in exon regions, whereas distribution of AT-rich trinucleotides SSRs were found throughout the genome (coding sequences, untranslated regions, introns, and intergenic spaces) (Temnykh et al., 2001). SSRs are codominant, multi-allele genetic markers that are highly reproducible and transferable among related species (Mason, 2015). As a result, it has been the most widely used marker for genotyping and other breeding purposes (Senthilvel et al., 2008). Identification of new SSRs will provide the necessary impetus to the research community interested in genotyping, genetic mapping, and genetic diversity studies in various *Pennisetum* species (Senthilvel et al., 2008). A total of 305,759 SNPs were observed in the *P. glaucum* transcriptome data. Analysis of SNPs showed that 64.76% (581,800/898,460) of the nucleotide changes were transitions, while 35.24% (316,660/

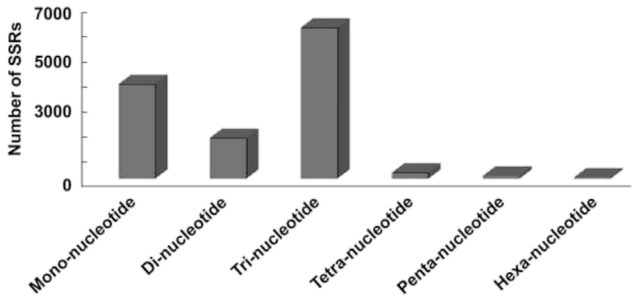

FIGURE 5 | Distribution of SSRs observed in this study. X-axis represents SSRs repeat motif type. Y-axis represents number of SSRs.

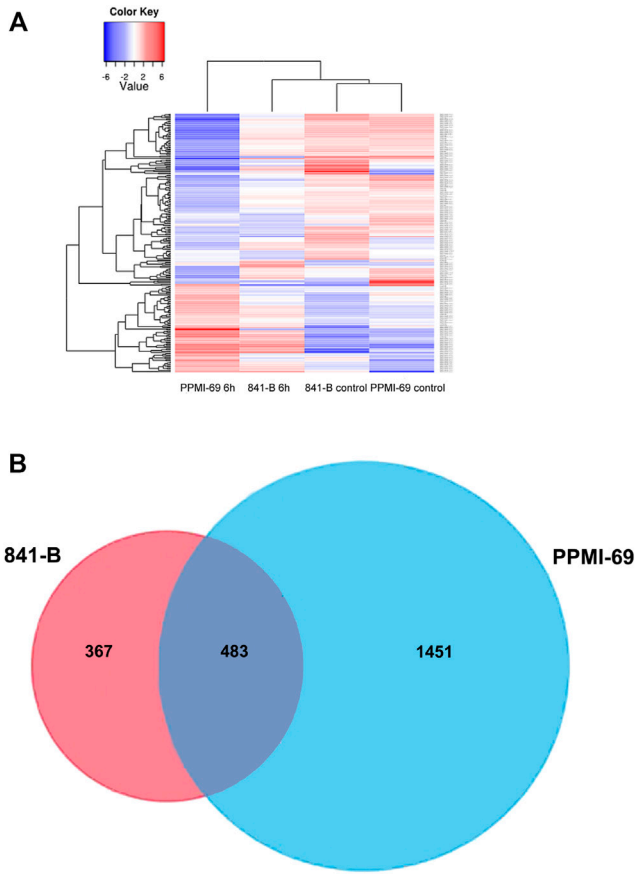

FIGURE 6 | Representation of heat stress responsive genes. **(A)**- Heatmaps of the significant differentially expressed genes with hierarchical clustering. **(B)**- Venn diagram of the differential expressed genes under heat stress conditions in 841-B and PPMI-69 genotypes.

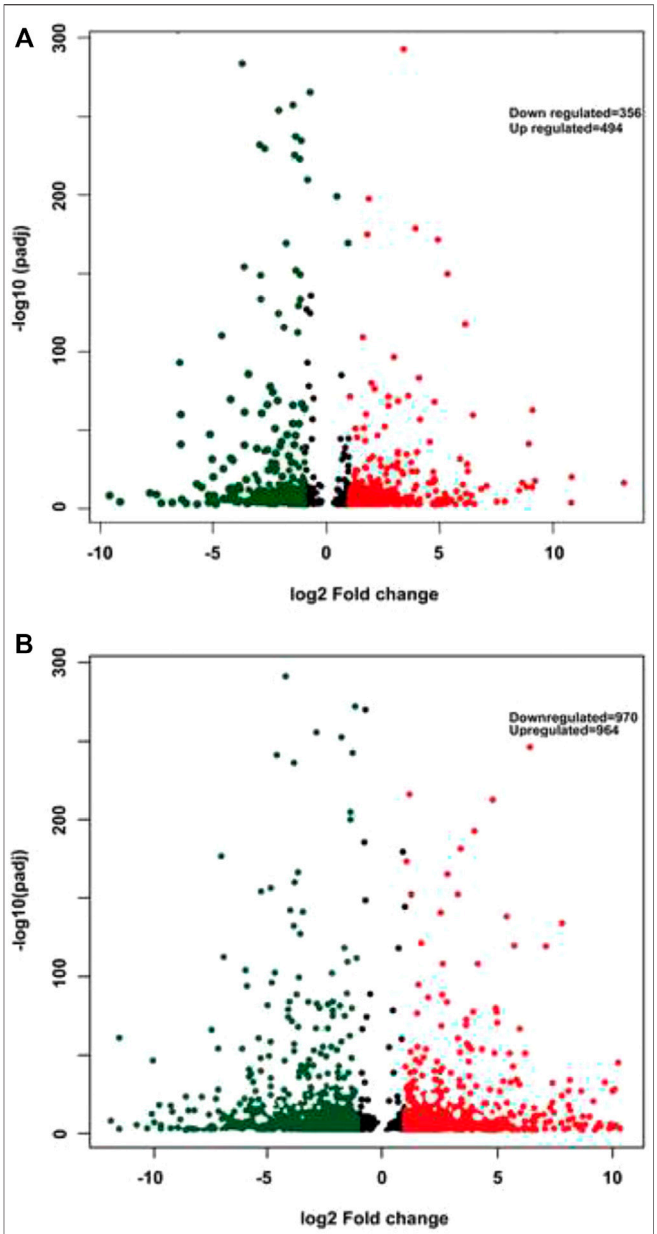

FIGURE 7 | Volcano plots displaying the differentially expressed genes. **(A)**- 841-B genotype. **(B)**- PPMI-69 genotype. Red color represents upregulated genes, green color indicates downregulated genes, while nonsignificant genes are shown as black dots.

898,460) were transversions. The observed transition: transversion (Ts/Tv) ratio is 1.84. The ratio of transition to transversion is expected to be more than one due to the mutational processes in plant genome. The ratio is lower than the estimates from maize (2.5) and *Arabidopsis* (2.4). In our studies, an average of one variant was observed on every 5,116 bases. The identified SNPs may be utilized as a genomic resource for *P. glaucum* improved by mining alleles of genes and genome assisted breeding for future genome-wide association studies.

Differential Gene Expression Analysis and Validation of Heat Stress-Responsive Genes

FPKM (Fragments per kilobase per million fragments) unit was used to calculate the expression levels of genes in *P. glaucum* flag

TABLE 3 | Statistics related to the number of significantly enriched differentially expressed genes (DEGs).

Comparisons	Up	Down	Total
841-B Control vs. PPMI_69 Control	323	355	678
841-B 6 h vs. 841-B Control	494	356	850
841-B 6 h vs. PPMI-69 Control	813	508	1,321
841-B 6 h vs. PPMI-69 6 h	748	422	1,170
PPMI-69 6 h vs. 841-B Control	970	936	1906
PPMI-69 6 h vs. PPM1-69 Control	964	970	1934

leaves transcriptome. Each sample reads were aligned separately to 47,310 unigenes. 75.02% reads got aligned (out of 72, 512, 870) for genotype 841-B control and 68.25% reads got aligned (out of 67, 530, 958) for genotype 841-B 6 h, 72.84% reads got aligned (out of 69, 132, 264) for genotype PPMI-69 control and 74.21% reads got aligned (out of 65, 544, 917) for genotype PPMI-69 6 h. After filtering, based on the adjusted *p*-value, less than 0.005 and (-1< log2 fold change >1) there were 850 DEGs identified between 841-B control and 841-B 6 h, of which 494 genes were upregulated and 356 genes were downregulated. Among 1,934 DEGs identified between PPMI-69 control and PPMI-69 6 h, 964 genes were upregulated and 970 genes were downregulated (**Figure 6B**).

Differential gene expression patterns across the treatments and comparison of differential expression patterns between the two different genotypes, were analyzed by cluster analysis with the hierarchical clustering method (**Figure 6A**) and the DEGs were visualized by volcano plots (**Figure 7**). The hierarchical cluster analysis of two different genotypes with heat stress treatment shows that the differential gene expression pattern of the transcriptome readily differentiates *P. glaucum* genotype PPMI-69 6 h from others (841-B control, 841-B 6 h, and PPMI-69 control) (**Figure 6A**), possibly indicating the variation in differential gene expression occurring between the genotypes in response to heat stress. The maximum number of DEGs (1,934 genes) was observed between PPMI-69 6 h and PPMI-69 control (**Table 3**). These DEGs between the samples 841-B 6 h and PPMI-69 6 h are grouped into two clusters (**Figure 6A**), representing that the genes involved in thermotolerance have different level of differential gene expression. In general, resemblance in the pattern of DEGs was observed for the samples 841-B 6 h, 841-B control, and PPMI-69. This suggests that 841-B 6 h has better ability to maintain homeostasis during heat stress as compared to PPMI-69 6 h. A comparative analysis between the differentially expressed genes in 841-B and PPMI-69 genotypes (**Figure 6B**) was performed to identify the common DEGs in both the genotypes under heat stress and those that are unique to each genotype of the *P. glaucum*. A total of 2,301 genes were differentially expressed, 20.99% (483 genes) of which were shared common by both the genotypes. 15.95% (367 genes) of the DEGs were unique to 841-B genotypes and 63.06% (1,451 genes) of the DEGs were unique to PPMI-69. However, the differential expression of genes between the two genotypes cannot be attributed exclusively to the treatments alone, as the two genotypes have inherently different levels of heat

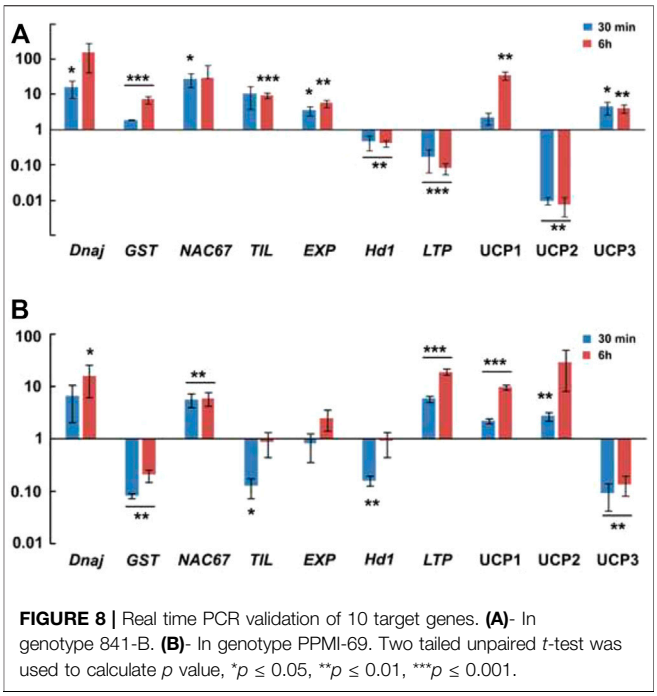

FIGURE 8 | Real time PCR validation of 10 target genes. **(A)**- In genotype 841-B. **(B)**- In genotype PPMI-69. Two tailed unpaired *t*-test was used to calculate *p* value, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

stress tolerance and therefore could have different gene expression profile. The sequencing data generated in this study is being utilized for mining and validation of heat stress responsive genes in different varieties of pearl millet including 841-B and PPMI-69 varieties (Sankar et al., 2021).

In order to investigate the temporal expression patterns by qRT-PCR analysis, 10 target genes were selected based on the fold changes and adjusted *p*-values. The selected genes displayed varying patterns in response to different durations of heat stress (**Figure 8**). The validation of selected 10 genes (seven known and three uncharacterized) by qRT-PCR deciphered their significant role in heat stress management in *P. glaucum*. These genes might play essential role(s) in the amelioration of heat stress in *P. glaucum*.

Heat stress induces change in protein conformation and increase improper folding of the native proteins. As a result, accumulation of many heat shock proteins is triggered to counterbalance the negative effect of heat stress in plants (Schlesinger, 1990). Among the many heat shock proteins, DnaJ or Hsp40 is known to play significant role in plant development, signal transduction, and abiotic stresses response, either by itself or in association with Hsp70 (Yang et al., 2010; Shen et al., 2011; Sankar et al., 2021). *PgDnaJ* expression show significant upregulation, about 15 folds in 841-B in response to 30 min heat stress, and 16 folds in PPMI-69 (**Figure 8**). However, this result indicates the important role of *DnaJ* in maintaining cellular protein homeostasis during heat stress (42°C). Plasma membrane acts as the primary sensor of the cell when the cell is subjected to heat stress (Murata and Los, 1997). Consequently, membrane properties undergo a number of changes in its composition, ion concentration, and ion channels in response to heat stress (Saidi et al., 2010; Prasad, 1996). Peroxidation of lipid membrane

is one of the most important change that occurs in the cell in response to various stresses (Thompson et al., 1998). MDA content is a direct indicator of lipid peroxidation. These byproducts of lipid peroxidation, such as electrophiles or xenobiotics, are detoxified by reactive oxygen species (ROS)-scavenging enzymes such glutathione S-transferases (GST), superoxide dismutases, catalases, and ascorbate peroxidases (APX). The expression of *PgGST* in our study was significantly upregulated (7 folds) in response to heat stress in 841-B but significantly downregulated in PPMI-69 (**Figure 8**), indicating its role in the heat stress tolerance pathway. This result correlates with MDA content (**Supplementary Figure S2**), as it was observed to be higher in PPMI-69 compared with 841-B. NAC transcription factors (TFs) (NAM, No apical meristem; ATAF, Arabidopsis transcription activation factor and CUC, Cup-shaped cotyledon) play important role in plant growth and development and in regulating response to abiotic or biotic stresses (Souer et al., 1996; Nuruzzaman et al., 2013). Among these NAC genes, *NAC67* has a role in imparting tolerance to multiple abiotic stress such as drought, salt, and cold stresses. *NAC67* has been reported to be involved in conferring tolerance to abiotic stresses in rice (Rahman et al., 2016). However, so far, there has been no report indicating its role in heat stress tolerance. In this study, *PgNAC67* expression was found to be significantly upregulated (27 folds) in 841-B in response to 30 min and six folds in PPMI-69 in response to 6 h of heat stress (**Figure 8**). It shows that *PgNAC67* plays a role in heat stress response in *P. glaucum*. Temperature-induced lipocalins (TILs), a plasma membrane protein, have an important role in basal and acquired thermotolerance in plant. TILs alleviate the heat induced lipid peroxidation in membrane. *PgTIL* expression was observed to be significantly upregulated (nine folds) in response to 6 h of heat stress in 841-B but significantly downregulated in PPMI-69 (**Figure 8**). This might be the reason why 841-B is able to maintain a low MDA content (**Supplementary Figure S2**) as compared to PPMI-69. *PgEXP* expression shows five folds significant upregulation in 841-B in response to 6 h of heat stress (**Figure 8**). Association of expression genes and heat stress tolerance in some plants has been reported (Xu et al., 2007; Kuluev et al., 2016). Overexpression of the *EXP1* gene exhibits low electrolyte leakage, decrease in membrane lipid peroxidation but higher chlorophyll content, net photosynthetic rate, relative water content, and activity of antioxidant enzyme in transgenic plants (Xu et al., 2014). Heading 1 (*Hd1*) and early heading 1 (*Ehd1*) are mainly known for the regulation of flower development and flowering, leading to either induction or suppression corresponding to the particular photoperiod (Endo-Higashi and Izawa, 2011). Environmental factors such as day length and abiotic and biotic stresses regulate the expression of these genes. Previous studies show that inhibition of early heading 1 (*Ehd1*) in response to drought stress delays flowering in rice (Cho et al., 2017). In our studies, *PgHd1* expression shows significant downregulation in both the genotypes in response to 30 min and 6 h heat stress (**Figure 8**). *PgLTP* expression shows significant (19 folds) upregulation in response to 6 h of heat stress in PPMI-69 but significantly downregulated in 841-B (**Figure 8**), indicating the involvement of high activity with regard to transfer of lipid molecules in the cell. This shows active regulation of membrane fluidity in PPMI-69 in response to heat stress. Lipid Transfer Protein (LTP) are reported to be involved with growth and development, response to abiotic and biotic stresses but their functions remain unclear (Debono et al., 2009; Guo et al., 2013; Yu et al., 2013; Pan et al., 2016). Moreover, LTPs has the ability to facilitate the transfer of phospholipids between membranes *in vitro* (Kader 1996). Among the differentially expressed contigs, uncharacterized ORFs share maximum proportion in our study. Uncharacterized genes with a predicted protein domain associated with zinc fingers (ZnF), ribonuclease (RN), and chaperone were validated for their expression during heat stress. Some of the uncharacterized genes were expressed uniquely either in 841-B or PPMI-69. For example, uncharacterized gene *PgUCP1,* with the predicted zinc fingers (ZnF) domain is significantly upregulated (34 folds) in 841-B and (10 folds) in PPMI-69 in response to heat stress (**Figure 8**). ZnF is known for the involvement in multiple stress response, but their exact molecular mechanism and their interaction is yet to be deciphered (Vij and Tyagi 2008; Bogamuwa and Jang, 2016; Maibam et al., 2019). On the contrary, uncharacterized gene *PgUCP2,* with the predicted ribonuclease (RN) domain is significantly downregulated in 841-B but significantly upregulated (29 folds) in PPMI-69 in response to heat stress (**Figure 8**). It has been reported that loss of ribonuclease function in *Arabidopsis* enhances its heat stress tolerance (Nguyen et al., 2015). However, uncharacterized gene *PgUCP3,* with the predicted chaperone domain show considerable upregulation (4 folds) in 841-B and is downregulated in PPMI-69 in response to heat stress. These uncharacterized genes could likely represent the important genes involved in imparting variation in thermotolerance among different genotypes. Furthermore, detailed investigation of these uncharacterized genes is required for understanding the role in response to stress.

CONCLUSION

This study investigated the transcriptome profile of pearl millet flag leaves in response to heat stress. In this study, high quality 47,310 unigenes were generated and annotated. This data will provide the foundation for research on gene expression, genomics, and functional genomics in pearl millet improvement program. Furthermore, the SSRs obtained in this study shall facilitate the research on genotyping, and diversity studies of this important crop. The candidate genes, whose expression patterns were validated by qRT-PCR, included seven genes known to have role in heat stress in Pennisetum and/or other crops and three uncharacterized genes whose role is yet to be established in heat stress. All these genes along with the pool of differentially expressed DEGs between two

genotypes comprise important resource that can be explored for their effective utilization in the development of transgenic crops tolerant to heat stress.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

AM: data curation and writing—original draft preparation, SL: formal analysis and writing—review and editing, KG: writing—review and editing, SN, AS, MS, SS, and MD: formal analysis, and JP: conceptualization, resources, project administration, writing—review and editing, and funding acquisition.

ACKNOWLEDGMENTS

Award of fellowship to the first author and third author by the Department of Biotechnology, Government of India, and by PG School-IARI, respectively, is duly acknowledged. The authors are grateful to the Director, ICAR-National Institute for Plant Biotechnology (ICAR-NIPB), New Delhi, for providing necessary facilities. The Director, ICAR-Indian Agricultural Research Institute (ICAR-IARI), is thanked for facilitating the use of National Phytotron Facility for growing seedlings. Thanks are also due to Dr. Tara Satyavathi Chellapilla, Division of Genetics, IARI, New Delhi, for providing the seed material.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.884106/full#supplementary-material

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frontiers | Frontiers in Genetics

REVIEW
published: 20 June 2022
doi: 10.3389/fgene.2022.883924

Cytokinin and Its Key Role to Enrich the Plant Nutrients and Growth Under Adverse Conditions-An Update

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Among the field crops, wheat is regarded as one of the most paramount cereal crops because it is widely grown, consumed as food across the world, and also known as the staple food for nearly 35 per cent of the world population. However, it is threatened by spot blotch disease causing considerable yield loss, with existing genotypes lacking the resistance and the necessary nutrients. Cytokinins (CKs) are key phytohormones that not only regulate the plant growth/development but also play an important role during stress and in the nutrient metabolic pathway of crop plants. Deficiency of important nutrients like zinc, iron, and vitamin A causes irreparable damage to the body, pressing the need to increase the accumulation of such micronutrients in the edible parts of the plant. Crop bio-fortification is one of the emerging approaches through which the quantities of these nutrients could be increased to an advisable amount. Cytokinin is observed to have a pivotal role in managing environmental stress/climate change and defense systems of plants, and apart from this, it is also found that it has an impact over Zn accumulation in cereal crops. Manipulation of the cytokine dehydrogenase (CKX) enzyme that degrades cytokinin could affect the yield, root growth, and important nutrients. Several instances revealed that an increment in the contents of Zn, S, Fe, and Mn in the seeds of cereals is a reflection of increasing the activity of CKX enzyme resulting the enhancement of the root system which not only helps in the absorption of water in a drought prone area but is also beneficial for scavenging nutrients to the deeper ends of the soil. Exploring micronutrients from the lithosphere *via* the root system helps in the uptake of the micronutrients and transporting them *via* the vascular system to the sink of crop plants, therefore, identification and incorporation of CKs/CKX linked gene(s) into targeted crop plants, exploring a bio-fortification approach including *CRISPR-Cas9* through conventional and molecular breeding approaches could be the most paramount job for improving the important traits and stress management in order to enhance the plant growth, productivity, and nutritional value of the wheat crops, which would be useful for mankind.

Keywords: wheat, spot blotch, nutrients, cytokinin (CK), CKX, biofortification, stress

OPEN ACCESS

Edited by:
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National Institute of Plant Genome Research (NIPGR), India

Reviewed by:
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *25 February 2022*
Accepted: *05 May 2022*
Published: *20 June 2022*

Citation:
Prasad R (2022) Cytokinin and Its Key Role to Enrich the Plant Nutrients and Growth Under Adverse Conditions-An Update. Front. Genet. 13:883924. doi: 10.3389/fgene.2022.883924

INTRODUCTION

Wheat (*Triticum aestivum* L.), belongs to the family Poaceae, and is one of the most economically important cereal crops in the world (Schnurbusch, 2019; Alzaayid and Aloush, 2021). Among the field crops, wheat is regarded as the most crucial cereal crop because it is widely grown and consumed as food around the world. It is also known as the staple food for nearly 35 per cent of the world population and demand for wheat is expected to grow faster than the other major crops (Pingali, 2000). It is being cultivated in a wide range of environmental conditions across the globe and it is rich in nutrition components and provides approximately 20% of protein in the human diet (Reynolds and Braun, 2019). Much of the success was caused by the combination of high rates of investment in crop research, infrastructure, market development, and appropriate policy support that took place during the first Green Revolution, but still there is need to improve the crop productivity to meet the demand of the rapidly growing population (Pingali, 2000). Wheat, however, is being cultivated at a large scale and there is maximum demand in the world, so in response to food security in 21st century, structural transformations are needed to improve the crop yield in order to meet the demand of needy people (Pingali, 2015). Instead of just being considered a staple food, wheat is also a staple source of nutrients for around 40% of the world (Giraldo et al. (2019). However, malnutrition is still a serious issue these days, hence, the development of the promising wheat genotypes through crop bio-fortification which helps the conventional and advance breeding approach is required for nutritional security in the 21st century (Sujatha et al., 2021). Approximately 90%–95% of the wheat produced in the world is common or bread wheat having $2n = 6x = 42$ composed of three sub genomes like A, B, and D diploid genomes which is a rich reservoir of genes determining yield and its contributing traits (Nadolska et al., 2017).

However, wheat crop is threatened by spot blotch disease caused by *Bipolaris sorokiniana* syn. *Helminthosporium sativum* syn. *Cochliobolus sativus* which is considered one of the most devastating diseases in Eastern India and South East Asia (Joshi et al., 2002); Kumar et al. (2009). Globally it is known as the most important disease, mainly in warm and humid regions of South Asia and South America (Kumar et al., 2019). As per challenges, efforts have been made and several resistance genotypes have been identified but availability of immune or near immune plants is lacking (Lillemo et al., 2012; Kumar et al., 2019). This disease was reported in the beginning of 19th century but gained more importance after the Green Revolution (Saari, 1998). Sensitive cultivars of barley and wheat are under severe attack from pathogens mainly at the time of late milking, dough stage, or at the time of flowering which badly disturbs the grain filling and eventually leads to lowering the yield of barley (Prasad et al., 2013) and wheat crops (Gupta et al., 2018). The application of fungicide can completely control/reduce the spot blotch disease severity (Videma and Kohli, 1998), but repeated application of such fungicides not only increases the cost of cultivation but also pollutes the environment, and is associated with emergence of fungicidal resistance in the target pathogen as well (Golembiewski et al., 1995). Hence, development of resistance cultivars by combining the conventional and advance molecular breeding approach is an effective and cost-effective strategy for combating the spot blotch problem. The availability of genetic information on spot blotch resistance genetics is scant as revealed by available literature and very limited genotypes have had their resistance level identified. Besides this disease, wheat crop also suffers from other biotic and abiotic factors as well as lacking the necessary nutrients for the human body. When the body does not get enough nutrients, it creates many problems including digestion, fatigue, dizziness, weight loss, and malnutrition which can cause physical or mental disability as well. Esra Koç and Belgizar Karayiğit (2022) stated that micronutrients are essential for physiological functions, and their deficiency causes serious health disorders, and it (Zn) is effective in many events such as reproduction and neurotransmission, especially the immune system. Similarly, Verma et al. (2021) reported that deficiency of micronutrients causes impairments to learning, physical growth, and reproductive health, decrease in immune resistance, and an increase in the rate of infection too.

Although the country has achieved total food grain production estimated at 296.65 million tonnes during 2019–20, this production is/was higher by 26.87 million tonnes than the average production of food grain of the previous 5 years. Similarly, wheat production is estimated at 107.59 million tones during 2019–20 (Anonymous). This bumper food grain production is primarily attributed to the production of high yielding genotypes. However, during the production of high yielding varieties, enough attention has not been given towards important nutrients; as a result, such improved genotypes are high yielding but have low concentrations of important nutrients as the standard recommended level. Thus, exploring the genetic information and identifying potential genotypes which confer a good source for resistance, are rich in important nutrients, and can harness the cytokinin hormone associated genes would be a wonderful approach for improving crop yield and other desirable traits using conventional and molecular breeding techniques including multidisciplinary approaches. In light of producing the high yielding wheat genotypes along with those rich in targeted nutrients and desirable for other agronomically traits, harnessing the cytokinin phyto hormones are one of the emerging approaches for the researchers under the changing climate. The changing climate results in an increase of the greenhouse gases which cause the reduction of crop production, yield, quality, and as result, nutritional deficiency in humans (Myers et al., 2014). Moreno-Jiménez et al. (2019) stated that increasing drought is because of changing the climatic and it lowers the availability of essential micronutrients, especially Fe and Zn. In order to supply the demand of the rapidly growing population, increasing the yield of crop plants is the prime objective of plant breeders/scientists in the 21st century as well as producing enough food, increasing the important nutrients level in the edible parts of crop plants, through either bio-fortification (producing crops that have higher levels of nutrition in their edible parts) or developing the superior genotypes along with rich in nutrients through modern breeding approaches is the current need. Since malnutrition is a challenging issue at a global level, efforts have

been made under the leadership of the Consultative Group on International Agricultural Research (CGIAR) and accordingly huge bio-fortified genotypes of different crop plants have been developed across the globe. In continuation, in the country, 28 bio-fortified wheat genotypes along with those better for other desirable traits have been developed and released (Yadava et al., 2022). Yield of wheat or targeted crop plants including other desirable traits can also be enhanced by two key methods, the first is developing the high yielding genotypes as well as those better for resistance and rich in important nutrients by incorporating all linked gene(s)/QTLs into a single cultivar, and the second is saving the potential yield loss of genotypes occurred by either biotic or abiotic factors.

HARNESSING THE CYTOKININ AND ITS ROLE FOR ENHANCING THE CROP YIELD AND OTHER TRAITS

Hormones are produced naturally by plants, while plant growth regulators are applied to plants by humans for specific purpose. Plant growth hormones are essential components and control the overall outcome of plant growth and development (Mishra et al., 2022). Plant hormones and growth regulators are chemicals that affect the flowering, ageing, root growth, distortion and killing of organs, prevention or promotion of stem elongation, color enhancement of fruit, prevention of leafing, leaf fall, etc. There are five main groups of plant-growth-regulating compounds such as cytokinin, auxin, gibberellin (GA), ethylene, and abscisic acid (ABA) which are studied by the researchers, however, out of five groups, cytokinin plays a very crucial role for the cell cycle and affects the plant growth, development (Honig et al., 2018), promoting cell division and other physiological processes including germination, flowering, seed development, and leaf senescence, etc. as also presented in **Figure 1**. CKs have a significant impact on regulation of plant growth, stabilization of photosynthetic machinery during stress and exogenous application and modulation of CK levels have a positive effect on drought tolerance (Rivero et al., 2007). Research findings shows that cytokinins can alleviate the damage to plants caused by a variety of abiotic stresses (Mi et al., 2017; Prerostova et al., 2018). ABA is also considered the most important hormone as it controlled the plant water loss, hence it plays a key role under water-limited conditions for plant survival (de Ollas and Dodd, 2016. So, hormones are being exploited for crop improvement for traits of interest under normal as well stressed conditions (Gan and Amasino, 1996; Haberer and Kieber, 2002). Unlike other hormones, cytokinins are found in both plants and animals and they are considered to be master regulators of plant growth and development as well as being involved in the regulation of many important physiological and metabolic processes in crop plants (Wu et al., 2020) as it is also shown in **Figures 1**, **2**, respectively. Chemically, natural cytokinins are N6-substituted purine derivatives. Isopentenyladenine (iP), zeatin (Z), and dihydrozeatin (DZ) are the predominant cytokinins found in higher plants. The free bases and their ribosides (iPR, ZR, DZR) are thought to be the biologically active compounds. Glycosidic conjugates play a role in cytokinin transport, protection from degradation, and reversible/irreversible inactivation. There are over 20 different forms of cytokinins that have been reported in wheat by Sykorova et al. (2008) and most of them are able to interconvert to release the active free bases.

Climate change is expected to continue posing either biotic or abiotic stresses globally, which leads to hampering the agricultural practices and limiting the yield of crop plants. The investments in exploring and harnessing the genes associated with desirable traits and beneficial roles of cytokinin phytoharmone for enhancing the plant production under stressed as well as efficient agricultural production systems are required to ensure the availability of enough food with nutrition security in the face of climate change. Stable or well adapted genotypes and the roles of cytokine hormones can thrive in challenging situations. Cytokinin plays diverse roles (**Figures 1**, **2**) in response to plant growth and development, influencing many agriculturally important processes, including growth, nutrient responses, and the response to biotic and abiotic stresses (Liu et al., 2020), but under adversity stress, mechanisms of cytokinin-alleviating stress are different under different stresses, and its levels in plants are regulated by biosynthesis and inactivation pathways in crop plants.

In addition to genetic approaches for enhancing the impotent/essential nutrients, grain Zn concentration in wheat can also be increased by applying Zn-containing fertilizers, a process termed agronomic bio-fortification or agro-fortification (Zhao et al., 2019). In a review of published field studies, Joy et al. (2015b) noted that foliar application of Zn ($ZnSO_4$) fertilizers, applied as a drench to field-grown wheat that can increase the whole-grain Zn concentration by a median of 63%. Soil-applied Zn fertilizers can also increase grain Zn concentrations, albeit to a much lesser extent than foliar-applied Zn fertilizers but may also increase crop yield (Zou et al., 2012). Similarly, Zia et al. (2020) carried out the field experiment and reported that *Zincol-2016*, a new variety of wheat, contains a higher concentration of Zn in its grain but other wheat genotypes responded to substantially increasing the grain Zn concentration when foliar Zn fertilizers are applied. However, application of soil Zn fertilizers had no significant effect on grain Zn concentration.

CYTOKININ GENES AND THEIR ASSOCIATION/EXPRESSION FOR YIELD AND YIELD RELATED TRAITS IN WHEAT

Szala et al. (2020) studied the effect of *TaCKX* wheat gene family members (GFMs) encoding the enzyme cytokinin oxidase/dehydrogenase (CKX), which irreversibly degrades cytokinins (Werner et al., 2006), and therefore strongly regulates cytokinin contents in different parts of plants. Their findings revealed the presence of natural variation in expressive levels of tested genes in controlled and normal field conditions which was very high for yield and its contributing traits, indicating the possibility of selection of beneficial wheat genotypes for breeding and enhancing the yield of crops. Cytokinins also play a diverse role in plant development

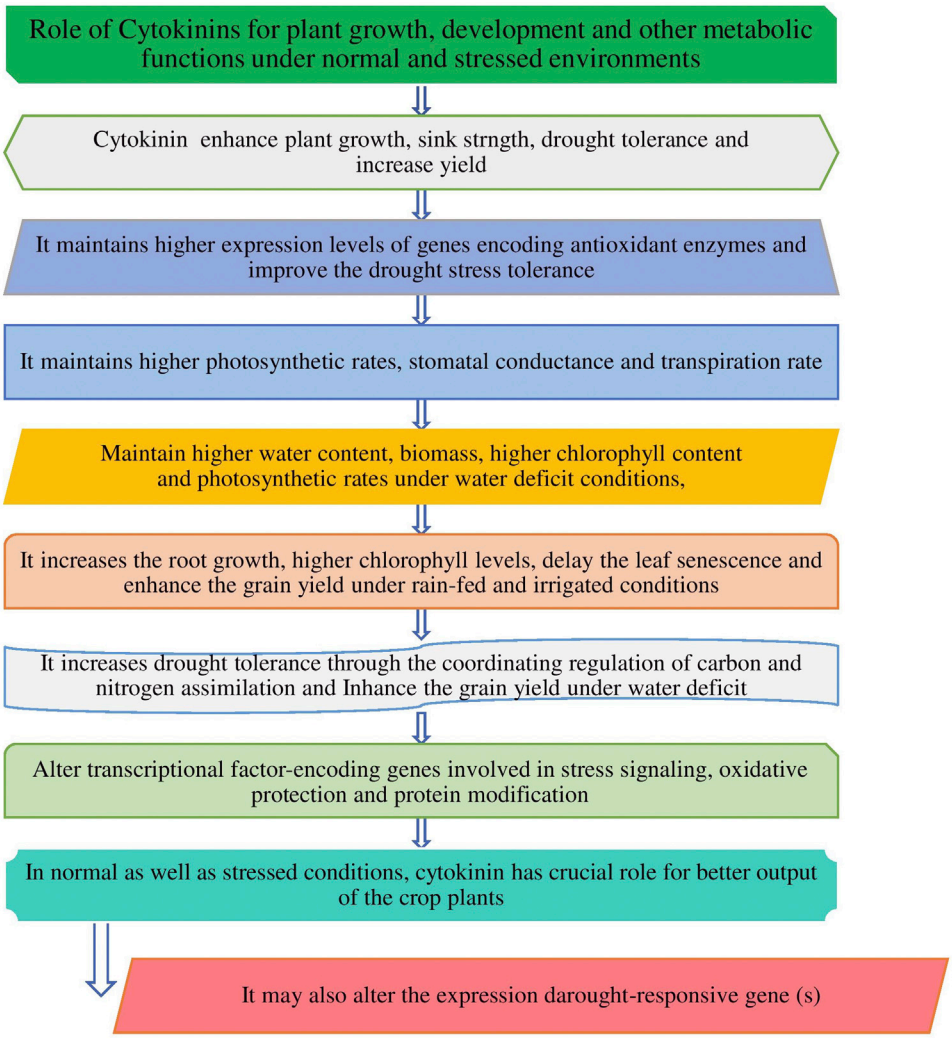

FIGURE 1 | Cytokinin and its major role in crop plants in normal and stressed conditions.

and affect a number of agriculturally important processes (Jameson and Song, 2016; Kieber and Schaller, 2018). In cereal crops, silencing of selected *CKX* is well documented in rice (Ashikari et al., 2005), in barley (Zalewski et al., 2010; Zalewski et al., 2012) and in wheat (Jablonski et al., 2020), leading to an increased level of cytokinins influencing the yield and its component traits. The number of *CKX* GFMs varies depending on species, however, in bread wheat 11 to 14 gene family members have been proposed (Ogonowska et al., 2019; Shoaib et al., 2019). Like wheat, in barley, Zalewski et al. (2014) studied the expression pattern of *HvCKX* and reported that these genes have a crucial role in the growth and reproductive development of barley crops. Similarly, Ogonowska et al. (2019) investigated the *CKX* genes in wheat with an aim to know the expression specificity of such genes for different developmental stages of crops of the plants and based on the expression of genes, they have classified such genes into four following groups:

1) Leaf-specific e.g., *TaCKX9*, *TaCKX5*, *TaCKX4*
2) Inflorescence specific and developing spike e.g., *TaCKX1* and *TaCKX2*
3) Seedling root-specific e.g., *TaCKX10*, *TaCKX7,* and
4) Expressed at various levels in all tested organs e.g., *TaCKX11*, *TaCKX3*, *TaCKX8.*

Jablonski et al. (2020) also studied the effect of *TaCKX1* silencing in wheat and reported that it was influenced by different modes of co-expression with other *TaCKX* GFMs and parameters of yield-related traits as well. Each of the tested yield-related traits was regulated by various up or downregulated *TaCKX* GFMs and phytohormones. According to Shanks et al. (2018), cytokinins regulate gene transcription in targeted organs and developmental stages of crops are associated with a wide range of transcription factors (TFs). One of the largest groups of plant TFs involved in cytokinin-dependent regulation is the family of NAC (for NAM, ATAF, and CUC) TFs. It has been

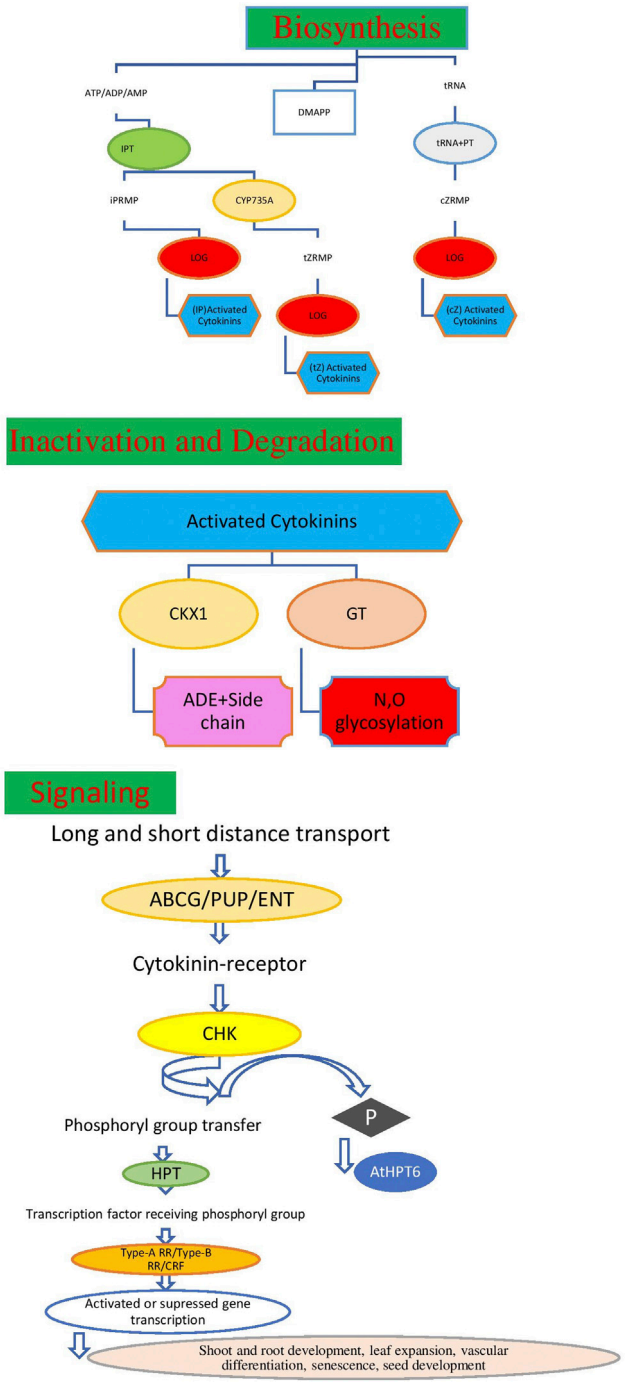

FIGURE 2 | Model of cytokinin biosynthesis, metabolism, degradation and signaling dransduction process.

documented that NACs are involved in the regulation of important agronomic traits. Alzaayid and Aloush (2021) conducted the field experiment with an aim to investigate the effect of spraying cytokinins of different concentrations on the growth and yield of wheat varieties and found that ten wheat cultivars showed a significant difference in growth, yield, and quality. Higher concentrations of cytokinins indicated a significant difference for the most of traits such as flag leaf area, number of grains, biological yield, and protein percentage respectively. Hence, identified genotypes may be used as a parent or donor under breeding programs for improving the yield and its attributing traits as well.

Cytokinins biosynthesis, metabolism, degradation, and signaling transduction processes of cytokinins are presented in **Figure 2**, which were already described by Wu et al. (2020). Gene(s) currently known to be involved in the cytokinin biosynthesis pathway and encode the *isopentenyl transferase* (IPT) and lonely guy (LOG) enzymes are reported by Takei et al., 2001 and Kurakawa et al., 2007. The initial step of cytokinin biosynthesis in higher plants is the formation of cytokinin nucleotides, namely, isopentenyladenosine 5′-tri-, di-, or monophosphate (iPRTP, iPRDP, or iPRMP, respectively from ATP, ADP, or AMP and dimethylallyl pyrophosphate (DMAPP) by IPTs5. LOGs, which encode phosphoribohydrolase-activating enzymes, directly convert a cytokinin nucleotide to an active free-base form of cytokinins in the final step of cytokinin biosynthesis while the levels of active cytokinins can be modulated *via* irreversible cleavage by cytokinin oxidase (*CKX*) enzymes (Schmulling et al., 2003) or through conjugation to glucose by cytokinin glycosyltransferases (Hou et al., 2004). Plants regulate the concentration of active cytokinins through either reversible or irreversible metabolism processes. Therefore, the precise maintenance of the homeostasis of cytokinins through these synthesis and inactivation enzymes is essential for plant development and adaptation. The full forms of the abbreviations used in **Figure 2** are DMAPP: dimethylallyl pyrophosphate; iPRMP: isopentenyladenosine-5-monophosphate; tZRMP, trans-zeatin riboside 5′-monophosphate; cZRMP, cis-zeatin riboside 5′-monophosphate; iP, N6-(Δ2-isopentenyl) adenine; tZ: trans-zeatin; cZ: cis-zeatin; Ade: adenine; IPT, isopentenyltransferases; tRNA-IPT, tRNA-isopentenyl transferase; CYP735A, cytochrome P450 monooxygenase; LOG, LONELY GUY; GT, glycosyltransferase; CKX, cytokinin oxidase/dehydrogenase; ABCG, g subfamily ATP-binding cassette; PUP, purine permeases; ENT, equilibrative nucleoside transporters; HKs, histidine kinase; HPTs, histidine phosphotransfer proteins; ARR, response regulator, CRF, cytokinin response factor, etc. Cytokinin signal transduction pathway is also presented in **Figure 2**, and to date it has been well studied by researchers. In microorganisms a two-component system (TCS) is applicable, which changes the gene expression levels and acts in response to various stimuli and improve their ability to recognize and adapt to environmental changes (Cheung and Hendrickson, 2010). This TCS includes the flowing proteins: histidine kinases (HKs) associated with the membrane and response regulators (RRs) in the cytoplasm, HKs detect the environmental input in the sensor area and transmit the generated signal to the cytoplasm as reported by Cheung and Hendrickson (2010). However, based on TCS, plants have evolved a multi-step phosphorylation system, including the following three components: HKs, histidine phosphotransfer proteins (HPs), and RRs presented in **Figure 2** and reported by Grefen and Harter (2004). Cytokinin uses this multi-step phosphorylation system for its signal transduction, including participation in cell division, leaf senescence, and apical dominance (Pils and Heyl, 2009). Mishra et al. (2022), stated that plant growth hormones are essential components

that control the overall outcome of the growth and development of the plant, while cytokinins are hormones that play an important role in plant immunity and defense systems. In order to show this, they have identified nine functional modules comprised of different hub genes 36) which contribute to the cytokinin signaling route. Out of 36 genes, 17 genes are associated with *QTLs* for salt, cold drought, and bacterial stress, and are therefore recommended to design the new stress-resistant cultivars which can provide sustainable yield in stress-specific conditions. Trans-Zeatin (tz) is an active form of cytokinin involved in managing environmental stress, the cytokinin pathway has been widely studied and a huge amount of gene expression data are available in public repositories (Edgar et al., 2002).

CYTOKININ AND LINKED MOLECULAR MARKER FOR YIELD AND YIELD RELATED TRAITS IN WHEAT

The main pigment in crop plants responsible for photosynthesis is chlorophyll, including chlorophyll a and b, and the key photosynthetic pigment in chloroplasts and its amount directly affects the plant's photosynthetic efficiency (Araus et al., 1997; Thomas et al., 2005). Its abundance and stability in the leaf significantly affects the grain filling, grain weight, and eventually the actual yield of the crops (Shao et al., 2013). Research findings reveal that cytokinin (*CTK*) can greatly increase leaf chlorophyll content, chloroplast stability, and net photosynthetic rate (Shao et al., 2012; Ding et al., 2013). However, in wheat crop little is known about the association of *Tackx* gene with chlorophyll content and grain weight. The cytokinin can effectively increase chlorophyll content and chloroplast stability, but it is irreversibly inactivated by cytokinin oxidase (*CKX*). Cheng et al. (2015) carried out an experiment with an aim to identify variations of wheat *TKX* (*Tackx*) genes and their association with grain weight and wheat chlorophyll level, and validating the effect of targeted *Tackx* gene on these two traits. Their findings indicated a variation of *Tackx4*, that was proven to be significantly associated with chlorophyll content and grain weight in the RIL populations and also identified two *Tackx4* patterns *viz*., one with two co-segregated fragments (*Tackx4-1*/*Tackx4-2*) containing 618 bp and 620 bp in size, and another with no PCR product. These two genotypes were designated as genotype-A and genotype-B, respectively. Their mapping analysis reveals that *Tackx4* was closely linked to *Xwmc169* on chromosome 3AL, as well as co-segregated with a major quantitative trait locus (QTL) for both grain weight and chlorophyll content and this *QTL* explained 8.9%–22.3% of the phenotypic variations for the two traits across the four cropping seasons, whereas previous researchers identified the multiple copies of *CKX* genes on chromosome 3DS of wheat, including *Tackx2.1 and Tackx2.2* (Zhang et al., 2011). Previous researchers have also identified the several *QTLs* for yield and yield related traits detected on wheat chromosome 3A which explain 4.1 to 14.27 percent of phenotypic variations in different environments (Kumar et al., 2007; Cuthbert et al., 2008; Wang et al., 2009). For chlorophyll content and photosynthesis, many more *QTLs* have been found on wheat chromosomes 1A, 1D, 2A, 2D, 3B, 4A, 5A, 5B, 5D, 6A, 6D, 7A, and 7D (Sukumaran et al., 2015). Such useful findings may be explored for improving the accuracy and effectiveness of marker assisted selection for chlorophyll level and grain weight in wheat breeding to develop promising genotypes.

HARNESSING THE GENETIC RESOURCES FOR IMPROVING THE NUTRIENTS, YIELD, AND OTHER DESIRABLE TRAITS

To continue the challenge, the improved wheat genotypes had satisfactory yield and see improvement in other traits but somehow lacked the necessary nutrients for the human body. In light of this happening in the recent past, about 28 bio-fortified wheat genotypes (**Figure 3**) have been developed by various agricultural institutes of the country having enough important nutrients and improvement in other desirable traits (Yadava et al., 2022) (**Table 1**). Besides this, many other studies have been carried out regarding the transport of micronutrients in crops and to enhance their content, such as Wang et al. (2014), Qin et al. (2016), Connorton et al. (2017), Singh et al. (2017), Beasley et al. (2019) and Liang et al. (2019). Hence, in order to improve wheat genotypes for nutrients and other desirable traits including resistance for biotic and abiotic stresses, these genotypes may be chosen as donor parents in crossing/breeding programmes. Besides this, published studies report that some genotypes have a unique genetic make-up that means if a foliar spray of chemical fertilizers is provided, then they are synthesizing enough nutrients into grains thus, genotypes could be explored through smart breeding at a molecular level to understand genetics of such targeted traits with aim to develop superior genotypes. Similarly, association of morpho-physiological traits with resistance to spot blotch in wheat such as leaf angle (Joshi and Chand 2002; Prasad et al., 2013), leaf tip necrosis (Joshi et al., 2004a), and stay green trait (Joshi et al., 2007a) have been studied and recommended to explore breeding of the promising genotypes as these phenotypic traits are strongly associated with resistance to spot blotch disease. Stay-green is a key trait of wheat can not only enhance the yield of wheat crops because of its efficiency in photosynthesis but is also able to contribute for resistance to heat, spot blotch and other stresses. This unique trait can also be used as a morphological marker for selecting the spot blotch disease resistance wheat among the large populations and explore the breeding of promising genotypes through hybridization. Cytokinins are well known as the most potent general coordinator between the stay-green trait and senescence of plant species. Schwarz et al. (2020) reported that expression of cytokinins can directly increase seed yield, grain numbers, and seed size of the concerned crops, and it has a significant response against environmental stressors as well (Cortleven et al., 2019). The examinations of endogenous cytokinin levels under various conditions reveals that cytokinin metabolism is highly regulated during the response to abiotic stress (Zwack and Rashotte, 2015). Because of their recognized effects on increasing seed number and seed size, and effect under stressed conditions and synthesizing important nutrients, the cytokinins may well be the hormone that underpins the second "Green Revolution" as highlighted by

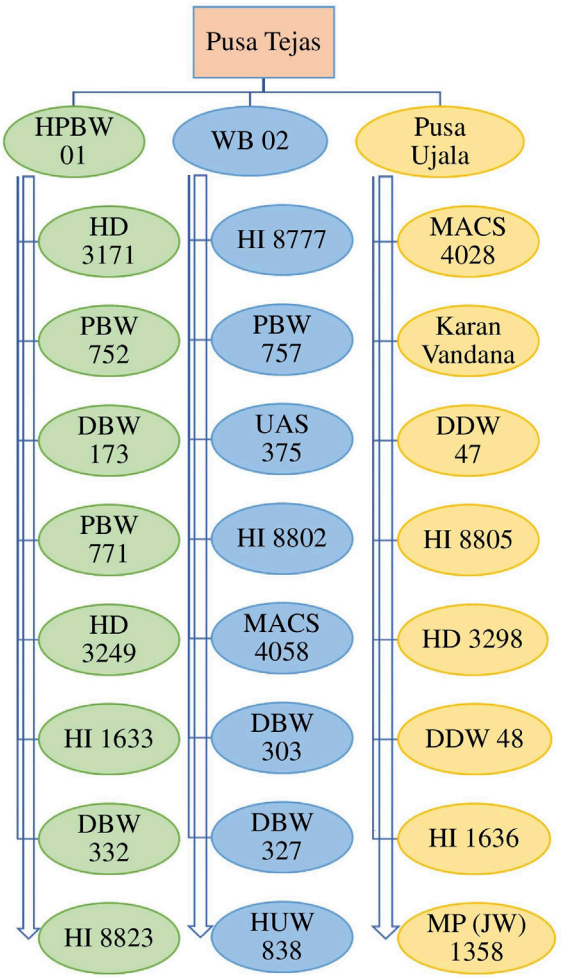

FIGURE 3 | Name of 28 Bio-fortified wheat varieties developed by research institutes in the country.

Lynch (2007). Virk et al. (2021), have summarized the targeted breeding for micronutrients contents is/was conceived by HarvestPlus which was the challenging international program of CGIAR. This is a biofortified breeding program including high-throughput micronutrient phenotyping, genomic selection coupled with speed breeding for accelerating genetic gains with an aim to develop the biofortified genotypes through biofortification for three important micronutrients, namely iron (Fe), zinc (Zn), and provitamin A (PVA), needed for human health as they have gained momentum in the 21st century. The plan of Harvest Plus, along with its global consortium partners, is to significantly increase the quantity of Fe, Zn, and PVA in staple crops as well as release bio-fortified wheat varieties with huge potential across the globe. Such bio-fortified genotypes could be used as parent/donors in future breeding programs for crop improvement. The key center of CGIAR *viz*., CIMMYT has an outstanding role to develop superior wheat genotypes which have enough zinc and other required nutrients (Yuan et al., 2019; Jigly et al., 2019).

IMPORTANT APPROCHES/TECHNOLOGY UTILISED FOR CROP BIO-FORTIFICATION

Reports indicate that more than 820 million people in the world are hungry and two billion people are suffering from micronutrient deficiencies (Kane et al., 2015; Boliko et al., 2019; UNEP. 2021). Most of the crop plants can accumulate micronutrients however; some main plants lack the adequate amounts of such nutrients *viz*., Fe and Zn in the edible parts (Wakeel et al., 2018), for instance, basic/staple crops such as rice, wheat, and maize contain low amounts of Zn and Fe (Shariatipour and Heidari 2020). In recent research, it is strongly stated that micronutrient deficiencies increase susceptibility to many infectious diseases, including Covid-19 (Akhtar et al., 2021). Therefore, more attention has been made to enhance such import nutrients in the crop plants through bio-fortification, which is an effective strategy to combat micronutrient deficiency. Different useful approaches of bio-fortification are being used to improve the nutritional value of plants, to overcome nutritional the problems presented in **Figure 4**, and also described by Koç and Karayiğit (2022), with a remark that bio-fortification is a cost-effective and sustainable agricultural strategy for increasing the bioavailability of essential elements/nutrients in the edible parts of plants and reducing malnutrition. Further, they have also pointed out that genetic bio-fortification based on genetic engineering such as increasing or manipulating the expression of genes that affect the regulation of metal homeostasis and carrier proteins that serve to increase the micronutrient contents and greater productivity through *CRISPR-Cas9* (bacterial Clustered Regularly Interspaced Short Palindromic Repeats) technology can be considered as a promising high-potential strategy or modern and very advanced GM technology for solving the micronutrient deficiency problem and this technique was reported for the first time by Jinek et al., 2012. By modifying the germ line cells, *Crispr-cas* technology has the potential to develop transgenics without involving transformation and tissue culture plants (Malik and Maqbool, 2020). Eckerstorfer et al. (2019) stated that, across the globe, new genetic modification technique (*nGMs*) approaches, particularly genome editing, are used in basic and applied research. In parallel to classic genetically modified technology a wide range of *nGM* techniques are being developed for the (genetic) modification of organisms, including plants, for research purposes or for the development of crops for agricultural purposes. These *nGMs* are also referred to as "new techniques" or "new breeding techniques" for improving targeted traits (Lusser et al., 2012; SAM, 2017).

ROLE OF CYTOKININS UNDER A-BIOTIC STRESS RESPONSE

Besides the important role of cytokinins for plant growth and development in normal conditions, they play a very crucial role for crop plants under abiotic stressed conditions such as heat stress, drought stress, cold stress, and salt stress (Liu et al., 2020) which is summarized as follows

TABLE 1 | List of 28 bio-fortified wheat varieties released in country better for nutrients quality parameters.

Variety	Descriptions
WB 02	WB 02 wheat variety is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal which is released in 2017. It is rich in iron (40.0 ppm) and zinc (42.0 ppm) in comparison to 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. The grain yield of this variety is 51.6 q/ha, matured in142 days, suitable for irrigated timely sown conditions in *rabi* and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan, Western Uttar Pradesh and oter states
HPBW 01	HPBW 1 wheat variety is developed by Punjab Agricultural University, Ludhiana under ICAR-All India Coordinated Research Project on Wheat & Barley which is released in 2017. It is rich in iron (40.0 ppm) and zinc (40.6 ppm) in comparison to 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. This variety yielded 51.7 q/ha, matured in 141 days, suitable for irrigated timely sown conditions and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states of the country
Pusa Tejas (HI 8759)	Pusa Tejas wheat variety is also known as HI 8759 durum wheat, developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2017. It is rich in protein (12.0%), iron (41.1 ppm) and zinc (42.8 ppm) in comparison to 8%–10% protein, 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. Its yield is 57.0 q/ha, matured is 117 days, suitable for irrigated timely sown conditions in *rabi* and recommended for its cultivation in Madhya Pradesh, Chhattisgarh, Gujarat, Rajasthan and Uttar Pradesh
Pusa Ujala (HI 1605)	Pusa Ujala wheat variety is developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore which is released in 2017. It is rich in protein (13.0%) and iron (43.0 ppm) in comparison to 8%–10% protein and 28.0–32.0 ppm iron in popular varieties, having grain yield 30.0 q/ha, maturity time 105 days, suitable for timely sown restricted irrigated conditions in rabi season and recommended for cultivation in Maharashtra and Karnataka
HD 3171	HD 3171 wheat variety is developed by ICAR-Indian Agricultural Research Institute, New Delhi and released in 2017. This is rich in zinc (47.1 ppm) in comparison to 30.0–32.0 ppm in popular varieties. The grain yield of this variety is 28.0 q/ha, maturity time is 122 days, suitable for timely sown rainfed conditions in rabi and recommended for cultivation in Eastern Uttar Pradesh, Bihar, Jharkhand, Odisha, West Bengal, Assam and plains of North Eastern States
HI 8777	HI 8777 is durum wheat developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2018. It is rich in iron (48.7 ppm) and zinc (43.6 ppm) in comparison to 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. The grain yield of this variety is 18.5 q/ha, maturated in 108 days, suitable for timely sown rain-fed conditions in *rabi* season and recommended for its cultivation in Maharashtra, Karnataka and plains of Tamil Nadu
MACS 4028	MACS 4028 is a durum wheat developed by Agharkar Research Institute, Pune under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2018. It is rich in protein (14.7%), iron (46.1 ppm) and zinc (40.3 ppm) in comparison to 8%–10% protein, 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. It gives grain yield 19.3 q/ha, maturated in 102 days, suitable for rainfed, low fertility, timely sown conditions in *rabi* and recommended for cultivation in Maharashtra and Karnataka
PBW 752	PBW 752 wheat variety is developed by Punjab Agricultural University, Ludhiana under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2018. It is rich in protein (12.4%) in comparison to 8–10% in popular varieties, having grain yield 49.7 q/ha, matured in 120 days, suitable for late sown irrigated conditions in *rabi* season and recommended to cultivate for Punjab, Haryana, Delhi, Rajasthan and other states
PBW 757	It is developed by Punjab Agricultural University, Ludhiana under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2018. Contains high zinc (42.3 ppm) in comparison to 30.0–32.0 ppm zinc in popular varieties. Yield of this variety is 36.7 q/ha, maturity time is 104 days, suitable for very late sown irrigated conditions in *rabi* season and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
Karan Vandana (DBW 187)	Karan Vandana wheat variety is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal and released in 2018 and 2020. It is rich in iron (43.1 ppm) in comparison to 28.0–32.0 ppm in popular varieties, having grain yield 48.8 q/ha in North Eastern Plains Zone (NEPZ), 61.3 q/ha in North Western Plains Zone (NWPZ) and 75.5 q/ha in high fertility conditions. Variety in matured in 120 days (NEPZ), 146 days (NWPZ) and 158 days (Highfertility) conditions. Suitable for timely sown irrigated and fertlity conditions in *rabi* season and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
DBW 173	DBW 173 is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal released in 2018. It is rich in protein (12.5%) and iron (40.7 ppm) in comparison to 8%–10% protein and 28.0–32.0 ppm iron in popular varieties, having grain yield 47.2 q/ha, matured in 122 days, suitable for late sown irrigated conditions in rabi season and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
UAS 375	This wheat variety is developed by University of Agricultural Sciences, Dharwad under ICAR-All India Coordinated Research Project on Wheat & Barley, released in 2018, rich in protein (13.8%) in comparison to 8%–10% in popular varieties. Produces 21.4 q/ha grain yield, matured in 103 days, suitable for timely sown rainfed conditions in rabi season and recommended for cultivation in Maharashtra and Karnataka
DDW 47	DDW 47 is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal, released in 2020P. Variety rich in protein (12.7%) and iron (40.1 ppm) in comparison to 8%–10% protein and 28.0–32.0 ppm iron in popular varieties, having grain yield 37.3 q/ha, maturity time 121 days, suitable for timely sown restricted irrigated conditions in rabi season and recommended for cultivation in Madhya Pradesh, Gujarat, Rajasthan and Chhattisgarh
PBW 771	PBW 771 variety is developed by Punjab Agricultural University, Ludhiana under ICAR-All Indian Coordinated Research Project on Wheat & Barley and released in 2020. It is rich in zinc (41.4 ppm) in comparison to 30.0–32.0 ppm in popular varieties. It has 50.3 q/ha grain yield, matured in 120 days, suitable for late sown irrigated conditions in *rabi* season and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
HI 8802	It is durum wheat developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2020. It is rich in protein (13.0%) in comparison to 8%–10% in popular varieties, having grain yield: 29.1 q/ha, matured in 109 days, suitable for timely sown in rain-fed and recommended for cultivation in Maharashtra, Karnataka and plains of Tamil Nadu

(Continued on following page)

TABLE 1 | (*Continued*) List of 28 bio-fortified wheat varieties released in country better for nutrients quality parameters.

Variety	Descriptions
HI 8805	HI 8,805 (durum wheat) is developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2020. It is rich in protein (12.8%) and iron (40.4 ppm) in comparison to 8%–10% protein and 28.0–32.0 ppm iron in popular varieties. It has grain yield 30.4 q/ha, matured in 105 days, suitable for timely sown in rainfed conditions and recommended for its cultivation in Maharashtra, Karnataka and plains of Tamil Nadu
HD 3249	HD 3,249 variety is developed by ICAR-Indian Agricultural Research Institute, New Delhi and released in 2020. It is rich in iron (42.5 ppm) in comparison to 28.0–32.0 ppm in popular varieties. It has yielded 48.8 q/ha, matured in 122 days, suitable for timely sown irrigated conditions in rabi season and recommended for cultivation in Eastern Uttar Pradesh, Bihar, Jharkhand, West Bengal (excluding Hills), Odisha, Assam and plains of North Eastern States
MACS 4058	MACS 4,058, is durum wheat which is developed by Agharkar Research Institute, Pune under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2020. It is rich in protein (14.7%), iron (39.5 ppm) and zinc (37.8 ppm) in comparison to 8%–10% protein, 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties, it has 29.6 q/ha grain yield, matured in 102 days, suitable for timely sown restricted irrigated conditions in *rabi* season and recommended for cultivation in Maharashtra and Karnataka
HD 3298	HD 3,298 is developed by ICAR-Indian Agricultural Research Institute, New Delhi which is of released in 2020. It is rich in protein (12.1%) and iron (43.1 ppm) in comparison to 8%–10% protein and 28.0–32.0 ppm iron in popular varieties, having grain yield 43.7 q/ha, matured in 103 days, suitable for very late sown n irrigated conditions and recommended for cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
HI 1633	This is developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2020. It is rich in protein (12.4%), iron (41.6 ppm) and zinc (41.1 ppm) in comparison to 8%–10% protein, 28.0–32.0 ppm iron and 30.0–32.0 ppm zinc in popular varieties. Variety has 41.7 q/ha grain yield, matured in 100 days, suitable for late sown irrigated conditions and recommended for cultivation in Maharashtra, Karnataka and plains of Tamil Nadu
DBW 303	DBW 303 is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal and released in 2020. It is rich in protein (12.1%) in comparison to 8%–10% protein in popular varieties. Variety produces 81.2 q/ha grain yield, matured in 156 days, suitable for irrigated early sown and high fertility conditions in rabi and recommended for its cultivation in Punjab, Haryana, Delhi, Rajasthan and other states
DDW 48	DDW 48 is durum wheat, developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal and released in 2020. It is rich in protein (12.1%) in comparison to 8%–10% protein in popular varieties, having grain yield 47.4 q/ha, matured in 111 days, suitable for timely sown irrigated conditions in rabi season and recommended for cultivation in Maharashtra, Karnataka and plains of Tamil Nadu
DBW 332	Variety is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal which released in 2021. This variety is rich in protein (12.2%) and zinc (40.6 ppm) in comparison to popular variety. Its yield capacity is 78.3 q/ha, maturity time is 156 days and suitable for early sown irrigated conditions in *rabi* season for different states of the country
DBW 327	This variety is developed by ICAR-Indian Institute of Wheat & Barley Research, Karnal and released in 2021. Its contains high zinc (40.6 ppm), yielded 79.4 q/ha, matured in 155 days and suitable for its cultivation early sown irrigated conditions in Punjab, Haryana, Delhi, Rajasthan, Western Uttar Pradesh, etc.
HI 1636	It is developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2021. Variety contains high zinc (40.4 ppm), has 56.6 q/ha grain yield, matured in 119 days and suitable for timely sown irrigated conditions in *rabi* for Madhya Pradesh, Chhattisgarh, Gujarat, Rajasthan, etc.
HI 8823	HI 8823 wheat variety is developed by ICAR-Indian Agricultural Research Institute, Regional Station, Indore and released in 2021. Rich in protein (12.1%) and zinc (40.1 ppm) in comparison to popular varieties. It gives 38.5 q/ha grain yield, maturity in 122 days and suitable for timely sown irrigated conditions in *rabi* for the states like Madhya Pradesh, Chhattisgarh, Gujarat, Rajasthan, etc.
HUW 838	HUW 838 wheat variety is developed by Banaras Hindu University, Varanasi under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2021. It contains high zinc (41.8 ppm), yielded 51.3 q/ha grain yield, matured in 148 days and suitable for early sown irrigated conditions in *rabi* for the states such as Punjab, Haryana, Delhi, Rajasthan, Western Uttar Pradesh, etc.
MP (JW) 1,358	This wheat variety is developed by Jawahar Lal Nehru Krishi Viswavidhyalaya, Zonal Agricultural Research Station, Powarkheda under ICAR-All India Coordinated Research Project on Wheat & Barley and released in 2021. It is rich in protein (12.1%) and iron (40.6 ppm) in comparison to popular wheat varieties. Its grain yield is 56.1 q/ha, maturity time is 105 days and suitable for early sown irrigated conditions in for Maharashtra, Karnataka and plains of Tamil Nadu.

It is known that high temperatures (heat/terminal heat) can reduce chlorophyll content and photochemical efficiency of plant leaves, resulting in negative impact on the photosynthetic process (Zhang et al., 2017). Higher temperature also increases the production of reactive oxygen species (ROS) and protease activity, leading to leaf senescence (Hu et al., 2020). It increases the production of ROS, and elevated cytokinins can stimulate the antioxidant system to remove ROS (Xu et al., 2009). According to Skalak et al. (2016), hormonal analysis of the proteome and transcriptome also confirms that cytokinins play an important role in plant resistance to heat stress, and most of the heat shock (HS) proteins which are upregulated by increasing the cytokinins. Since accumulation of endogenous cytokinins can maintain normal plant growth under high temperature, heat stress tolerance of plants can be improved by increasing the content of endogenous cytokinins. Insertion of *isopentenyl transferase* (*IPT*) in *Arabidopsis* at the seedling stage which significantly improves the level of endogenous cytokinin and thus enhances the tolerance in high temperatures (Skalak et al., 2016). Drought is one of the major factors which can inhibit the plant physiological functions, including reduction of photosynthesis, crop yield, and accelerated senescence (Liu et al., 2012). The possibility of improving the drought tolerance

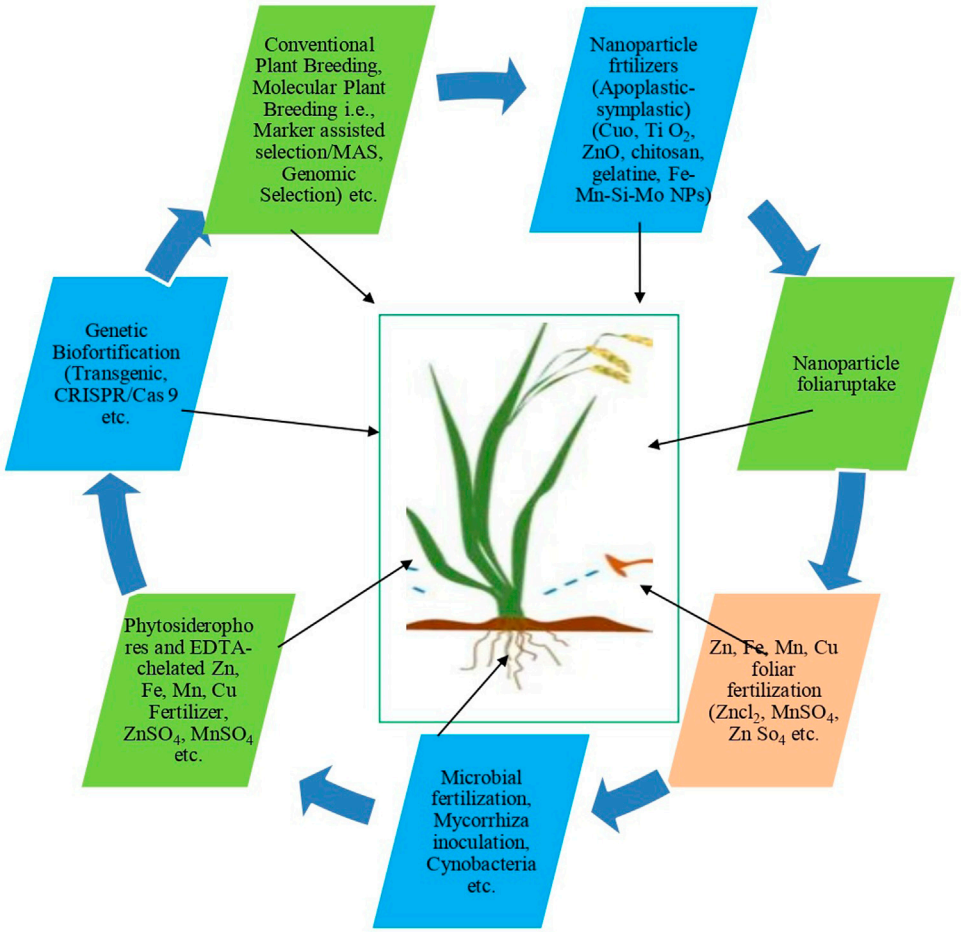

FIGURE 4 | Important approches/technology uses for crop bio-fortification.

of plants by regulating cytokinin levels depends on stress duration, soil water potential, and plant dehydration rate (Veselov et al., 2017). In response to drought, up and downregulation of endogenous cytokinins can enhance the degree of drought tolerance (Werner et al., 2010). Some findings reveal that during drought stress, the accumulation of plant endogenous cytokinins is reduced, and this reduction can enhance the plant drought tolerance *via* various physiological responses including stomatal closure (Naidoo and Naidoo 2018). Cytokinin is downregulated, leading to the expansion of the root system and a high root to shoot ratio, which increases the water absorption area of roots. Relatively small shoot and leaf area as compared to roots can effectively decrease transpiration rate (Lubovska et al., 2014) and therefore, the whole plant can maintain high relative water content and improve drought tolerance. Cold (low temperature) is another stress which can affect the plant cells by hardening the membrane system and interfering with all membrane-related processes (Liu et al., 2016), low temperatures can also lead to the accumulation of ROS, due to the decrease of antioxidant enzyme activity making the ROS scavenging system unable to work normally, and in turn, the excessive accumulation of ROS will have harmful effects on the membrane, resulting in leakage of ions and cell metabolism disorder (Sui, 2015). Like other stress, salt stress can hamper the physiological and biochemical processes of crop plants. Sodium-ion (Na+) accumulation in plants can lead to the disorder of ion homeostasis, the imbalance of potassium ion (K+)/Na + ratio, and Na + ion toxicity (Liu et al., 2017) and cause oxidative stress, which damages the cell membrane, causes ion leakage, or direct damage to proteins and other macromolecules, leading to cytotoxicity and even cell death (Lin et al., 2018). Avalbaev et al. (2016) reported that pretreatment of wheat with exogenous methyl jasmonate (*MeJA*) can maintain the high content of cytokinin by decreasing the *CKX* transcription level induced by salt stress, and enhance salt tolerance level. Therefore, by spraying the exogenous cytokinins onto plants, the salt tolerance property can be enhanced.

CONCLUSION

Wheat is considered one of the most economically important cereal crops in the world. Its productivity is high, but increasing consumption and changing climate indicates the need for further

improvement in its yield potential. Climate change is expected to continue posing biotic/abiotic stresses, and if current trends continue, many parts of the planet will become hostile to agriculture. On the other hand, because of the rapidly growing population and the changing climate, demand for wheat is expected to grow faster than the other major crops. Therefore, investments in exploring and harnessing existing genetic resources including bio-fortified wheat and information regarding the role of cytokinins under normal and adverse conditions for increasing the yield, grains containing enough nutrients and having a sufficient defense response, impact of morphological markers *viz*., stay green traits, leaf tip necrosis, leaf angle molecular markers, and other useful genetic information in order to produce enough food grains that are also rich in the required nutrients to ensure food security in the 21st century is the need of the day.

Since cytokinins are the most important endogenous substances moderating the physiological and molecular responses, thay have a key role during completion of the life cycle of plants in order to give a satisfactory yield. So, plant breeders could directly target the cytokinins to improve targeted traits by utilizing minimum input, as cytokinins are known to be a key driver of seed yield and it may well be the hormone that underpins the second green revolution. The Green Revolution boosted crop yields during the mid 20th century by introducing dwarf genotypes of wheat capable of responding to a higher dose of fertilization and enough irrigation without lodging. Now there is need of a second Green Revolution to meet out the demand of a rapidly growing population. The Green Revolution was based on crops responsive to high soil fertility however, now there is need to develop the genotypes of wheat crops which can perform better under low input, low soil fertility, under stressed conditions including heat, terminal heat, drought, metal toxicity, and under biotic stresses as well. By keeping the above facts in mind, exploring genetic resources, harnessing the cytokinin key hormones, and applying updated molecular breeding approaches, plant breeders can develop the superior and stable genotypes which will be able to cater to the food demand of the needy population.

AUTHOR CONTRIBUTIONS

Manuscript is completely written by RP.

ACKNOWLEDGMENTS

I, gratefully acknowledge the Banaras Hindu University, Varanasi (India) for awarding the Incentive Grant (research grant) under IoE Scheme.

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REVIEW
published: 14 July 2022
doi: 10.3389/fgene.2022.932859

CRISPR-Based Genome Editing for Nutrient Enrichment in Crops: A Promising Approach Toward Global Food Security

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The global malnutrition burden imparts long-term developmental, economic, social, and medical consequences to individuals, communities, and countries. The current developments in biotechnology have infused biofortification in several food crops to fight malnutrition. However, these methods are not sustainable and suffer from several limitations, which are being solved by the CRISPR-Cas-based system of genome editing. The pin-pointed approach of CRISPR-based genome editing has made it a top-notch method due to targeted gene editing, thus making it free from ethical issues faced by transgenic crops. The CRISPR-Cas genome-editing tool has been extensively used in crop improvement programs due to its more straightforward design, low methodology cost, high efficiency, good reproducibility, and quick cycle. The system is now being utilized in the biofortification of cereal crops such as rice, wheat, barley, and maize, including vegetable crops such as potato and tomato. The CRISPR-Cas-based crop genome editing has been utilized in imparting/producing qualitative enhancement in aroma, shelf life, sweetness, and quantitative improvement in starch, protein, gamma-aminobutyric acid (GABA), oleic acid, anthocyanin, phytic acid, gluten, and steroidal glycoalkaloid contents. Some varieties have even been modified to become disease and stress-resistant. Thus, the present review critically discusses CRISPR-Cas genome editing-based biofortification of crops for imparting nutraceutical properties.

Keywords: biofortification, biofortified crops, CRISPR-cas system, genome editing, hidden hunger, malnutrition, micronutrients

OPEN ACCESS

Edited by:
Santosh Kumar Gupta, National Institute of Plant Genome Research (NIPGR), India

Reviewed by:
Jaindra Nath Tripathi, International Institute of Tropical Agriculture (IITA), Kenya
Priti Sharma, Punjab Agricultural University, India

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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *30 April 2022*
Accepted: *08 June 2022*
Published: *14 July 2022*

Citation:
Kumar D, Yadav A, Ahmad R, Dwivedi UN and Yadav K (2022) CRISPR-Based Genome Editing for Nutrient Enrichment in Crops: A Promising Approach Toward Global Food Security. Front. Genet. 13:932859. doi: 10.3389/fgene.2022.932859

INTRODUCTION

Malnutrition is becoming a rapidly increasing and serious problem as the world's population grows. The world's population will reach 8.3 billion in 2030, up from 7.8 billion at present. According to estimates, almost 800 million people are malnourished worldwide, with 98 percent living in underdeveloped countries (Sinha et al., 2019). Undernutrition (wasting, stunting, and being underweight), insufficient vitamins and minerals, obesity, and the consequent diet-related non-communicable disorders are all examples of malnutrition. Furthermore, more than 340 million people suffer from one or more micronutrient deficiencies, including deficiencies in vitamin A, iron, iodine, and zinc (UNICEF, 2021).

Fortification and biofortification are food enrichment technologies that differ in their approach. In the former method, fortificants are added directly to the food during processing, but the latter involves fortification at the crop production level. In comparison to fortification, biofortification is cost-effective as it is a one-time investment to develop a biofortified crop and recurrent costs are low (Shoab and Hefferon, 2022). Biofortified crops hold a brighter future to address nutritional challenges (Yadav et al., 2020; Buturi et al., 2021; Mushtaq and Nazir, 2021; Ziarati et al., 2021). Biofortification is considered a sustainable and long-term solution to provide micronutrient-rich crops to people (Bouis and Welch, 2010; Bouis and Saltzman, 2017; Garg et al., 2018; Heck et al., 2020; Van Der Straeten et al., 2020).

The crops are biofortified for desired nutrients through nutrient treatments as well as breeding (Garg et al., 2018). Agronomic biofortification involves the deliberate use of mineral fertilizers to increase the concentration of a target mineral in edible portions of crops (Adu et al., 2018). Advanced agronomic biofortification includes engineered nanoparticles attached with fertilizers (nano fertilizers) and PGPR (plant growth–promoting rhizobacteria) (Nayana et al., 2020). Moreover, crucial quantitative trait loci (QTLs) are also utilized in crop breeding programs to improve crop nutrient profiles (Gangashetty et al., 2016). Nevertheless, plant breeding, especially polyploid crop breeding, is a time-consuming and laborious method for improving crop productivity (Parry et al., 2009; Nagamine and Ezura, 2022). Some crops have also been biofortified for desired nutrients through transgenic technology-based genetic alterations (Pérez-Massot et al., 2013). Disadvantages of genetically modified (GM) crops include allergic reactions in humans and reduced nutrition. Also, they cause environmental impact by releasing toxins in the soil, induce pest resistance, and disruption of crop biodiversity. Several ethical concerns are associated with GM crops.

In view of the disadvantages of GM crops, genome editing (GE) technology offers distinct advantages (Gaj et al., 2013; Xiong et al., 2015; Aglawe et al., 2018; Fiaz et al., 2021; Nagamine and Ezura, 2022). Thus, genome editing produces predictable and inheritable mutations in specified regions of the genome, with minimal off-target effects and no external gene sequence integration (Bhattacharya et al., 2021). Deletions, insertions, single-nucleotide substitutions, and extensive fragment substitutions are used for GE-mediated DNA alterations. Systems such as homing endonucleases or meganucleases (HEs) (Daboussi et al., 2015), Zinc-Finger Nucleases (ZFNs) (Urnov et al., 2010), and transcription activator-like effector nucleases (TALENs) (Joung and Sander, 2013) were engaged as genome editing tools before the discovery of CRISPR-associated protein (Cas) (Wang et al., 2016). The brief details of previously used genome editing methods are described below.

Meganucleases, also known as homing endonucleases, are rare-cutting enzymes found in all microbial genomes. These enzymes identify and cleave lengthy DNA sequences (usually 18–30 base pairs), resulting in double-strand DNA breaks (DSBs). Various designed meganuclease variants are available to cleave unique DNA targets for genomic changes for creating important characteristics in crop species (Daboussi et al., 2015). Homing endonucleases technology has suffered from technical problems in the manufacture of these nucleases and designing vectors for their entrance into cells and off-targeting consequences (Jin et al., 2016; Rey-Rico and Cucchiarini, 2016).

Zinc-finger nucleases (ZFNs) are "nucleases" consisting of engineered zinc-finger DNA-binding domains paired with a nuclease, most often the FokI nuclease. ZFN-induced double-strand breaks are exposed to cellular DNA repair processes, resulting in remarkably targeted mutagenesis and targeted gene replacement (Carroll, 2011). The zinc-finger domains consist of four to six 30 amino acid domains that may bind to trinucleotide sequences, limiting total DNA-binding domain specificity to 12–18 nucleotide sequences (Davies et al., 2017). However, ZFN technology has disadvantages such as complex design (which requires customized protein for each DNA sequence), low engineering feasibility, low specificity, normal efficacy, and inability to gene knockout and RNA editing (Zhang J.-H. et al., 2016; Zhang B. et al., 2016; Callaway, 2016; Wang et al., 2016; Salsman and Dellaire, 2017; Sun et al., 2018).

Transcription activator-like effector nucleases (TALENs) are a nonspecific DNA-cleaving nuclease linked to a DNA-binding domain that could be tailored to target specific sequences. TALENs are made up of an engineered array of TALE repeats fused to the FokI nuclease domain and are used for editing the genome (Reyon et al., 2013). However, TALENs also have disadvantages such as complex design, lower engineering feasibility, lower specificity, low efficacy, and inability to gene knockout and RNA editing (Zhang J.-H. et al., 2016; Zhang B. et al., 2016; Callaway, 2016; Wang et al., 2016; Salsman and Dellaire, 2017; Sun et al., 2018).

Thus, CRISPR-Cas technology has emerged as a promising genome editing tool overcoming many of the abovementioned disadvantages of HEs, ZFNs, and TALENs. Therefore, it is currently the most extensively used genome editing technique worldwide because of its simple design, cost-effectiveness, high efficiency, good reproducibility, high engineering feasibility, ability to create gene knockout, RNA editing, and quick cycle (Asmamaw and Zawdie, 2021).

Thus, the present review critically discusses CRISPR-Cas genome editing-based biofortification of crops with respect to enhancement of carbohydrate, protein, fatty acids, secondary metabolite contents, as well as imparting disease and stress-resistance.

OVERVIEW OF THE CRISPR-CAS SYSTEMS

Most bacteria and Archaea have an adaptive defense system called CRISPR-Cas that protects them from phages, viruses, and other foreign genetic material (Marraffini and Sontheimer, 2010). The main components of the CRISPR-Cas9 system are an RNA-guided Cas9 endonuclease and a single-guide RNA (sgRNA). Type II CRISPR-Cas9 is one of the best-defined and most commonly utilized categories in multiple CRISPR-Cas systems (Jiang and Doudna, 2017). Cas9 endonucleases HNH domain cuts one strand of sgRNA, while the RuvC-like domain cuts the opposite strand of dsDNA, resulting in double-strand breaks (DSBs). As a

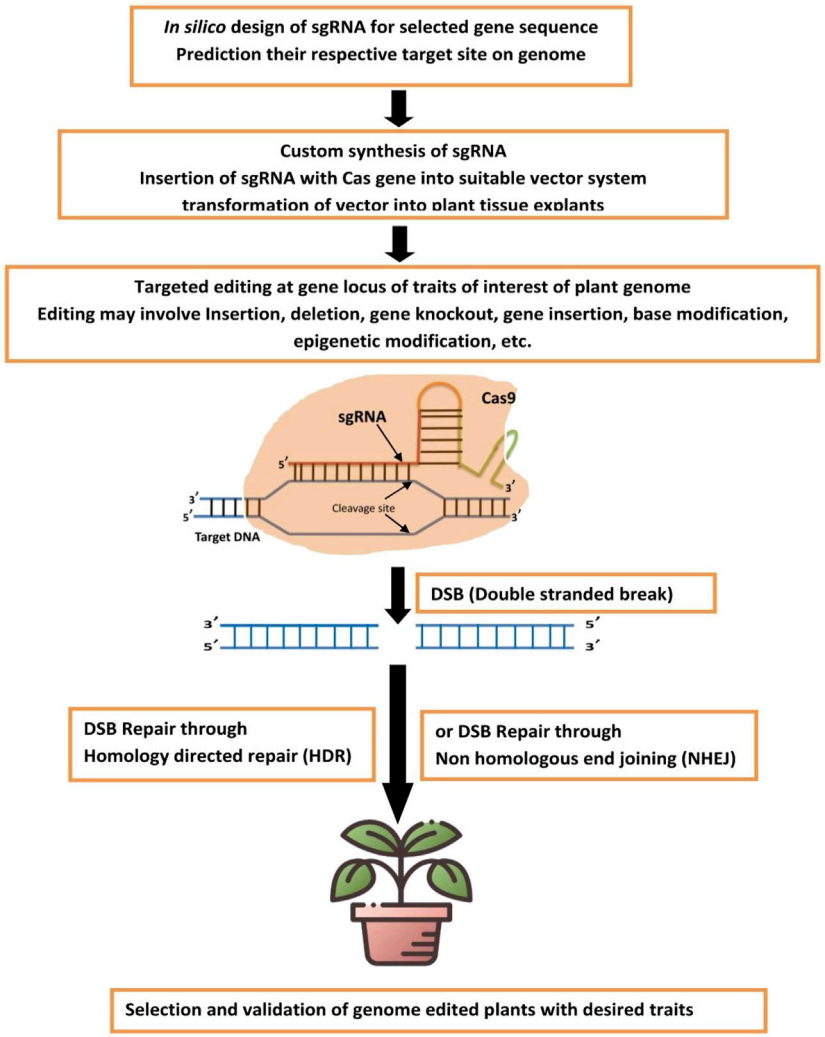

FIGURE 1 | Schematic representation for CRISPR-Cas genome editing methodology for developing genetically edited biofortified plants.

result, the plant endogenous repair system automatically repairs DSBs *in vivo*, utilizing error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR), resulting in massive insertion or fragment replacement (Liu X. et al., 2017).

The schematic representation of the CRISPR-Cas genome editing methodology for developing genetically edited biofortified plants is shown in **Figure 1**.

Efforts have been made by researchers to modify the CRISPR-Cas system to make its application more efficient and specific as depicted in **Table 1**.

DEVELOPMENT OF BIOFORTIFIED CROPS THROUGH CRISPR-CAS GENOME EDITING APPROACH

The CRISPR-Cas system allows rapid, site-specific genome modification in a single cell or a whole organism. It regulates transcription, changes epigenomes, runs genome-wide screens, and imaging chromosomes. The CRISPR-Cas system is now increasingly used for developing edited crops due to its diverse applications in genome editing. Using the CRISPR-Cas, research groups have engineered several crop systems for disease resistance (Schenke and Cai, 2020), drought, salinity, and thermotolerance (Chennakesavulu et al., 2021). CRISPR-Cas is aiding in developing climate-ready crops (Razzaq et al., 2021) and improving crop quality parameters such as appearance, palatability, nutritional components, and other preferred traits (Liu Q et al., 2021). This study has reviewed the nutritional enrichment of important crops using the CRISPR-Cas genome editing method.

Vitamin A Enriched Crops

Carotenoids are widely distributed isoprenoid pigments essential for photosynthetic organisms. Humans do not produce carotenoids *de novo*, but they require them in their

TABLE 1 | Modifications in the CRISPR-Cas system.

CRISPR-Cas system	Modification	Effect	References
$Cas9^{D10A}$ nickase	By point mutations D10A of RuvC domain of Cas9	For minimizing off-target effects and cleaving the single strand	Ran et al. (2013)
Dead Cas9 (dCas9)	By two-point mutations H841A and D10A, into HNH and RuvC nuclease domain of Cas9, respectively CRISPRa-fusing transcriptional activators and dCas9 CRISPRi-fusing transcriptional repressors and dCas9	For transcriptional activation, inhibition, and epigenetic modification	Qi et al. (2013), Dominguez et al. (2016)
Base editing system	Cytidine base editor (CBE) developed by fusion of APOBEC1 to dCas9 Adenine base editor (ABE) developed by fusion of TadA to dCas9 CRISPR-BETS (base editing to stop)	For site-directed mutagenesis as CBE causes C_G to T_A point mutations, ABE causes A_T to G_C, and CRISPR-BETS causes stop codon	Komor et al. (2016), Gaudelli et al. (2017), Wu et al. (2021)
Cas 9 variants	xCas9 developed by the phage-assisted continuous evolution (PACE) system, SpCas9-NG developed the PACE-system such as SpCas9-NRRH, SpCas9-NRCH, SpCas9-NRTH, SpG, SpRY, etc.	xCas9 recognizes the multiple PAMs other than NGG-PAM (NG, GAA, GAT, etc.), SpCas9-NG recognizes relaxed NG PAMs and SpG recognize near-PAM less and SpRY, recognizes all PAMs (NRN and NYN PAMs)	Hu et al. (2018), Nishimasu et al. (2018), Miller et al. (2020), Walton et al. (2020)
Prime editing system	Composed of pegRNA and reverse transcriptase fused to the C terminus of Cas9 (H841A) nickase	For targeted insertions, deletions, and base-to-base conversions without using donor DNA templates or double-strand breaks (DSBs)	Scholefield and Harrison (2021)
CRISPR tissue–specific knockout (CRISPR-TSKO)	CRISPR-TSKO is generated by expression of Cas9 only in particular tissues using specific promoters	For discovery and analysis of gene functions in specific tissues	Decaestecker et al. (2019)
RNA editing system	CRISPR-Cas13 binds to target single-stranded RNA (ssRNA) and cleaves the target. REPAIR RNA base editor (fusion of dcas13 to adenosine deaminase) RESCUE RNA base editor (fusion of dCas13 to cytidine deaminase	For RNA knockdown, transcript labeling, splicing regulation, and virus detection	Abudayyeh et al. (2017), Tang et al. (2021)

food, notably as β-carotene and vitamin A precursors (Maoka, 2020). Vitamin A is necessary for biological functions that include light transduction in vision, embryonic development, immunological operation, and overall health maintenance in human beings (Timoneda et al., 2018). Vitamin A deficiency (VAD) is one of the most severe worldwide health issues, resulting in a variety of symptoms such as xerophthalmia, night blindness, pediatric blindness, and an increased risk of morbidity and mortality, particularly in young children (Sommer, 2008; Reddy et al., 2022). Given this, carotenoid-enriched staple crops (golden crops), either through traditional breeding or genetic engineering, were initiated to combat VAD (Zheng et al., 2020). Among all the genetic engineering techniques, the CRISPR-based genome-editing technique seems the most efficient and widespread method, making rapid, DNA/transgene-free, and targeted multiplex genetic modification of organisms: a reality for developing "golden" staple crops (Zheng X. et al., 2021). Through CRISPR-Cas-based systems, various genome-edited golden crops, that is, carotenoid biofortified crops have been produced to combat VAD (**Table 2**). For instance, the Golden rice cultivar Kitaake has been developed by Knock-in, a 5.2-kb carotenogenesis cassette consisting of *CrtI* and maize *PSY* genes. The variety contains 7.9 μg/g dry weight (DW) β-carotene in the endosperm (Dong et al., 2020).

Vitamin E–Enriched Crops

Vitamin E (tocopherol) is a potent lipid-soluble antioxidant and an essential component of the human diet. Many human diseases, such as cardiovascular disease and certain cancers, are associated with insufficient vitamin E intake (Rizvi et al., 2014). The daily requirement of vitamin E for humans lies between 15 and 30 mg (Ungurianu et al., 2021).

Through CRISPR-Cas9 technology, significant increase in tocopherols and tocotrienol content was achieved by targeted overexpression of Hordeum vulgare *homogentisate* phytyltransferase (*HvHPT*) and Hordeum vulgare homogentisate geranylgeranyltransferase (*HvHGGT*) (Zeng et al., 2020). These genes can be utilized for enhancing vitamin E content in other crops.

Iron-Enriched Crops

Iron is involved in various metabolic activities such as oxygen transport and electron transport chain. Iron metabolism disorders cause the most frequent diseases in humans, encompassing multiple conditions with various clinical presentations, ranging from anemia to neurodegenerative diseases (Abbaspour et al., 2014). The iron need in humans can be fulfilled through dietary and crop biofortification (Liberal et al., 2020). CRISPR-Cas9 is responsible for the latter type of biofortification. This system has been reported to disrupt

TABLE 2 | Vitamin A enriched biofortified crops *via* CRISPR-Cas systems.

Vitamin A enriched biofortified crops	CRISP-Cas systems	Targeted genes	References
Golden flesh Melon	CRISPR base editors	Arg to- His mutation in the melon orange protein gene (CmOr) gene	Tzuri et al. (2015)
Tomato	CRISPR-Cas9	*STAYGREEN (SGR)* gene, a negative regulator of carotenoid biosynthesis, was knockdown	Li A. et al. (2018)
Golden Banana	CRISPR-Cas9	lycopene epsilon-cyclase (LCYε) was knockdown by creating indels	Kaur et al. (2020)
Golden rice	CRISPR-Cas9	*phytoene desaturase CrtI* and maize *Phytoene synthase* (*PSY*) genes were knocked in	Dong et al. (2020)

the *Inositol pentakisphosphate 2- kinase 1* (*IPK1*) gene causing iron biofortification in wheat (Ibrahim et al., 2021).

Zn-Enriched Crops

The mineral Zn is involved in numerous cellular metabolic processes and catalytic activity of approximately 100 enzymes (Sandstead, 1994). It plays a role in immune functions (Prasad, 1995; Solomons, 1998), protein synthesis (Prasad, 1995; Heyneman, 1996), wound healing (Heyneman, 1996), DNA synthesis, and cell division (Prasad, 1995). Zinc keeps our immune system healthy (Maggini et al., 2010). Zinc maintains cell normal development and activation for innate and adaptive immune responses. It facilitates the integrity of epithelial barriers, which are essential for protecting organisms and preventing pathogen entry (Sturniolo et al., 2002; Finamore et al., 2008; Maares and Haase, 2016). Moreover, Zn can modulate the development and activity of T cells and hence be used as an immunomodulatory candidate. There is only one report on Zn enhancement in wheat, to the best of our knowledge. In this crop, the CRISPR-Cas system disrupts *Triticum aestivum Inositol Pentakisphosphate 2-kinase 1 (TaIPK1)* that reduces phytic acid to cause improvement in zinc accumulation in wheat grains (Ibrahim et al., 2021). More crops are needed to be biofortified with Zn through the CRISPR-Cas system to make available the products worldwide.

Biofortification Through Targeting Cytokinin Metabolism

Plants absorb a range of mineral elements essential for growth, including C, H, O, N, Fe, Zn, K, Na, and others, in various forms. C, H, and O are acquired from gases or water, and their uptake pathways are straightforward and well-known (Reid and Hayes, 2003). In contrast, other elements are primarily classified as mineral elements and are mainly taken from soil in terrestrial plants or water in aquatic plants (Reid and Hayes, 2003). Root system architecture (RSA), which constitutes the structure of root length, spread, number, and length of lateral roots, is a critical developmental and agronomic characteristic that affects overall plant architecture, growth rate and yield, abiotic stress resistance, nutrient absorption, and developmental flexibility in response to environmental changes (Jung and McCouch, 2013). Phytohormones are communicators between soil and RSA, regulating root development processes, extending from organogenesis to creating higher-order lateral roots (LRs) *via* various mechanisms (Sharma et al., 2021). The hormones cytokinin (CK) and auxin (IAA), along with ethylene, are essential regulators of root growth, vascular differentiation, and root gravitropism (Aloni et al., 2006). Cytokinin negatively regulates root elongation and branching and crucially shapes RSA (Ramireddy et al., 2014). The enzymes isopentenyl transferase (IPT) present in plants regulates cytokinin levels, which are later destroyed by cytokinin oxidase/dehydrogenase (CKX) and inactivated by glucosylation through cytokinin glucosyl transferases (CGTs) (Chen et al., 2021). Since cytokinin level in roots negatively correlates with crop yields. The reduced cytokine level induced by the enzymes mentioned above increases root growth and uptake of mineral nutrients, particularly Zn and Fe (Chen et al., 2020). Therefore, overexpression of *CKX* and *CGTs* in the root zone increases the crop yield.

Genetically edited plants have been developed with indigenously lowered cytokinin levels that favor enrichment of P, Ca, S, Cu, Mn, Fe, and Zn in plant biomass (Ramireddy et al., 2018a). Ramireddy et al. (2018b) developed Zn-fortified field-grown barley which breaks down plant cytokinin through transgenics. In the additional study carried out by the author, the grain yield of barley was increased by knocking out CKX genes through an RNA-guided Cas9 system to generate ckx1 and ckx3 mutant lines with knockout mutations in the HvCKX1 and HvCKX3 genes, respectively. Reduced CKX activity in the ckx1 lines induced longer roots, increased surface area, and a higher number of root hairs. In contrast, enhanced CKX activity in the ckx3 mutants had opposite results. The authors' findings show that the control of cytokinin activity is complicated, where alterations in just a single component might have unexpected consequences (Gasparis et al., 2019). In another study, silencing the OsCKX4 gene or knockout of the homologous gene OsCKX2 resulted in decreased Zn concentrations in brown rice. However, CRISPR-Cas9 mediated knockout of CYP735A involved in the formation of trans-zeatin (tZ-type) cytokinins elevates Zn concentrations (Gao et al., 2019). In yet another study, Karunarathne et al. (2022) developed the barley abnormal cytokinin response 1 repressor (HvARE1) mutants with high nitrogen content in shoots in nitrogen-deficient soil through Agrobacterium-mediated genetic transformation of immature embryos (cv. Golden Promise) with sgRNAs targeting HvARE1. Such crop types possess nitrogen use efficiency (NUE) and can reduce fertilizer input in soils, rendering them a cost-effective alternative that can prevent environmental pollution due to excessive fertilizer applications.

Quality Improved Crops

In addition to developing nutrient-enriched crops, many crops are improved to boost production, biotic and abiotic stress resistance, and quality and nutritional value. Over several decades, innovative agricultural technology has considerably

TABLE 3 | Summary of CRISPR-Cas-based genome-edited quality improved crops.

Quality improved crops	CRISPR-Cas systems	Targeted genes	Function	References
Soybean	CRISPR-Cas9	*FAD2-1A and FAD2-1B*	Knock-down of genes *FAD2-1A* and *FAD2-1B* altered high oleic acid and low linoleic acid content	Haun et al. (2014)
Soybean	CRISPR-Cas9	*FAD2-1A and FAD2-1B*	Knock-down of genes *FAD2-1A* and *FAD2-1B* high oleic acid and low linoleic acid content	Demorest et al. (2016)
Rice	CRISPR-Cas9	*DEP1*	Negative regulator for dense erect panicles	Li et al. (2016)
Grape	CRISPR-Cas9	*IdnDH*	Knock-down of IdnDH decreased tartaric acid content	Ren et al. (2016)
Rice	CRISPR-Cas9	*GW2*, *GW5*, and *TGW6*	Negative regulator for grain length and width	Xu et al. (2016)
Rice	CRISPR-Cas9	*GW5*	Negative regulator for grain width	Liu J. et al. (2017)
Rice	CRISPR-Cas9	Starch branching enzyme (*SBEI*) and *SBEIIb*	Generation of targeted mutagenesis in *SBEI* and *SBEIIb* to create high-amylose rice	Sun et al. (2017)
Rice	CRISPR-Cas9	*OsHAK-1*	Knock down of OsHAK-1 for reduced uptake Cs^+ from the roots	Nieves-Cordones et al. (2017)
Rice	CRISPR-Cas9	*OsNramp5*	Low Cd accumulation	Tang et al., 2017
Rice	CRISPR-Cas9	*OsNRAMP2*	Remobilization and distribution of Fe and Cd	Chang et al. (2022)
Tomato	CRISPR-Cas9	*fas, lc*	Loss-of-function mutant for regulating three major productivity traits in tomatoes; fruit size, inflorescence branching, and plant architecture	Rodríguez-Leal et al. (2017)
Tomato	CRISPR-Cas9	*ALC*	Mutagenesis and replacement in the *ALC* gene generated long-shelf-life	Yu et al. (2017)
Tomato	CRISPR-Cas9	*SlGAD2 and SlGAD3*	Deletion of the autoinhibitory domain of SlGAD2 and SlGAD3 to increase GABA content in tomatoes	Nonaka et al. (2017)
Tomato	CRISPR-Cas9	*RIN*	Knockdown of *RIN* decreased volatile organic compounds	Ito et al. (2017)
Tomato	CRISPR-Cas9	*SlAN2*	Targeted mutagenesis of *SlAN2* to understand anthocyanin biosynthesis and regulation in purple tomato cultivar "Indigo Rose"	Čermák et al. (2015), Zhi et al. (2020)
Rice	CRISPR-Cas9	Fatty acid desaturase (*OsFAD2-1*)	Targeted mutagenesis in the OsFAD2-1 gene for producing high oleic/low linoleic in rice bran oil (RBO)	Abe et al. (2018)
Rice	CRISPR-Cas9	*GS9*	The *gs9* null mutant with slender grains transformed into round grains	Zhao et al. (2018)
Wheat	CRISPR-Cas9	*α-gliadin* genes	Two sgRNAs target the *α-gliadin* gene for α-gliadin reduction, which results in low-gluten meals	Sánchez-León et al. (2018)
Wheat	CRISPR-Cas9	*TaGW2*	Negative regulator for grain size and weight	Wang et al. (2018)
Barley	CRISPR-Cas9	GBSS1 and protein targeting to Starch 1 (*PTST1*)	Generation of gbss1 and ptst1 mutants to produce starch-free endosperm	Zhong et al. (2019)
Potato	CRISPR-Cas9	steroid 16α-hydroxylase (*St16DOX*)	Knockdown of the *St16DOX* reduced the steroidal glycoalkaloids (SGA)	Nakayasu et al. (2018)
Tomato	CRISPR-Cas9	*OVATE*, *Fas*, *Fw2.2*, and *CLV3*	Edited six significant loci for yield and productivity in tomato crop lines	Zsögön et al. (2018)
Tomato	CRISPR-Cas9	*SGR1, LCY-E, Blc, LCY-B1, and LCY-B2*	Knock down of *SGR1*, *LCY-E*, *Blc*, *LCY-B1*, and *LCY-B2* increase the lycopene content	Li A. et al. (2018)
Sorghum	CRISPR-Cas9	*k1C* gene family	Knock down of genes *k1C* increased digestibility and protein quality	Li X. et al. (2018)
Carrot	CRISPR-Cas9	*F3H*	Knock down of F3H decreased anthocyanin acid content	Klimek-Chodacka et al. (2018)
Rapeseed	CRISPR-Cas9	FAD2	Knock down of *FAD2* increased oleic acid content and decreased linoleic and linolenic acid contents	Okuzaki et al. (2018)
Cassava	CRISPR-Cas9	*GBSSI*	Knock down of GBSSI decreased amylose contents	Bull et al. (2018)
Apple	CRISPR-Cas9	*IdnDH*	Mutation in *IdnDH* significantly accumulated tartaric acid	Osakabe et al. (2018)
Rice	CRISPR-Cas9	*GS3* and *Gn1a*	Negative regulator for grain size and length	Shen et al. (2018)
Lettuce	CRISPR-Cas9	*LsGGP2*	Overexpression of *LsGGP2* increased n ascorbate content	Zhang et al. (2018)
Rice	CRISPR- ABE	*GL2/OsGRF4* and *OsGRF3*	Positive regulator for grain size	Hao et al. (2019)
Wheat	CRISPR-Cas9	*TaGW7*	Negative regulator for grain width, shape, and weight	Wang D. et al. (2019)
Rice	CRISPR-Cas9	*OsGS3*, *OsGW2*, and *OsGn1a*	Negative regulator for grain size, width and weight, and number	Zhou et al. (2019)
Rice	CRISPR-Cas9	(Phospholipase D) *OsPLDα1*	Generation of mutations in the phospholipase D (OsPLDα1) gene to reduced phytic acid content as compared to their wild-type parent	Khan et al. (2019)
Maize	CRISPR-Cas9	(*Shrunken-2 gene*) *SH2*, and WX	Two sgRNAs were designed to target SH2 and WX for the generation of sweet corn and waxy corn	Dong et al. (2019)
Potato	CRISPR-Cas9 and CBE	*St*GBSS	Loss of function of the *StGBSSI* protein is related to impaired amylase biosynthesis	Veillet et al. (2019)
Sweet Potato	CRISPR-Cas9	*GBSSI*	Knockdown of *GBSSI* decreased the decreased amylase content	Wang H. et al. (2019)
Tomato	CRISPR-Cas9	*PL*, *PG2a*, and *TBG4*	Generation of mutants in the enzymes PL, PG2a, and TBG4 for increasing the shelf life	Wang W. et al. (2019)

(Continued on following page)

TABLE 3 | (*Continued*) Summary of CRISPR-Cas-based genome-edited quality improved crops.

Quality improved crops	CRISPR-Cas systems	Targeted genes	Function	References
Tomato	CRISPR-Cas9	*HYS*	Knockdown of *HYS* decreased anthocyanin content	Qiu et al. (2019)
Peanut	CRISPR-Cas9	*FAD2A* and *FAD2B*	Knock down of genes *FAD2A* and *FAD2B* increased oleic acid content	Yuan et al. (2019)
Pomegranate	CRISPR-Cas9	*PgUGT84A23* and *PgUGT84A24*	Knock down of PgUGT84A23 and PgUGT84A24 accumulated gallic acid 3-0- and 4-o-glucosides	Chang et al. (2019)
Rice	CRISPR-Cas9	*GS3* and *GL3.1*	Negative regulator for grain size and length	Yuyu et al. (2020)
Rice	CRISPR-Cas9	*OsAAP6* and *OsAAP10*	Knockout of OsAAP6 and OsAAP10 mutants were generated in japonica varieties which reduces the high grain protein content (GPC)	Wang et al. (2020)
Rice	CRISPR-Cas9	*OsGBSSI* (granule-bound starch synthase I) or waxy (Wx) gene	Downregulation of Wx expression associated with fine-tuning grain amylose content	Huang et al. (2020)
Rice	CRISPR-Cas9	*OsBADH2*	Creation of novel alleles of OsBADH2, leading to the introduction of aroma into an elite non-aromatic rice variety ASD16	Ashokkumar et al. (2020)
Rice	CRISPR-Cas9	(glutamate decarboxylase 3) *OsGAD3*	Trimming the coding region of the CaMBD domain from the *OsGAD3* gene produces higher gamma-aminobutyric acid (GABA) content	Akama et al. (2020)
Maize	CRISPR-Cas9	*waxy* gene	For fine-tuning amylase content	Gao et al. (2020)
Potato	CRISPR-Cas9	Polyphenol Oxidases (*stPPO2*)	Knockdown of the *stPPO2* reduced the browning of the tuber	González et al. (2020)
Tomato	CBE	*SlDDB1, SlDET1, SlCYC-B*	Upregulation of *SlDDB1*, *SlDET1*, *and SlCYC-B* increased carotenoid, lycopene, and β carotene	Hunziker et al. (2020)
Tomato	CRISPR-Cas9	*SlANT2, SlAN2-like*	Knockdown of *SlANT2*, *SlAN2-like* decreased anthocyanin content	Zhi et al., 2020; Yan et al., 2020
Tomato	CRISPR-Cas9	*FLORAL4*	Mutagenesis of *FLORAL4* increased phenylalanine-derived volatile content	Tikunov et al. (2020)
Tomato	CRISPR/ LbCpf1	Salt-tolerant SlHKT1; 2 allele	Homology-directed repair (HDR)–based genome editing for salt tolerance	Vu et al. (2020)
Tomato	CRISPR-Cas9	*EXCESSIVE NUMBER OF FLORAL ORGANS* (*ENO*)	The *ENO* gene mutation gives rise to plants that yield larger multilocular fruits due to an increased size of the floral meristem	Yuste-Lisbona et al. (2020)
Soybean	CRISPR-Cas9	*Gly mBd 28 K* and *Glym Bd30 K*	Knock down of *genes GlymBd 28 K* and *GlymBd 30 K* produces hypoallergenic soybean plants	Sugano et al. (2020)
Eggplant	CRISPR-Cas9	*PP04*, *PPOS*, and *PP06*	Knock down of *PP04*, *PPOS*, and *PP06* decreased browning	Maioli et al. (2020)
Brassica rapa	CRISPR-Cas9	*BrOG1A and BrOG1B*	Knock down of *BrOG1A and BrOG1B* decreased fructose and glucose and increased sucrose contents	Jiang et al. (2020)
Rapeseed	CRISPR-Cas9	*SFAR4* and *SEARS*	Knock down of SFAR4 and SEARS increased oleic acid content and decreased linoleic and linolenic acid contents	Karunarathna et al. (2020)
Rapeseed	CRISPR-Cas9	*BnTT8*	Knock down of *BnTT8* altered the oil content and fatty acid composition	Zhai et al. (2020)
Rapeseed	CRISPR-Cas9	*BnITPK*	Knock down of *BnITPK* decreased phytic acid content	Sashidhar et al. (2020)
Tomato	CRISPR-Cas9	*SlAN2*	Targeted mutagenesis of *SlAN2* to understand anthocyanin biosynthesis and regulation in purple tomato cultivar “Indigo Rose”	Zhi et al. (2020)
Rice	CRISPR-Cas9	*GW2*	Negative regulator for grain length and width	Achary and Reddy, (2021)
Rice	CRISPR-Cas9	*OsSPL16/qGW8*	Negative regulator for the grain size	Usman et al. (2021)
Maize	CRISPR-Cas9	*CLE genes*	Engineered *CLE* genes are used to increase the yield	Liu L. et al. (2021)
Maize	CRISPR-Cas9	*ZmBADH2a* and *ZmBADH2b*	Generation of *zmbadh2a* and *zmbadh2b* single mutants and the *zmbadh2a-zmbadh2b* double mutant for popcorn-like scent in seeds from the double mutant, but not from either single mutant or in wild type	Wang Y. et al. (2021)
Potato	CRISPR-Cas9	sterol side chain reductase 2 (*StSSR*)	Successfully edited the *StSSR2* gene of tetraploid cultivated potatoes to reduce toxic SGA.	Zheng Z. et al. (2021)
Potato	CRISPR-Cas9	Starch branching enzyme (*Sbe*)	Generation of mutation in one or two *Sbe* genes to develop an increased amylose ratio and long amylopectin chains	Zhao et al. (2021)
Tomato	CRISPR-Cas9	*SlINVINH1* and *SlVPE5*	Knock down of genes (*SlINVINH1* and *SlVPE5*) increased glucose, fructose, and total soluble solids (TSS)	Wang B. et al. (2021)
Camelina	CRISPR-Cas9	CsFAD2	Knock down of CsFAD increased oleic acid contents	Lee et al. (2021)
Banana	CRISPR-Cas9	*MaACO1*	Knock down of *MaACO1* increased shelf life	Hu et al. (2021)
Rice	CRISPR-Cas9	*OsNRAMP2*	Remobilization and distribution of Fe and Cd	Chang et al. (2022)
Rice	CRISPR-Cas9	(betaine aldehyde dehydrogenase 2) *OsBADH2*	Loss of function (*OsBADH2*) affects the biosynthesis of 2-acetyl-1-pyrroline (2-AP), which is responsible for the aroma in fragrant rice	Hui et al. (2022)

enhanced crop productivity. Consumers are more concerned about crop quality because it is linked to human health by delivering nutrients such as proteins, fiber, vitamins, minerals, and bioactive substances (Slavin and Lloyd, 2012). Compared to conventional breeding, CRISPR-based systems have increased the quality of staple, oilseed, and horticultural crops with significant accuracy and efficiency (Ku and Ha, 2020). To the best of the literature review, through CRISPR-Cas mediated genome editing, various crops have been reported for improvement in their diverse categories of quality (**Table 3**).

CONCLUSION AND FUTURE PROSPECTS

The CRISPR-Cas system is an efficient, convenient, and cost-effective genome editing tool through which major crops can be biofortified for deficient vitamins or minerals. Furthermore, due to the non-insertion of foreign DNA and lesser regulatory restrictions, the products of this technology are easily acceptable by people in society. This genome editing tool has immense potential to eliminate the nutrient deficiency of crops and provide food security for the ever-increasing population. So far, CRISPR technology has been specifically utilized to modify a single gene for crop improvement. However, it holds the potential to manipulate several genes simultaneously, either by assembling multiple sgRNA expression cassettes in a single CRISPR vector or by producing more RNA through an endogenous RNA processing system.

There are several challenges to the widespread CRISPR-based agriculture revolution which include varying legislation and regulatory frameworks for gene-edited crops, delivery of CRISPR-Cas payloads, and off-target activity in CRISPR-Cas systems. In this direction, the United States, Argentina, Brazil, Chile, and Colombia have established product-based regulations by illustrating that gene-edited crops are free from GMO monitoring, provided the end products contain no exogenous DNA. Furthermore, 13 World Trade Organization countries have issued a declaration favoring gene editing for agricultural innovation, marking the first step toward drafting a worldwide regulatory framework.

Recently in India, the Department of Biotechnology, Ministry of Science and Technology has come up with "*Guidelines for Safety Assessment of Genome Edited Plants, 2022*" (https://ibkp.dbtindia.gov.in/Page Content/ ShowBrowsedFile? FileName=20220521202 445079_ Final11052022Annexure%20I,%20Genome_Edited_Plants_2022_ Hyperlink.pdf&FPath=E:\\DBT_Content_Test\CMS\Guidelines/20220521202445079_Final11052022Annexure%20I,%20Genome_Edited_Plants_2022_Hyperlink.pdf), which provides a road map for the development and sustainable use of genome editing technologies in India, specifying the biosafety and/or environmental safety concerns and describing the regulatory pathways to be adopted while undertaking the genome editing of plants.

Another limitation of CRISPR technology is off-target changes in the host genome, which is needed to be rectified through alteration in the current CRISPR-Cas methodology to minimize off-target binding for optimizing sgRNA, Cas proteins, and delivery methods. Inclusion of highly specific sgRNAs may lower off-target rates. Other strategies, including the extension of the sgRNA sequence and 3′-terminal cleavage of the sgRNA, may reduce the off-targeting effect[1].

Despite the persisting challenges, several essential crops have been altered using CRISPR-Cas for nutrition, quality, and productivity enhancement. Nonetheless, additional study is needed to investigate more crop diversity in terms of nutrition, quality, and productivity enrichment to identify effective biofortification targets and optimize CRISPR delivery methods. The CRISPR-based agricultural genome editing is the future of crop fortification as it can design genes for increased vitamin synthesis, crop quality features, and crop production qualities. CRISPR-based genome editing has a great potential to achieve the 2030 goal of eradicating hunger, food insecurity, and all forms of human malnutrition.

AUTHOR CONTRIBUTIONS

KY and UND conceptualized the manuscript. DK and KY drafted the manuscript. UND, AY, and RA proof-read the manuscript. All authors agreed to the final manuscript.

ACKNOWLEDGMENTS

DK acknowledges Junior and Senior Research Fellowships by the University Grants Commission (UGC), Government of India, New Delhi. KY acknowledges financial assistance under the Department of Science and Technology (DST-SERB), the Council of Science and Technology (CST), UP, and the Center of Excellence and Research and Development projects from the Department of Higher Education, Government of Uttar Pradesh, Lucknow, India.

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frontiers | Frontiers in Genetics

TYPE Review
PUBLISHED 18 July 2022
DOI 10.3389/fgene.2022.941595

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Genomics,
a section of the journal
Frontiers in Genetics

RECEIVED 11 May 2022
ACCEPTED 29 June 2022
PUBLISHED 18 July 2022

CITATION
Sharma A, Prakash S and Chattopadhyay D (2022), Killing two birds with a single stone—genetic manipulation of cytokinin oxidase/dehydrogenase (CKX) genes for enhancing crop productivity and amelioration of drought stress response.
Front. Genet. 13:941595.
doi: 10.3389/fgene.2022.941595

Killing two birds with a single stone—genetic manipulation of cytokinin oxidase/dehydrogenase (*CKX*) genes for enhancing crop productivity and amelioration of drought stress response

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The development of high-yielding, bio-fortified, stress-tolerant crop cultivars is the need of the hour in the wake of increasing global food insecurity, abrupt climate change, and continuous shrinking of resources and landmass suitable for agriculture. The cytokinin group of phytohormones positively regulates seed yield by simultaneous regulation of source capacity (leaf senescence) and sink strength (grain number and size). Cytokinins also regulate root-shoot architecture by promoting shoot growth and inhibiting root growth. Cytokinin oxidase/dehydrogenase (CKX) are the only enzymes that catalyze the irreversible degradation of active cytokinins and thus negatively regulate the endogenous cytokinin levels. Genetic manipulation of *CKX* genes is the key to improve seed yield and root-shoot architecture through direct manipulation of endogenous cytokinin levels. Downregulation of *CKX* genes expressed in sink tissues such as inflorescence meristem and developing seeds, through reverse genetics approaches such as RNAi and CRISPR/Cas9 resulted in increased yield marked by increased number and size of grains. On the other hand, root-specific expression of *CKX* genes resulted in decreased endogenous cytokinin levels in roots which in turn resulted in increased root growth indicated by increased root branching, root biomass, and root-shoot biomass ratio. Enhanced root growth provided enhanced tolerance to drought stress and improved micronutrient uptake efficiency. In this review, we have emphasized the role of *CKX* as a genetic factor determining yield, micronutrient uptake efficiency, and response to drought stress. We have summarised the efforts made to increase crop productivity and drought stress tolerance in different crop species through genetic manipulation of *CKX* family genes.

KEYWORDS

cytokinin oxidases/dehydrogenase, cytokinin homeostasis, root-shoot architecture, bio-fortification, sink strength, source capacity

1 Introduction

Demand for staple food crops is expected to increase by up to 60% as the global human population is expected to reach the 9.6 billion mark by 2050 (Zhu et al., 2020). According to the latest estimates, around 928 million people were severely food insecure and 768 million people were undernourished in 2020 (FAO, 2021). The breeding programs have primarily focused on increasing crop yield and productivity but an equally important objective in the breeding programs is the improvement of micronutrient composition and density which has been largely overlooked to date (Gao et al., 2019). Increased risk of climate change has escalated the adverse effects of abiotic stresses on crop production. Heat and drought stress has proved to be the two most prominent constraints on the growth and production of crop plants (Fahad et al., 2017). Drought conditions cause yield losses up to 30%–90% depending on crop species, crop growth stage at the onset of drought, and type of harvested agriculture product (Dietz et al., 2021). As the rates of yield increase brought about by the 'Green revolution' have started to decline, there is an immediate and urgent need to increase crop productivity both in terms of quality and quantity by developing robust high yielding, bio-fortified and, stress-tolerant crop cultivars to meet growing food demands in the face of abrupt climate change, increased risk of biotic and abiotic stress and decreased availability of resources and landmass suitable for agriculture (Chen et al., 2020a; Zhu et al., 2020).

The 'Green Revolution' was focused on the signaling associated with phytohormone gibberellins. It was reported that the semi-dwarf cereal varieties compromised in either gibberellin biosynthesis (rice) or gibberellin response (wheat and maize) had semi-dwarf phenotypes, less prone to lodging, and high seed yield. The reason behind the higher seed yield of these varieties was the reallocation of a greater proportion of photoassimilates to the reproductive tissues instead of vegetative tissues (Chen et al., 2020a; Liu et al., 2020a). Moreover, the cultivation of these varieties is resource-intensive, requiring huge inputs of fertilizers, water, and pesticides (Chen et al., 2020a; Jameson and Song, 2020a). Yield enhancement brought about by the 'Green Revolution' has started to flatten due to climate change and resource limitations (Zhu et al., 2020). Cytokinins are poised to underpin the second "Green Revolution" because of their recognized effects on increasing seed yield and overall plant architecture. Additionally, the possibility to alter the endogenous cytokinin levels through genetic manipulation of genes associated with cytokinin homeostasis might be useful in minimizing the effects of various environmental stresses and nutrient deficiencies (Jameson and Song, 2020a). The cytokinin group of phytohormones is involved in the regulation of multiple aspects of plant growth and development (Kieber and Schaller, 2018a), many of which have direct implications for crop improvement, such as the regulation of root-shoot architecture (Jing and Strader, 2019; Kurepa and Smalle, 2022), regulation of inflorescence meristem activity and seed yield output (Ashikari et al., 2005; Bartrina et al., 2011a; Schwarz et al., 2020a), regulation of leaf senescence and photosynthesis (Hönig et al., 2018), nutrient uptake (Gao et al., 2019; Kurepa and Smalle, 2022) and responses to biotic and abiotic stresses (Cortleven et al., 2019; Liu et al., 2020b; Li et al., 2021b; Salvi et al., 2021). Thus, there are clear implications of endogenous cytokinin level manipulation in altering plant architecture for the development of robust stress-tolerant crop varieties having optimum root-shoot architecture along with high yield output (Jameson and Song, 2020a). Cytokinin oxidase/dehydrogenase (CKX) is the only enzyme of cytokinin homeostasis maintenance that catalyzes the irreversible degradation of active cytokinins and thus, is an important negative regulator of endogenous cytokinin concentration. Genetic manipulation of genes encoding cytokinin oxidase/dehydrogenase is the key to spatiotemporal regulation of endogenous cytokinin levels. This review aims to summarise the efforts made to establish *CKX* as a genetic target for yield improvement, enhanced bio-mineral accumulation, and drought stress tolerance. Starting with a brief introduction to cytokinin biosynthesis and homeostasis we further describe the structure, function, and evolution of cytokinin oxidases/dehydrogenases enzymes along with a brief review of the *CKX* gene family in flowering plants. Next, the role of *CKX* as a genetic target for yield improvement, root-shoot architecture improvement, drought stress tolerance, and enhanced micronutrient acquisition is discussed in detail followed by a conclusion and future directions.

1.1 Cytokinin homeostasis: A regulatory web to regulate local cytokinin concentration

Cytokinins are N^6-substituted adenine-derived phytohormones having either isoprene or aromatic side chain as N^6 substitution (Kieber and Schaller, 2018a). Dihydrozeatin (DHZ), Isopentenyl adenine (iP), *cis*-zeatin (cZ), and *trans*-zeatin (tZ) are the most common forms of cytokinins present in plants. Isopentenyl adenine transferases encoded by *ADP/ATP IPT* genes catalyze the transfer of the isoprenyl side chain from Dimethyl allyl diphosphate (DMAPP) and (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP) to adenine ring of either ATP or ADP resulting in the synthesis of isopentenyl adenine ribonucleotide triphosphate/diphosphate (iPRTP/iPRDP) and trans-zeatin ribonucleotide triphosphate/diphosphate (tZRTP/tZRDP) respectively which are further converted into respective monophosphates (iPRMP and tZRMP) (Miyawaki et al., 2006; Frébort et al., 2011a). iPRTP/iPRDP/iPRMP can also be converted into respective trans-Zeatin counterparts by cytokinin trans-hydroxylases encoded by *CYP735A* genes (Takei et al., 2004). Enzymes encoded by *t-RNA IPT* genes catalyze the addition of isoprenyl side chain

to adenine ring in specific tRNA molecules and are thought to be responsible for the synthesis of cis-Zeatin (cZ) type of cytokinins (Miyawaki et al., 2006; Hluska et al., 2021a). Cytokinin ribotides (iPRMP/tZRMP/cZRMP) are converted into bioactive free base forms (iP, tZ, and cZ) by specific phosphoribohydrolases encoded by *LONELY GUY (LOG)* genes (Kurakawa et al., 2007; Kuroha et al., 2009; Frébort et al., 2011a). Sugars can be attached to hydroxyl side chains of cZ, tZ, and DHZ by *O*-glucosyltransferase (*ZOG*) resulting in reversible inactivation (Martin et al., 1999; Veach et al., 2003). The resulting sugar conjugates can be again converted back into active forms by β-glucosidases encoded by *GLU* genes (Brzobohatý et al., 1993; Kieber and Schaller, 2014). The addition of sugars to N^7 and N^9 position of the adenine ring of active cytokinins by N-glucosyltransferases renders them permanently inactive (Šmehilová et al., 2016). Cytokinin oxidase/dehydrogenase encoded by *CKX* genes facilitates the irreversible degradation of both free base and nucleotide forms of cytokinins (Figure 1) (Kieber and Schaller, 2014; 2018b). Many genes involved in cytokinin homeostasis maintenance such as *IPT* (Wang et al., 2020a), *LOG*(Chen et al., 2022), *CKX* (Chen et al., 2020b), glucosyltransferases (*ZOG*) (Chen et al., 2021), and cytokinin trans-hydroxylase (*CYP735A*) (Takei et al., 2004) are present as small multigene families in plants. Different members often exhibit distinct spatio-temporal expression patterns and encode gene products having distinct substrate specificity, subcellular localization, and physico-chemical activities facilitating very fine spatio-temporal regulation of different cytokinins metabolites.

1.2 Cytokinin oxidases/dehydrogenases—structure, function, and evolution

Cytokinin oxidase/dehydrogenases (CKX, EC 1.5.99.12) are the flavoprotein oxidoreductase enzymes capable of catalyzing the irreversible cytokinin degradation by cleaving the isoprenyl side chain attached to the N^6 position of the adenine ring (Avalbaev et al., 2012). Cytokinin oxidases/dehydrogenases degrade freebase active forms such as iP, tZ, cZ, and their respective nucleoside and nucleotide forms by catalyzing the secondary amine group and the resulting imine is non-enzymatically hydrolyzed into adenine or adenosine and corresponding aldehydes (Frébort et al., 2011b). DHZ and cytokinins having aromatic side chains such as benzylaminopurine (BAP) and kinetin are not degraded by CKX. Crystal structures of ZmCKX1 (ZmCKO1), ZmCKX2 (ZmCKO2), ZmCKX3 (ZmCKXO3), and ZmCKX4 (ZmCKO4) from maize and AtCKX7 from *Arabidopsis thaliana* has been resolved (Malito et al., 2004; Bae et al., 2008; Kopečný et al., 2016). Structures of both maize and *Arabidopsis* CKX exhibit a two-domain topology of vanillyl oxidase family proteins consisting of an N-terminal FAD-binding domain and C-terminal substrate binding domain (Malito et al., 2004; Bae et al., 2008; Gouda et al., 2020; Frébortová and Frébort, 2021). In ZmCKX1, the FAD-binding domain spans between amino acid residues 40–244 and 492–534 while residues 245–491 constitute the substrate binding domain (Malito et al., 2004; Kopečný et al., 2016). In AtCKX7, the FAD-binding domain is composed of residues 34–237 and 480–514 while residues 238–479 constitute the substrate binding domain (Bae et al., 2008). The active site of CKX enzymes is bipartite in structure consisting of a funnel-shaped region on the surface that binds adenine moiety of cytokinin substrates and an internal cavity situated above the isoalloxazine ring of the FAD cofactor serving as the binding site for the aliphatic side chain. Asp169-Glu288 pair is an important element in the active site which polarises the N10 atom of the side chain which in turn facilitates the transfer of two protons and one hydrogen atom from C11 of the side-chain to N5 of the FAD cofactor. The intermediate imine product is further hydrolyzed to yield reaction products. Reduced $FADH_2$ is re-oxidized by electron acceptors such as quinones (dehydrogenase mode) or molecular oxygen (oxidase mode). However, the existence of any tunnel or cavity serving as the binding site for electron acceptor in the vicinity of the FAD cofactor remains elusive (Malito et al., 2004; Kopečný et al., 2016). Amino acid residues pertaining to cofactor binding and substrate binding are conserved between maize and *Arabidopsis* CKXs, indicating that the catalytic mechanism is conserved between dicot and monocot plants (Bae et al., 2008). Cytokinin oxidases/dehydrogenases are encoded by small multigene families in higher plants and different members of gene families often exhibit distinct spatio-temporal expression patterns and their gene products exhibit different subcellular localization, substrate specificities, and physic-chemical properties (Joshi et al., 2018; Chen et al., 2020a; Gouda et al., 2020). There are 7 *CKX* homologs in the model dicot plant *A. thaliana* (Schmülling et al., 2003), 11 in rice (Ashikari et al., 2005; Rong et al., 2022), 13 in maize (Zalabák et al., 2014), 11 in barley (Gasparis et al., 2019), 11–14 in bread wheat (Chen et al., 2020a), 10 in chickpea (Khandal et al., 2020), 17 in soybean (Nguyen et al., 2021a), 9 in model legume *Medicago truncatula* (Wang et al., 2021a), 23 in *Brassica napus* (Liu et al., 2018; Schwarz et al., 2020a), 34 in *Brassica olearacea* (Zhu et al., 2022), 11 in C_4 model plant *Setaria italica* (Wang et al., 2014a), *8* in *Vitis vinifera* (Yu et al., 2021) and 7 in biodiesel plant *Jatropha curcas* (Cai et al., 2018) (Table 1).

There is an apparent functional dichotomy in *CKX* gene family members in flowering plants. Recent reports propose that two categories of *CKX* genes exist in flowering plants, which are ancient and non-ancient *CKXs*. The differences between these two groups have been reported in terms of their subcellular localization, biochemical properties, roles in growth and development, and respective substrate specificities. Ancient *CKX* genes are confined to 1–2 gene copies in each species

TABLE 1 Features of *CKX* gene family members in selected plant species.

S.No	Plant species	Number of *CKX* genes	Range of ORF size/gene size (base pairs)	Range of encoded polypeptide length (number of amino acids)	Range of molecular weight of encoded polypeptides (KDa)	spatio-temporal expression pattern	References
Model plant							
1	*Arabidopsis thaliana*	7	1,506–1728	501–575	—	Inflorescence meristem and floral meristem: *AtCKX3* and *AtCKX5* Vascular tissue, transmitting tissue, and embryo sac: *AtCKX7*	Schmülling et al. (2003); Bartrina et al. (2011a); Köllmer et al. (2014)
Cereal crops							
2	*Oryza sativa*	11	1,506–1863	501–620	—	*Inflorescence meristem*: *OsCKX2* Senescing, leaves and developing grains: *OsCKX11,* Leaf-blade, roots, and shoot base: *OsCKX4*	Ashikari et al. (2005); Gao et al. (2014); Zhang et al. (2021); Rong et al. (2022a); Mameaux et al. (2012)
3	*Hordeum vulgare*	At least 11	699–1842	233–614	—	14 *DAP spikes*: HvCKX1, HvCKX9, HvCKX4, and HvCKX11, Leaves: HvCKX9, HvCKX5, and HvCKX11	Zalewski et al. (2014)
4	*Zea mays*	13	804–2066	267–568	—	*Constitutive expression*: *ZmCKX6, ZmCKX10,* and *ZmCKX1,* Young and mature leaves: *ZmCKX2* and *ZmCKX3, Reproductive organs: ZmCKX4* and *ZmCKX4b*	Mameaux et al. (2012); Gu et al. (2010)
Millets							
5	*Setaria italica*	11	720–1,620	239–539	—	Expression of all the *SiCKX* genes induced in germinating embryo when treated with 6-BAP. Induced by salinity and drought treatment: SiCKX1, SiCKX3, SiCKX5, and SiCKX8	Wang et al. (2014b)
Legume crops							
6	*Medicago truncatula*	9	1,530–1,644	509–547	56.8–62.2	Flowers; Medtr7g090920 and Medtr4g126150/MtCKX2. Leaves: Medtr4g044110. Roots and root nodules: Medtr4g126160	Wang et al. (2021a)
Oilseed crops							
7	*Brassica napus*	23	1,011–2,307	336–768	33–87	Systematic/constitutive: *BnCKX5-1, BnCKX5-2* and, *BnCKX7-3.* Silique pericarp: *BnCKX3-2* and *3–3. Buds: BnCKX3-1.* Inflorescence meristem, floral meristem, and developing gynoecia: *BnCKX3* and *BnCKX5*	Liu et al. (2018); Schwarz et al. (2020a)

(Continued on following page)

TABLE 1 (*Continued*) Features of *CKX* gene family members in selected plant species.

S.No	Plant species	Number of *CKX* genes	Range of ORF size/gene size (base pairs)	Range of encoded polypeptide length (number of amino acids)	Range of molecular weight of encoded polypeptides (KDa)	spatio-temporal expression pattern	References
8	*Glycine max*	17	1,329–1,659	442–552	49–62	Reproductive organs: *GmCKX7-1, GmCKX1-1, GmCKX1-2, GmCKX5-2, GmCKX7-3* and, *GmCKX3-1*. Seeds: *GmCKX7-1*	Nguyen et al. (2021a)
Cash crops							
9	*Jatropha*	7	1,332–1,650	443–549	—	Roots: *JcCKX1* and *JcCKX7*. Female flowers: *JcCKX1, JcCKX2* and *JcCKX4*. Male flowers: *JcCKX3*. Seeds: *JcCKX4* and *JcCKX2*. Leaves: *JcCKX4* and *JcCKX5*. All tissues: *JcCKX6*	Cai et al. (2018)
Fruits crops							
10	*Vitis vinifera*	8	1,279–1961	424–632	47–70	Inflorescence: VvCKX4 and VvCKX8	Yu et al. (2021)
11	*Malus domestica*	12	942–2,535	313–844	34–96	Leaves: MdCKX4, MdCKX5 and, MdCKX8. Root: MdCKX1, MdCKX7, MdCKX9 and, MdCKX11/12	Tan et al. (2018)

and their encoded proteins are believed to play housekeeping roles, preferentially degrade cZ, exhibit nearly uniform expression across tissues, and are localized to cytosol while non-ancient *CKX* genes are present in variable numbers across different species and the proteins encoded by them are involved in the regulation of organ development and stress responses, preferentially degrade iP and tZ type of cytokinins, exhibit differential spatio-temporal expression induced rapidly under environmental stresses and are found to be present in cellular compartments such as ER, vacuole and apoplast. *AtCKX7, ZmCKX10,* and *OsCKX11* are the representatives of ancient *CKXs* in *Arabidopsis*, maize, and rice respectively (Wang et al., 2020b). Interestingly similar functional dichotomy was also observed among *IPT* gene family members (Wang et al., 2020c). A recent report indicates that this apparent duality is a characteristic feature of cytokinin metabolism (Hluska et al., 2021b).

CKX genes are present in angiosperms, non-vascular and vascular seeded plants, some groups of bacteria, and a few fungi species. Surprisingly, they are altogether absent in algae. Although homologs of cytokinin biosynthesis genes (*IPT* and *LOG*) were reported in a few archaeal species, there is no information available about *CKX* homologs in any archaeal species (Wang et al., 2020a; Frébortová and Frébort, 2021). Initial reports hypothesized that plants acquired *CKX* genes by lateral transfer from cyanobacteria through chloroplast (Frébort et al., 2011b). Recent reports suggest the transfer of *CKX* genes into the plants from an ancient chlamydial endosymbiont ancestor that resided inside the common ancestor of plants (Wang et al., 2020b). Another group hypothesized that bacterial and plant *CKXs* might have originated independently from ancient and omnipresent FAD-linked oxidases. Their model indicates that algae might have derived FAD-linked oxidases from either the proteobacteria (modern mitochondria) or cyanobacteria (modern chloroplast) or even directly from the most common ancestor of eukaryotes. *CKX* genes present in proto-mitochondria and proto-chloroplast were lost during algal evolution, explaining the absence of *CKX* homologs in algae. FAD-linked oxidase genes derived from mitochondria or chloroplast or common ancestors later on duplicated, diverged, and expanded to give rise to modern plant *CKX* gene families, independent of bacterial *CKX* homologs (Dabravolski and Isayenkov, 2021).

1.3 Cytokinin oxidases as a genetic target for crop yield improvement

Cytokinins positively regulate both source capacity (photosynthetic efficiency) and sink strength (number of ovules formed in gynoecia as well as the number and weight of seeds) by directly regulating leaf senescence and

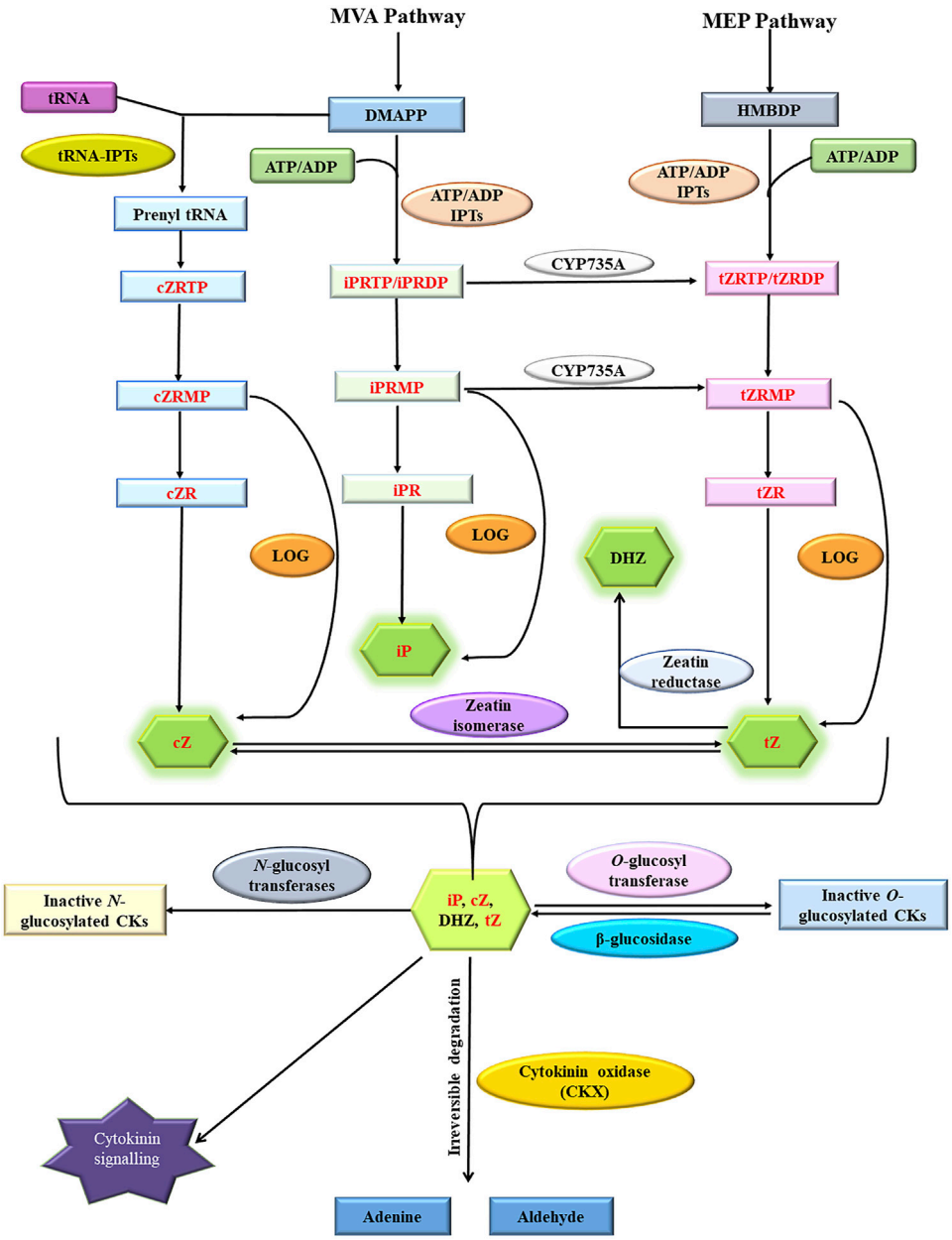

FIGURE 1
Cytokinin homeostasis maintenance pathway involving *in situ* cytokinin biosynthesis (catalyzed by ATP-ADP IPTs and tRNA-IPTs), activation (catalyzed by specific phosphoribohydrolases encoded by *LOG* genes), irreversible inactivation (catalyzed by *N*-glucosyltransferases), reversible inactivation (catalyzed by *O*-glucosyltransferases), reactivation (catalyzed by *β*-glucosidases) and irreversible degradation (catalyzed by cytokinin oxidase/dehydrogenases). Cytokinin oxidase/dehydrogenases substrates are highlighted in red (Modified from Frébort et al., 2011b; Kieber and Schaller, 2014; Hluska et al., 2021b).

inflorescence meristem activity and thus they are key regulators of yield output (Schwarz et al., 2020a). Endogenous cytokinin levels of different crops might be manipulated by different approaches based on whether a crop is source limited or sink limited, to increase the yield output (Jameson and Song, 2016a). Besides regulating inflorescence meristem activity, cytokinins are also indispensable for seed development indicated by a sharp increase in endogenous cytokinin levels in cereals crops such as maize, wheat, barley, and rice shortly after anthesis (Wheeler, 1972a; Yang et al., 2000, 2003; Chen et al., 2020a). These rising endogenous cytokinin levels coincide with the

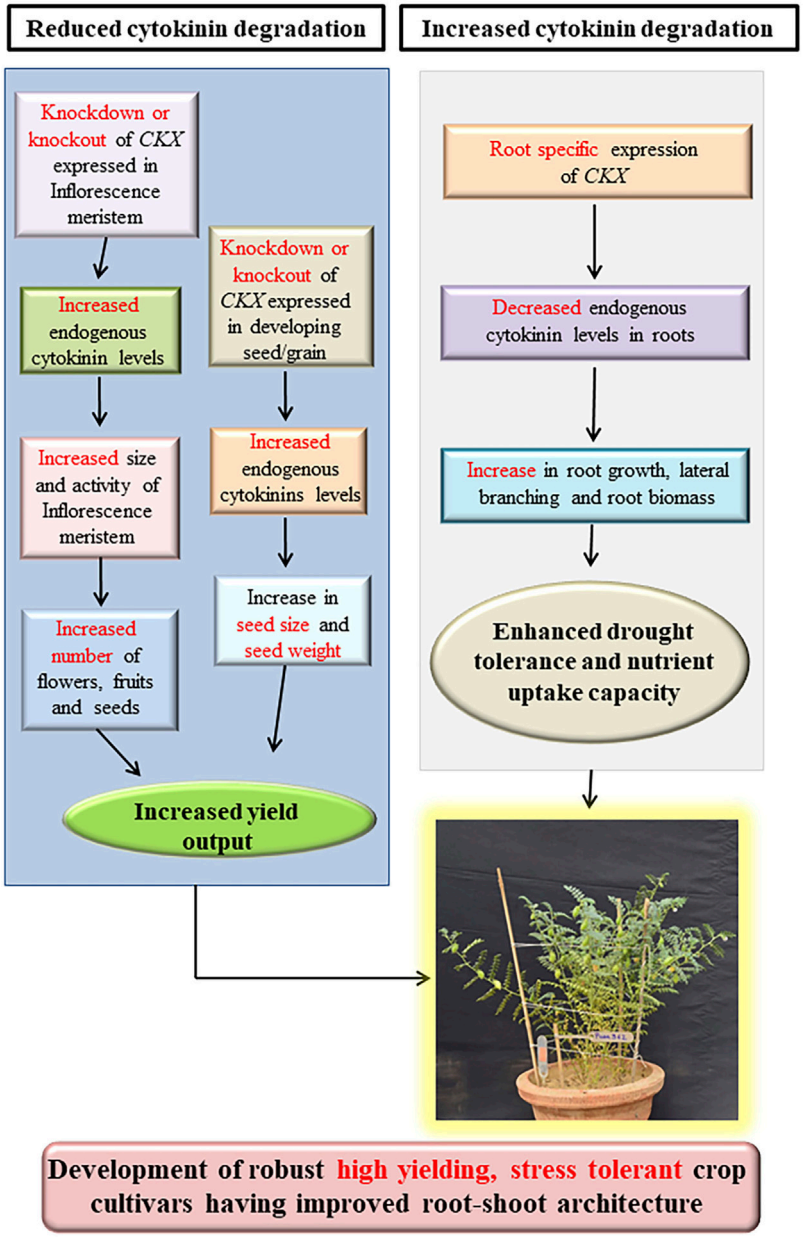

FIGURE 2
Combined strategy for the development of high yielding, biofortified, drought-tolerant crop cultivars through alteration of endogenous cytokinin by genetic manipulation of cytokinin oxidase/dehydrogenase family genes (Modified from (Jameson and Song, 2020b).

increased cell division rates and cell number in developing grain endosperm during grain filling stages resulting in the formation of a strong "Sink" which eventually enhances photoassimilate migration and accumulation during grain development (Wheeler, 1972b; Yang et al., 2000, 2003; Jameson and Song, 2016a). Cytokinins positively regulate inflorescence meristem activity and seed yield (Bartrina et al., 2011b; Jameson and Song, 2016b). As CKXs catalyze the irreversible degradation of cytokinins and thus negatively regulate inflorescence and floral meristem activity (Bartrina et al., 2011b; Schwarz et al., 2020b). Genetic manipulation of *CKX* genes (knockdown and knockout) expressed in reproductive organs and developing seeds provides us with an opportunity for increasing seed yield through endogenous cytokinin level alteration. Thus, *CKX* becomes an ideal target for yield improvement (Chen et al., 2020b) (Figure 2). Several studies have established the role of *CKX* as a major regulator of seed yield as reviewed below (Table 2).

TABLE 2 Genetic manipulation of *CKX* genes in different plant species for increasing seed yield output.

S.No.	Name of *CKX* gene	Source species	Target species	Type of genetic manipulation	Observed changes in yield-related parameters	Comments	References
1	*OsCKX2*	*Oryza sativa*	*Oryza sativa*	Natural genetic variation	Panicle branches ↑ grain number per panicle ↑ Total grain number ↑		Ashikari et al. (2005)
				RNAi mediated gene knockdown	Number of tillers ↑ Number of panicles ↑ Grains per plant ↑ TGW ↑ Total grain yield per plant ↑	Delayed Leaf senescence (DLS) phenotype in transgenic lines	Yeh et al. (2015)
					Plant height ↑ Filled grains per panicle ↑ Panicle number ↑ Harvest index ↑ TGW ↑ Leaf senescence ↑	Reduced yield loss in transgenic lines under salinity stress compared to control lines	Joshi et al. (2018)
				CRISPR/Cas9 mediated gene knockout	Plant height ↑ Panicle length ↑ Flower number per panicle↑	Effect on seed yield output not reported	Li et al. (2016)
				EMS mutagenesis is followed by Map-based cloning	Panicle length ↑ Panicle branches ↑ Filled grains per plant ↑ Grain length ↑ TGW ↑ Seed setting rate ↑		Tsago et al. (2020)
2	*OsCKX11*	*Oryza sativa*	*Oryza sativa*	CRISPR/Cas9 mediated gene knockout	Tiller's number ↑ Grains per panicle ↑ Grains per plant ↑ Fertility rate ↓ TGW ↓ Leaf senescence ↓	*OsCKX11* is involved in the regulation of both leaf senescence and seed yield	Zhang et al. (2021)
3	*TaCKX1*	*Triticum aestivum*	*Triticum aestivum*	RNAi mediated gene knockdown	Number of spikes ↑ Grain yield ↑ TGW ↓		Li et al. (2018)
4	*HvCKX1*	*Hordeum vulgare*	*Hordeum vulgare*	RNAi mediated gene knockdown	Spikes number ↑ Grains per plant ↑ Overall grain yield ↑ TGW ↓		Holubová et al. (2018)

(Continued on following page)

TABLE 2 (*Continued*) Genetic manipulation of *CKX* genes in different plant species for increasing seed yield output.

S.No.	Name of *CKX* gene	Source species	Target species	Type of genetic manipulation	Observed changes in yield-related parameters	Comments	References
5	*HvCKX1 and HvCKX3*	*Hordeum vulgare*	*Hordeum vulgare*	CRISPR/Cas9 mediated gene knockout	In one *ckx3* mutant line Spikes number ↑ In other mutant lines Grain number ↓ Grain weight ↓	Activation of strong cytokinin homeostatic response resulted in reduced expression of CK biosynthetic genes and increased CK inactivation through *O*-glucosylation	Gasparis et al. (2019)
6	*CKX3/CKX5*	*Arabidopsis thaliana*	*Arabidopsis thaliana*	*ckx3ckx5* double mutant generated by random T-DNA mutagenesis	Flowers number per plant ↑ Flower size ↑ Gynoecium size ↑ Inflorescence meristem size ↑ Ovules per gynoecium ↑ Siliques per plant ↑ Seed number per silique ↑ Overall seed yield ↑	*ckx3* or *ckx5* mutations alone were not sufficient to alter yield components significantly	Bartrina et al. (2011a)
		Brassica napus	*Brassica napus*	*ckx3ckx5 sextuple* mutant generated by random T-DNA mutagenesis and TILLING.	Inflorescence meristem size ↑ Floral primordia number ↑ Flower number per plant ↑ Gynoecium size ↑ Ovules per gynoecium ↑ Seed weight and number ↑ TGW ↑	The number of seeds per silique was equivalent to wild type because of high seed mortality	Schwarz et al. (2020)
7	*MtCKX3*	*Medicago truncatula*	*Medicago truncatula*	*Tnt1* retrotransposon tagged insertion mutants	Length of primary root ↑ Lateral roots number ↓	Single mutants of any other *CKX* gene did not exhibit significant deviation from wild-type phenotype, indicating functional redundancy	Wang et al. (2021)

[#]'↑' and '↓' symbols respectively indicate increase and decrease in corresponding parameter.

1.3.1 Rice

Rice grain number locus *Gn1a* is encoded by *OsCKX2*and it was found to be responsible for higher yield i.e., increased panicle branching, increased grain number per panicle as well as per plant in two high yielding rice varieties Habataki and 5150. There was reduced expression of *OsCKX2* in Habataki inflorescence meristem as compared to the Koshikari variety while in the case of 5150 there was no expression of *OsCKX2* in inflorescence meristem. Sequence comparison between Koshikari and Habataki alleles indicated that the Habataki allele had a 16bp deletion in the 5′-UTR, a 6bp deletion in the first exon, and 3 nucleotide changes causing amino acid sequence variation in exons 1 and 4 while the 5150 allele had 11 bp deletion in the coding region resulting in the generation of a premature stop codon. Among these 3 varieties, 5150 exhibited the highest yield followed by Habataki and Koshikari, indicating that *OsCKX2* negatively regulates yield output in rice (Ashikari et al., 2005). Downregulation of *OsCKX2* using RNAi resulted in delayed leaf senescence, increased tiller number (15%–43%), increased

panicle number (27%–81%), increased grain number per plant (44%–67%), increased total grain weight per plant (58%–75%) and increased thousand-grain weight (TGW) (5%–15%) in homozygous T_3 transgenic lines (Yeh et al., 2015). Gene knockout of *OsCKX2* using CRISPR/Cas9 resulted in a nearly two-fold increase in the number of flowers per plant as well as increased plant height and panicle length in T_2 homozygous lines having frameshift mutations. Although the effect on yield output *per se* was not reported (Li et al., 2016). Transgenic lines developed through downregulation of *OsCKX2* utilizing RNAi exhibited increased yield output and reduced yield penalty under salinity stress. Homozygous T_2 transgenic lines showed significantly reduced senescence, increased panicle branching, increased panicle number per plant (50%), increased TGW (10%), increased number of filled grains per panicle (69.7%), and higher harvest index (HI) (25.4%) as compared to the non-transgenic control lines under normal conditions while under salinity stress conditions transgenic lines had increased panicle number (80%), increased TGW (26%), increased number of filled grains per panicle (196.4%), and increased HI (136.4%) compared to non-transgenic control lines. Transgenic lines retained higher chlorophyll and carotenoids content, relative water content (RWC), net photosynthetic rate (NPR), stomatal conductance, intercellular CO_2 concentration, electron transport rate (ETR), and maximal photosystem II efficiency (Fv/Fm ratio) relative to wild type control lines under salinity stress conditions which might have contributed to reduced yield penalty under salinity stress (Joshi et al., 2018). A mutant *OsCKX2-2* identified in rice through random mutagenesis and map-based cloning had an increased number of panicle branches and increased grain length, grain weight, TGW, and yield per plant compared to their wild-type counterparts (Tsago et al., 2020). Recently *OsCKX11* gene-knockout lines generated through CRISPR/Cas9 exhibited significantly delayed leaf senescence along with an increased number of tillers (14.83%–27.07%), increased grains per panicle (15.11%–27.96%), and increased grains per plant (21.62%–27.29%), while fertility and TGW decreased as compared to wild type plants. Small-scale field tests based on two independent plots showed that grain yield increased significantly (by 7.47% and 7.58%) in *osckx11* mutant lines compared to WT control lines. Leaves of osckx*11* knockout lines retained higher cytokinin levels, chlorophyll content, maximal photosystem II efficiency (Fv/Fm), and accumulated lower ABA levels compared to control lines during the onset of senescence stage. Expression of ABA biosynthetic genes, senescence-associated genes, and chlorophyll catabolic genes was reduced while expression of ABA degradation genes was elevated in senescing leaves of knockout lines compared to control plants. OsCKX11 might positively regulate leaf senescence by modulating the expression of ABA, cytokinin, and chlorophyll metabolism genes at the transcription level. The results established the dual role of *OsCKX11* in the regulation of both leaf senescence (source capacity) and the number of grains (sink strength) simultaneously (Zhang et al., 2021).

1.3.2 Barley

RNAi mediated gene silencing of *HvCKX1* which is predominantly expressed in spikes and roots lead to increased grain production (154–168 grains in transgenic lines compared to 117 in control lines), increased TGW (32 g in transgenic lines compared to 26 g in control lines), increased average yield per plant (5–5.5 g in transgenic lines as compared to 3.35 g in controls) as well as increased root mass (28.4–31.2 mg in transgenic lines as compared to 23.1 mg in control lines) in T_0 barley transgenic lines (Zalewski et al., 2010). Silencing of *HvCKX2* led to an increase in grain number to 144%–163% and total grain yield was 135%–167% higher as compared to control plants in T_0 barley transgenic lines. Homozygous T_1 transgenic lines also exhibited increased productivity marked by increased TGW, increased plant height (1–4.5 cm higher than control), and an increased number of spikes (0.5-2 spikes higher per plant as compared to control lines) (Zalewski et al., 2012). *HvCKX1* knockdown and knockout lines were generated by short hairpin RNAi and CRISPR/Cas9 respectively. Gene knockdown lines exhibited increased plant productivity under controlled conditions marked by the production of 10% more spikes, 40% more grains per plant, and overall yield up to 120% of the control plants (Holubová et al., 2018). Two knockdown lines grown under field conditions exhibited increased yield per unit area up to 120%–139% in 2016 and 118%–136% in 2017 as compared to control lines. However, the yield parameters of knockout lines were not reported (Holubová et al., 2018). Recently *HvCKX1* and *HvCKX3* knockout lines generated through CRISPR/Cas9 exhibited inconsistent, ambiguous, and unexpected phenotypes in terms of yield, all but one *ckx3* line exhibited a significantly lower number of spikes and grains as well as lower TGW while *ckx1* lines were equivalent to control lines in terms of yield parameters. Gene expression analysis and RNA seq data indicated that expression of cytokinin biosynthesis (*IPT*), activation (*LOG*) and, reactivation (*GLU*) genes were decreased while expression of cytokinin inactivating genes (*ZOG*) was increased in *HvCKX1* and *HvCKX3* knockout lines compared to control lines. Thus activation of strong cytokinin homeostasis maintenance response nullified the effect of *HvCKX1* and *HvCKX3* knockout (Gasparis et al., 2019b). RNAi-mediated gene knockdown might not have triggered the homeostasis response because contrary to gene knockout, gene knockdown does not result in complete abolishment of gene function (Gasparis et al., 2019c). *HvCKX3* was found to be involved in the maintenance of inflorescence architecture in barley under high-temperature stress. Barley MADS-box protein HvMADS1 binds to A-tract-rich CArG-box motifs in

the promoter of the *HvCKX3* gene with high affinity at higher temperatures and activates its expression to regulate local cytokinin levels. Enhanced expression of *HvCKX3* under high-temperature results in reduced cytokinin response which causes repression of meristematic cell divisions, ultimately stabilizing meristem determinacy and facilitating the development and maintenance of unbranched spike architecture at elevated temperatures (Li et al., 2021a).

1.3.3 Wheat

In wheat, *TaCKX6-D1* (recently renamed as *TaCKX2.2.1-3D*) situated on chromosome 3D was found to be an orthologue of *OsCKX2*. An 18bp deletion in the second intron of *TaCKX6-D1* was found to be associated with higher TGW based on an association analysis across 115 diverse cultivars. Varieties having haplotype *TaCKX6-D1b* (wild type *TaCKX6-D1* allele) exhibited 1.4-5.4fold higher expression as compared to those having haplotype *TaCKX6-D1a* (*TaCKX6-D1* allele having 18bp deletion in 2nd intron near 3′ splice site). Differences in promoter diversity such as methylation state between *TaCKX6-D1b* and *TaCKX6-D1a*allele, change in alternative splicing pattern caused due to 18 bp deletion or an upstream gene or cis-factor might be causing the observed difference in expression between *TaCKX6-D1b* and *TaCKX6-D1a* allele as no other polymorphism were detected between *TaCKX6-D1b* *TaCKX6-D1a* alleles. Although the exact molecular basis is still not known. Association analysis indicated that the mean TGW of varieties having haplotype *a* was 3.94 g higher than type b. Thus there was a significant negative correlation between *TaCKX6-D1* expression and TGW (Zhang et al., 2012). Allelic variation in *TaCKX6a02* (renamed as *TaCKX2.1-3D*) located on chromosome 3D was found to be significantly correlated with grain size, grain weight, and grain filling rate (GFR) based on the analysis of grain traits of the recombinant inbred lines (RILs) generated by crossing high yielding and low yielding wheat varieties (Lu et al., 2015a). The *TaCKX6a02a* allele having 29 bp insertion in 3′ UTR was found to be significantly associated with increased grain yield as a result of association analysis across 102 cultivars (Lu et al., 2015b). Although UTRs mediated regulation of gene expression is a very well-established phenomenon (Mayr, 2018; Srivastava et al., 2018), the exact molecular basis behind the association of 29 bp insertion in 3′UTR of *TaCKX6a02a* allele is not known (Lu et al., 2015a). *TaCKX2.4* gene knockdown through shRNA mediated RNAi resulted in a 5.8%–12.6% increase in the number of grains per spike in T_3 homozygous transgenic lines as compared to wild type control lines while the number of spikes, number of spikelets per spike, kernel length and width as well as TGW were identical to control lines (Li et al., 2018). Expression of *TaCKX2.4* was drastically reduced in the spikes of transgenic lines as compared to the non-transgenic lines indicating a negative correlation between *TaCKX2.4* expression and the number of grains per spike (Li et al., 2018). RNAi mediated strong gene silencing of *TaCKX1*; an orthologue of *HvCKX1* increased several yield parameters in T_2 transgenic lines including a higher number of spikes, higher number of grains, increased grain yield, and seedling root weight. On the other hand, plant height, length of the spike, and TGW were decreased as compared to control lines. Knockdown of *TaCKX1* resulted in simultaneous strong down-regulation of *TaCKX11* and *TaCKX1* and up-regulation of *TaCKX2.2, TaCKX5,* and *TaCKX9* in T_2 homozygous lines resulting in overall CKX activity being identical to control lines because of the cumulative contribution of isozymes encoded by different *CKX* genes. Still, cytokinin concentrations were increased by 23%–76% in transgenic lines as compared to control lines. As different CKX enzymes have different substrate preferences, the composition of the active cytokinin pool and phenotypic traits of the modified plants were changed accordingly (Jabłoński et al., 2020).

1.3.4 Dicotyledonous plant species

Cytokinin status and its regulation by CKX is a very well-established determinant of yield in crops such as rice, barley, and wheat but very few reports are available on cytokinin-mediated regulation of yield in dicot plant species (Schwarz et al., 2020a). The First study on cytokinin-mediated regulation of yield through the activity of CKX was reported in the model dicot plant species *A. thaliana*. Double knockout lines (*ckx3ckx5*) of *CKX3* and *CKX5* which are expressed in the organizing center of inflorescence meristem and floral meristem at different growth stages as well as developing gynoecia primordia and placenta tissues, exhibited a stronger stem, enlarged inflorescence meristem having increased number of cells, larger flowers, larger gynoecium having nearly as twice as many ovules as wild type, 40% increase in the number of siliques formed and 55% increase in yield output as compared to wild-type control plants (Bartrina et al., 2011b). *WUSCHEL* encodes a homeodomain transcription factor that positively regulates stem cell population in the shoot apical meristems and floral meristems and thus acts as the master regulator of shoot and floral meristem size and integrity in flowering plants (Jha et al., 2020). RNA *in situ* hybridization revealed that the expression domain of *CKX3* overlaps with *WUSCHEL* expression domain and is significantly enlarged in *ckx3ckx5* indicating that increased endogenous cytokinin might be modulating the size and activity of inflorescence meristem through the *WUSCHEL-CLAVATA3* regulatory loop (Bartrina et al., 2011a). In an excellent example of translational research sextuple *ckx3ckx5* mutant was developed in allotetraploid *B. napus* using random EMS mutagenesis followed by TILLING and backcrossing for four generations. *ckx3ckx5* sextuple mutant exhibited a 52% increase in the number of flowers, 47% increase in the number of pods, 16% increase in the diameter of inflorescence meristem, 24% increase in the number of floral primordia, 32% increase in the number of ovules, 20% increase in total seed weight, increase in the total number of seeds per plant as well as TGW. Although the total

seed number was increased as compared to the wild-type controls the number of seeds per pod was equivalent to wild-type plants because of high seed mortality in *ckx3ckx5* mutant (Schwarz et al., 2020a). In both *A. thaliana* and *B. napus* mutations in *ckx3* or *ckx5* alone were not sufficient to alter yield parameters significantly indicating that contrary to their monocot counterparts, simultaneous silencing of more than one *CKX* gene might be required to increase the yield parameters significantly. Recently, three SNPs resulting in non-synonymous substitutions in *GmCKX7-1* which is the most highly expressed *CKX* member during pod and seed developmental stages were found to be associated with high yielding phenotype, and cultivars having these 3 SNPs in *GmCKX7-1* had high seed weight (OAC Wallace, DH420, IA1010LF) and high yield (DH748) (Nguyen et al., 2021b). Among the 3 SNPs identified, H105Q is thought to alter the FAD cofactor binding site critical for enzyme function and thus negatively affects the enzyme's structural integrity which might result in up to 100-fold lower enzyme activity which in turn will result in increased endogenous cytokinin content during seed development stages effectively increasing the yield output. Indeed among all the cultivars analyzed, the concentration of CK-nucleotide and free base forms during pod and seed development was highest in high-yielding cultivars DH420 and OAC Wallace (Nguyen et al., 2021a). Analysis of Tnt1 retrotransposon tagged mutants in model legume *M. truncatula* revealed that mutant lines having insertion in a single *CKX* gene were similar to wild-type control lines at both vegetative and reproductive stages (Wang et al., 2021b). The only exception was the *ckx3* mutant which exhibited a decrease in primary root length and an increase in the number of lateral roots. These results suggest that there is functional redundancy among different *MtCKX* gene family members in terms of regulation of root-shoot architecture and yield output which further indicate the necessity of downregulating more than one *CKX* gene in dicots to get a distinct detectable phenotype (Wang et al., 2021a).

1.4 *CKX* genes as a genetic target for improvement of root-shoot architecture, stress tolerance, and micronutrient acquisition

Root-system architecture (RSA) is primarily responsible for nutrient and water uptake, biotic rhizospheric interactions, and soil anchorage in flowering plants (Lombardi et al., 2021). RSA is quite flexible as root and shoot growth is differentially favored in response to varying environmental conditions (Koevoets et al., 2016). Under optimum environmental conditions having plenty of water and minerals, growth of shoot is promoted and root growth is maintained at a level sufficient to sustain shoot growth while under water and mineral deficient conditions extensive root growth is promoted to trap more water and minerals and shoot growth is restrained to avoid excessive water loss and mineral depletion. This root-shoot architecture is majorly controlled by the antagonistic effects of two phytohormones auxin and cytokinins (Kurepa and Smalle, 2022). Cytokinins directly repress the formation and development of lateral root primordium and negatively regulate the transcription of genes encoding PIN transporter proteins, effectively disrupting PIN-dependent auxin maxima required for lateral root development (Jing and Strader, 2019). The presence of "Casparian strips" and suberin lamellae in the endodermal cell layer negatively regulates apoplastic and transcellular pathways of radial ion transport into the central vascular cylinder (Bao et al., 2019). The endodermis is occasionally interrupted by cells known as "Passage cells" having reduced suberin deposition which leads to a compromised ability to restrict transcellular ion transport (El-Showk and Mähönen, 2018). Auxin-mediated repression of cytokinin signaling was found to impart passage cell fate to endodermal cells adjacent to the xylem pole indicating that cytokinin negatively regulates the passage cell formation and thus also negatively regulates ion uptake through the transcellular pathway (Andersen et al., 2018). Asymmetric cytokinin signaling at the upper part of nascent lateral roots reduces the growth at the upper part and thus acts as an anti-gravitropic signal counteracting against gravity-induced, auxin-dependent cell elongation at the lower root part, ultimately determining the gravitropic set-point angle (GSA) which itself is a key determinant of soil exploration capacity of RSA (Waidmann et al., 2019; Waidmann and Kleine-Vehn, 2020; Lombardi et al., 2021). Cytokinins are also known to negatively regulate nutrient uptake by repressing the expression of micronutrient transporter genes for several minerals such as zinc (Gao et al., 2019), nitrate (Kiba et al., 2011), sulfate (Maruyama-Nakashita et al., 2004), phosphate (Martín et al., 2000) and, iron (Séguéla et al., 2008). Cytokinins also negatively affect plant's response to water deficiency and drought stress through a plethora of diverse mechanisms including promotion of shoot growth and inhibition of root growth, increased stomata density and transpiration (Farber et al., 2016), direct interference with ABA-mediated stomatal closure responses and repression of ABA-inducible stress-related genes (Salvi et al., 2021; Kurepa and Smalle, 2022). Increased cytokinin signaling in type B *ARR1* gain of function mutant indicated that cytokinins induce the expression of ribosomal protein genes *RPL4A* and *RPL4D* which resulted in an increase in the overall rate of global protein synthesis in *Arabidopsis* culminating in growth inhibition and hypersensitivity to osmotic stress which can be reversed by ABA treatment (Karunadasa et al., 2020). Type B-ARRs bind to promoters of cytokinin responsive genes after getting phosphorylated by Histidine phosphotransfer proteins (AHPs) and activate the transcription of cytokinin responsive genes in response to cytokinin signaling (Kieber and Schaller, 2018b). A recent report reveals the negative role of cytokinins in a plant's defense response to salinity stress as type B ARR1, 10, and

TABLE 3 Genetic manipulation of *CKX* genes for drought tolerance and enhanced micronutrient acquisition.

S.No.	Name of *CKX* gene	Source species	Target species	Type of genetic manipulation	Observed effects of genetic manipulation	Comments	References
1	*AtCKX1 and AtCKX2*	*Arabidopsis thaliana*	*Nicotiana tabacum*	Constitutive overexpression under *CaMV35S* promoter	Lateral and adventitious roots number ↑ Length of primary root ↑ Root biomass ↑	Dwarfed shoot phenotype and size of shoot apical meristem and number of flowers decreased	Werner et al. (2010)
2	*AtCKX1 and AtCKX3*	*Arabidopsis thaliana*	*Nicotiana tabacum, Arabidopsis thaliana*	Root specific expression under *WRKY6* and *PYK10* promoters	Lateral and adventitious roots number ↑ Length of the primary root ↑ Root biomass ↑ Root: shoot biomass ratio↑ Nutrient stress tolerance ↑ Micronutrient content in leaves ↑ Phytoremediation capacity ↑	No obvious adverse effect on shoot attributes	Werner et al. (2010)
3	*AtCKX2*	*Arabidopsis thaliana*	*Brassica napus*	Constitutive overexpression under *CaMV35S* promoter	Length of primary root ↑ Lateral root density ↑ Adventitious roots number ↑ Root biomass ↑ Tolerance to nutrient limitation ↑ Leaves micronutrient content ↑ Phytoremediation capacity ↑	No obvious adverse effects on shoot attributes	Nehnevajova et al. (2019)
4	*AtCKX1 and AtCKX2*	*Arabidopsis thaliana*	*Hordeum vulgare*	Root specific expression utilizing *pEPP* promoter	Root length ↑ Root surface area ↑ Root biomass ↑ Root-shoot biomass ratio ↑ Micronutrient content in leaves and seeds ↑ Tolerance to drought stress ↑	Shoot growth and development were not compromised in transgenic lines	Ramireddy et al. (2018)
5	*MsCKX7*	*Medicago sativa*	*Arabidopsis thaliana*	Constitutive overexpression under *CaMV 35S* promoter	Root length ↑ Lateral roots number ↑ Root fresh weight ↑ Tolerance to salinity stress ↑		Li et al. (2018)
6	*CaCKX6*	*Cicer arietinum*	*Cicer arietinum*	Root specific expression under *WRKY31* promoter	Root length ↑ Lateral roots numbers ↑ Root biomass ↑ Seed yield output ↑ Mineral content in seeds ↑ Tolerance to drought stress ↑	Stem growth and development were not affected and root nodulation was not compromised in transgenic lines	Khandal et al. (2020)
7	*OsCKX4*	*Oryzae sativa*	*Oryzae sativa*	Root specific expression under *RCc3* promoter	Root growth ↑ Root-shoot biomass ratio ↑ Zinc content in the root, shoot, and grain ↑ Overall yield output ↑	Shoot growth and development was not compromised in transgenic lines	Gao et al. (2019)

[#] '↑' and '↓' symbols respectively indicate increase and decrease in the corresponding parameter

12 are degraded through MPK3 and MPK6 mediated signaling cascade under salinity stress eventually increasing the salt stress tolerance in *Arabidopsis* (Yan et al., 2021). Triple mutants of genes encoding type-B RR - *arr1,10,12* triple mutant exhibited significant increase in drought tolerance (Nguyen et al., 2016). Defective cytokinin signaling in *Arabidopsis ahp2,3,5* and *arr1,10,12* mutants resulted in increased accumulation of primary metabolites (sugars and amino acids), secondary metabolites such as anthocyanins and flavonoids, and extensive lipid profile reprograming which might have contributed to enhanced salinity stress tolerance of mutant plants compared to wild type plants. Moreover, the accumulation of secondary metabolites, lipids, and sterols was strongly correlated with altered expression of genes pertaining to the biosynthetic pathway of concerned metabolites. These results indicate that cytokinin signaling negatively regulates plant's defense response to salinity stress through modulating the transcription of specific metabolic pathway genes (Abdelrahman et al., 2021). Similarly, loss of function mutants (*ahp2,3,5*) of genes encoding Histidine phosphotransfer proteins (AHPs) which are positive regulators of cytokinin signaling, resulted in a strong drought-tolerant phenotype (Nishiyama et al., 2013). In perfect alignment with the statements stated above, local or systemic decrease in active cytokinin pool through constitutive or tissue-specific overexpression of *CKX* genes encoding cytokinin degrading cytokinin oxidase/dehydrogenase resulted in improved root-shoot ratio, increased abiotic stress tolerance, and enhanced micronutrient acquisition in several plant species as reviewed below (See Figure 2; Table 3).

1.4.1 Model plants

Constitutive overexpression of AtCKX1 and AtCKX2 under CaMV35S promoter in *Nicotiana tabacum* established the opposite role played by cytokinin in root and shoot growth and development. Endogenous cytokinin content was reduced by 31%–63% in transgenic lines which in turn resulted in enhanced root growth marked by the increased size of root apical meristem (RAM), rapid elongation of the primary root, up to 60% increase in root diameter, increased production of lateral and adventitious roots resulting in overall ~60% increase in dry root biomass. However shoot growth was adversely affected in transgenic plants indicated by a reduction in shoot meristem (SAM) size, dwarfed growth habit, formation of lanceolate epinastic leaves having severely reduced surface area, delayed flowering with a reduction in the number of flowers formed per plant as compared to wild type plants (Werner et al., 2010). Root specific expression of *AtCKX1* in *N. tabacum* using root-specific *WRKY6* promoter validated the better feasibility of tissue-specific expression of *CKX* genes compared to ectopic expression for altering the root-shoot architecture as homozygous transgenic T2 lines exhibited up to a 50% increase in primary root elongation, 27%–39% increase in fresh root biomass and as a consequence root-shoot ratio increased by up to 40% in transgenic lines while the shoot phenotypic parameters (shoot height, number of leaves and time of flowering) were identical to wild type plants. Transgenic lines also exhibited a higher survival rate under drought stress and increased biomineral uptake capacity as indicated by increased accumulation of Zinc (57%), Sulphur (43%), Manganese (33%) and, Phosphorus (46%) in leaves of transgenic lines compared to wild type plants (Werner et al., 2010).

1.4.2 Monocot crop plants

Barley transgenic lines developed through the root-specific expression of *AtCKX1* under mild root-specific promoter *bGLU* derived from maize exhibited enhanced recovery rate and increased shoot biomass under mild and severe drought conditions (Pospíšilová et al., 2016). T_3 transgenic homozygous lines developed by root-specific expression of *AtCKX1* and *AtCKX2* in barley utilizing root-specific *pEPP* promoter system developed larger root systems characterized by a 24%–70% increase in root length, 12%–50% increase in root surface area, 47% increase in root biomass and 16%–50% increase in root-shoot biomass ratio while shoot growth and yield parameters were equivalent to wild type plants. Transgenic lines showed enhanced accumulation of several macro and micronutrients in leaves (13%–53% increase in phosphorus, 17%–45% increase in sulfur, 20%–28% increase in copper, 51%–70% increase in manganese and, 7%–54% increase in zinc) and seeds (increase in calcium, copper and 26%–49% increase in zinc). Enhanced root growth also facilitated improved tolerance to drought stress in transgenic lines indicating 25%–29% stomatal conductance (SC), 30%–32% relative water content (RWC), and 33%–45% CO_2 assimilation rate as compared to 11% SC, 18% RWC and 13% CO_2 assimilation rate in control lines. The endogenous ABA and Proline levels increased by 11 and 620 fold in wild-type plants compared to a corresponding increase of 4–5 folds and 20%–50 fold in transgenic lines (Ramireddy et al., 2018b). ABA is a master regulator of a plant's defense response against several abiotic stresses (Vishwakarma et al., 2017). Similarly, amino acid proline accumulates under a variety of abiotic stresses such as drought and oxidative stress conditions. Proline works as an excellent osmoprotectant, compatible solute, ROS scavenger and stabilizes membrane integrity and protein structures (Hayat et al., 2012). ABA and proline accumulation are the markers for drought stress. Relatively decreased accumulation of ABA and proline in transgenic lines under drought stress compared to control plants indicate that transgenic lines experience weaker stress levels as detrimental effects of increased ABA content on photosynthesis and growth are minimized (Ramireddy et al., 2018c). Field grown transgenic barley lines validated the data obtained under controlled conditions as they showed an 8%–30% increase in zinc concentration, 16%–32% increase in iron, and 22%–36% increase in manganese concentration compared to wild type plants grown under the same conditions (Ramireddy et al., 2018a). In rice, a dominant gain of function mutant *ren1-D* was identified through enhancer mediated activation tagging

having an enlarged root system marked by an increased number of crown roots, increased root length, and increased root dry weight however shoot height was reduced as compared to control plants. Eventually, based on functional genetic analysis the mutant phenotype was attributed to enhanced expression of *OsCKX4* in mutant lines. Root-specific expression of *OsCKX4* under root-specific *RCc3* promoter led to enhanced root growth without any adverse effect on shoot phenotype eventually increasing the root-shoot biomass ratio of transgenic lines (Gao et al., 2014). Recent evidence indicates that crown root growth and development is negatively regulated by the *OsNAC2* transcription factor in rice. *OsNAC2* overexpression lines were marked by increased expression of *OsCKX4* and *OsCKX5* genes and decreased expression of *OsIPT3, OsIPT5,* and *OsLOG3* genes compared to control lines, effectively decreasing the number of crown roots and root length in *OsNAC2* overexpression lines. Moreover, OsNAC2 was shown to directly interact with the *OsCKX4* promoter along with several auxin-related genes establishing itself as a central integrator of auxin-cytokinin crosstalk involved in rice root development (Mao et al., 2020). In Rice, exogenous cytokinin application decreased the Zn-uptake under normal and Zn-limited conditions through downregulation of *OsZIP1* and *OsZIP5* which encode the metal transporters involved in Zn-uptake. Transgenic lines expressing *OsCKX4* under root-specific *RCc3* promoter accumulated 45%–67% more Zn in roots and 60%–68% more Zn in shoots as compared to wild-type control lines under both controlled and field conditions. Moreover, a 57%–61% increase in Zn concentration was reported in brown rice derived from transgenic lines along with an overall 10.9%–11.2% increase in crop yield per plot indicating the presence of cytokinin dependent regulatory module for Zn uptake in rice (Gao et al., 2019).

1.4.3 Dicot crop plants

Transgenic *Brassica rapa* seedlings generated by constitutive overexpression of *AtCKX2* under *CaMV35S* promoter exhibited a 25%–35% increase in primary root length, 70%–100% increase in lateral root density, 40%–50% increase in adventitious roots as compared to wild type lines under *in-vitro* growth conditions as well as up to 50% increase in dry root biomass when grown in soil or hydroponic culture. Moreover, there was no evident growth penalty on shoot development except for the partial release of apical dominance as lateral buds in transgenic lines formed up to 2 small leaves at maturity. Leaves of transgenic plants accumulated 13%–16% more Phosphorus, 41%–56% more calcium, 42%–75% more sulfur, 29%–32% more magnesium, 26%–32% more zinc, 28%–29% more copper, and 15%–20% more manganese while 11%–18% reduction in iron concentration was reported as compared to non-transgenic lines (Nehnevajova et al., 2019). Transgenic chickpea lines generated through the root-specific expression of *CaCKX6* under root-specific *CaWRKY31* promoter formed a larger root system marked by a 1.8-fold increase in lateral root numbers, 1.5–1.85 fold increase in root length, 1.5–2 fold increase in root biomass and 1.7 fold increase in root-shoot biomass ratio. Moreover, transgenic lines showed up to a 20% increase in shoot biomass and a 15%–25% increase in the number of seeds formed per plant. There was no difference in root nodule formation frequency between wild-type and transgenic lines. Owing to higher mineral uptake capacity imparted by larger root systems, transgenic lines accumulated higher amounts of minerals in their seeds as indicated by accumulation of 27–62% more zinc, 26–61% more copper, 22–48% more iron, 13–22% more magnesium, 11–27% more potassium and 5–19% more phosphorus. Transgenic lines also showed improved tolerance to long-term drought stress marked by relatively less decrease in relative water content (RWC), stomatal conductance, transpiration rate, and CO_2 assimilation rate as compared to control line subjected to similar drought conditions (Khandal et al., 2020).

1.5 Cytokinin oxidase/dehydrogenase inhibitors—A potential alternative to genetic manipulation of *CKX* genes

Besides molecular biology-based cytokinin oxidase inhibition through genetic manipulation of *CKX* genes, exogenous application of chemical-based cytokinin oxidase inhibitors can also be used to suppress CKX enzyme activity and increase endogenous cytokinin levels (Arora and Sen, 2022) Thidiazuron (*TDZ*), diphenylurea and its derivatives like CPPU, DCPPU were the first cytokinin oxidase inhibitors. However, these exhibit cytokinin activity as well which is usually remarkably higher than CKX inhibitory activities (Kopečný et al., 2010). HETDZ and 3FMTDZ derived from TDZ exhibited significantly lesser intrinsic cytokinin activity and 15-fold lower IC_{50} values as compared to TDZ for AtCKX2, ZmCKX1, and ZmCKX4a indicating higher CKX inhibiting potential of HETDZ and 3FMTDZ (Nisler et al., 2016). Recently developed CKX inhibitors derived from diphenylurea, CPPU and DCPPU did not exhibit any intrinsic cytokinin activity and one of these novel CKX inhibitors named 1-[2-(2-hydroxyethyl) phenyl]-3-[3-(trifluoromethoxy)phenyl] urea increased seed yield and stress tolerance in *Arabidopsis* as well as increased grain yields in field-grown rapeseed, wheat, and barley crops (Gupta et al., 2021; Nisler et al., 2021). CKX inhibitors have a wide range of applications in horticulture, agriculture, and plant biotechnology as they improve grain yield, enhance abiotic stress tolerance, and improve organogenesis and regeneration in plant tissue culture (Gupta et al., 2021). Chemical CKX inhibitors are attractive potential alternatives for CKX inhibition strategies based on genetic engineering approaches.

2 Conclusion and future perspectives

Optimum root-shoot ratio, improved root system architecture, and increased seed yield are desirable traits for crop plants for efficient uptake and utilization of limited soil and water resources but their incorporation into crop plants is rather complicated, laborious, and inefficient because these traits are generally regulated by multiple genes. Genetic manipulation of a single or couple of dominant master regulator genes such as *CKX* genes provides us with an unmatched opportunity to alter these multigenic traits for the development of improved crop cultivars having increased yield and enhanced resilience to adverse environmental fluctuations. Although theoretically feasible, genetic manipulation of *CKX* genes poses several practical challenges because of the activation of a strong homeostatic regulatory mechanism. Activation of homeostatic regulatory mechanisms might prevent the delivery of anticipated results or even necessitate the simultaneous knockdown or knockout of more than one *CKX* gene to get the desirable phenotype as observed in dicot crop plants. Endogenous cytokinin level alteration through genetic manipulation of *CKX* genes is analogous to dealing with a "double-edged" sword since cytokinins regulate root and shoot growth in an opposite and antagonistic manner. Thus, careful selection of target *CKX* genes and choice of tissue-specific transgene expression system is crucial to avoid undesirable pleiotropic effects. *CKX* genes exist as small multigene families in flowering plants, different members of which are more often than not diverged among different species and thus different *CKX* genes play different roles in different species. Genome-wide identification and functional characterization of the *CKX* gene family is an essential prerequisite for the identification of suitable target *CKX* genes in a species-specific manner. Genetic manipulation of *CKX* genes specifically gene knockout and knockdown is a tricky task in polyploid crops as it requires simultaneous knockout or knockdown of all the homologs of a specific *CKX* gene in all the sub-genomes of a polyploid crop. This tremendous feat has been achieved through a set of specific techniques such as random mutagenesis, TILLING, and crossing in allotetraploid oilseed rape. CRISPR/Cas9 mediated gene editing is a versatile method of mutating genes as it can induce mutations at multiple genomic sites simultaneously. Designing the sgRNA from a highly conserved region that targets all the gene copies simultaneously is the most feasible strategy to achieve the knockout of multiple homologs of specific *CKX* genes present in the complex polyploid genomes. Although CRISPR/Cas9 mediated gene editing has been optimized to edit genes in several polyploid crops (as reviewed in Schaart et al., 2021), there are no reports of CRISPR/Cas9 mediated knockout of *CKX* family genes in any polyploid crop species to date. Understanding the precise molecular mechanism behind enhanced micronutrient uptake capacity of *CKX* overexpression lines would help design strategies for the development of bio-fortified crop cultivars enriched in specific nutrients. To date, genetic manipulation of *CKX* genes has been utilized independently for yield enhancement and root growth improvement. Thus, an interesting, future endeavor would be simultaneous upregulation (overexpression) of *CKX* in roots and downregulation (knockdown or knockout) in shoots or grains to develop high yielding, bio-fortified, and stress-tolerant crop cultivars. Another engrossing prospect open for exploration in near future is the simultaneous enhancement of sink strength and source capacity through downregulation of one or more *CKX* genes in shoots or grains and expression of cytokinin biosynthetic *IPT* genes in leaves which might ameliorate the negative effects of complete gene knockdown such as low seed setting rate, high seed mortality and lower TGW. In conclusion, the role of *CKX* genes in determining yield, nutrient uptake, and root-shoot architecture is evolutionary conserved and is of functional importance to most flowering plants thus careful genetic manipulation of specific target *CKX* genes might be the key to developing improved crop cultivars having high yield, improved nutrient status and enhanced tolerance to abiotic stress conditions.

Author contributions

AS: Primary draft, conceptualization, figures and revision. SP: Primary draft, tables and revision. DC: Concept, coordination, revision and supervision.

Funding

The project is funded by a core grant from the National Institute of Plant Genome Research, India.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Acknowledgments

AS acknowledges INSPIRE fellowship (DST/INSPIRE/03/2021/000430) from the Department of Science &

Technology (DST), Government of India. SP acknowledges fellowship from Council of Scientific and Industrial Research (CSIR), Government of India. DC acknowledges the J. C Bose fellowship (JCB/2020/000014) from the Science and Engineering Board (SERB)—Department of Science & Technology (DST), Government of India. DC is grateful to the DBT-eLibrary Consortium (DeLCON) for providing access to e-Resources.

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frontiers | Frontiers in Genetics

TYPE Review
PUBLISHED 09 August 2022
DOI 10.3389/fgene.2022.943025

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

RECEIVED 13 May 2022
ACCEPTED 30 June 2022
PUBLISHED 09 August 2022

CITATION
Mandal S, Ghorai M, Anand U, Samanta D, Kant N, Mishra T, Rahman MH, Jha NK, Jha SK, Lal MK, Tiwari RK, Kumar M, Radha, Prasanth DA, Mane AB, Gopalakrishnan AV, Biswas P, Proćków J and Dey A (2022), Cytokinin and abiotic stress tolerance -What has been accomplished and the way forward? *Front. Genet.* 13:943025. doi: 10.3389/fgene.2022.943025

Cytokinin and abiotic stress tolerance -What has been accomplished and the way forward?

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More than a half-century has passed since it was discovered that phytohormone cytokinin (CK) is essential to drive cytokinesis and proliferation in plant tissue culture. Thereafter, cytokinin has emerged as the primary regulator of the plant cell cycle and numerous developmental processes. Lately, a growing body of evidence suggests that cytokinin has a role in mitigating both abiotic and biotic stress. Cytokinin is essential to defend plants against excessive light exposure and a unique kind of abiotic stress generated by an altered photoperiod. Secondly, cytokinin also exhibits multi-stress resilience under changing environments. Furthermore, cytokinin homeostasis is also affected by several forms of stress. Therefore, the diverse roles of cytokinin in reaction to stress, as well as its interactions with other hormones, are discussed in detail. When it comes to agriculture, understanding the functioning processes of cytokinins under changing environmental conditions can assist in utilizing the

phytohormone, to increase productivity. Through this review, we briefly describe the biological role of cytokinin in enhancing the performance of plants growth under abiotic challenges as well as the probable mechanisms underpinning cytokinin-induced stress tolerance. In addition, the article lays forth a strategy for using biotechnological tools to modify genes in the cytokinin pathway to engineer abiotic stress tolerance in plants. The information presented here will assist in better understanding the function of cytokinin in plants and their effective investigation in the cropping system.

KEYWORDS

cytokinin (CK), CK metabolic genes, CK signaling genes, abiotic stress, crop resilience, genome editing

Challenging environmental factors and cytokinin

Climate change and rapid population growth pose enormous hurdles to achieving food security, which remains a primary concern for all stakeholders and governments (Muluneh, 2021; Dasgupta and Robinson, 2022). Feeding the rapidly expanding global population, which is anticipated to exceed 10 billion people by 2050 and needs 49% additional food, is a major challenge (Ahmar et al., 2020). There are already 820 million people in the world who are chronically undernourished, and this figure is expected to rise sharply in the future years, further compromising global food security (FAOSTAT, 2020). In addition, the hidden hunger is considerably worse today than it was a decade ago in Africa, Western Asia, and other developing nations (FAO et al., 2018). Furthermore, food production is being hampered by unusual weather circumstances connected to environmental degradation and rising land competition as a result of urbanization (Lobell and Gourdji, 2012; Lenaerts et al., 2019). Climate change is expected to raise the earth's temperature, resulting in global warming, irregular rain patterns, and the intensification of various abiotic and biotic pressures, all of which significantly reduce agricultural yields (Raza et al., 2019). In the future, changing climate is expected to become more prevalent and aggravate different stress, posing major concerns to agricultural yield (Ray et al., 2013). In order to ensure sustainable agricultural production in the face of changing climatic and growing population, yearly crop yields must be increased (Tilman et al., 2011; Ray et al., 2013). Therefore, to address these multi-dimensional challenges, agricultural production systems must undergo a significant transition (Lenaerts et al., 2019). Sustainable agricultural production may be aided by the use of sustainable resources to boost crop yield per unit area and the effective usage of fertilizer and water. In order to alleviate the hidden and chronic hunger, economic development is essential, but it may not be sufficient to eradicate hunger (Lenaerts and Demont 2021). For thousands of years, plant breeding has been one of the most important strategies to fulfil people's food needs *via* crop domestication (Ahmar et al., 2020).

Cytokinins are family of adenine-derived phytohormones characterised by the presence of an aromatic chain or isoprenoid at the N6 position of their adenine moiety (Mok and Mok, 2001). Cytokinins are typically defined as growth-promoting hormones, despite the fact that diverse substance with cytokinin action have been found to regulate wide range of developmental and physiological processes in plants. In the 1950s, Miller and Skoog identified the first cytokinin, kinetin, which was classified as a plant-derived molecule that accelerated cell division (Miller et al., 1956). A further investigation found that kinetin, in conjunction with auxin, was important for stimulating organ development and cell division in undifferentiated cells culture (Skoog and Miller, 1957). Despite the fact that the cytokinin study began in the mid-1900s, it is an ancient hormone, having emerged as one of the earliest hormones in photosynthetically competent organisms (Wang et al., 2015). According to evolutionary research, the genetic sequences that are orthologs to known components of the cytokinin signaling pathway may be found in the common ancestor of all land plants, charophytes (Wang et al., 2015). These findings indicate that cytokinin had a function in plants as far back as 450 million years. In addition, cytokinins are recognized for their role in plant growth, development, senescence delay, and modulation of biotic and abiotic stress tolerance (Kieber and Schaller, 2018; Cortleven et al., 2019).

Changes in chemoattractant, temperature, nutrient content, and osmotic conditions all activate cytokinin signaling cascades, which are evolutionarily connected to the two-component systems in unicellular organisms that engage in signal transduction (Hwang et al., 2002; Wolanin et al., 2002). Cytokinins perform critical and multifaceted functions in plant development and abiotic stress responses (Ha et al., 2012; Hwang et al., 2012; Zwack and Rashotte, 2015). Several research have shown that cytokinins have both positive and negative impacts on stress tolerance or resistance (Ghanem et al., 2008; Nishiyama et al., 2011). Plants, on the other hand, may experience both a short-term and long-term rise in cytokinin levels when they are exposed to extreme stress conditions (Alvarez et al., 2008; Dobra et al., 2010). For instance, cytokinin synthesis *IPT* genes (adenosine phosphate isopentenyl transferases) are up-regulated upon salt (NaCl) treatment, and a mutation in cytokinin biosynthesis leads to a robust salt-tolerant phenotype (Nishiyama et al., 2011). The impact of exogenous cytokinin administration on abiotic stress tolerance have been the subject of several investigations. *Triticum*

aestivum (wheat) seedlings that receive exogenous cytokinins application are more tolerant to salt stress, whereas a similar treatment on *Phaseolus vulgaris* (beans) result in more susceptible phenotype to the salt stress (Kirkham et al., 1974; Abdullah and Ahmad, 1990). Furthermore, Arabidopsis plants displayed enhanced ability to survivie freezing or dehydration after being treated with endogenous cytokinin (Jones et al., 2010; Kang et al., 2012).

Transgenic plants that overexpress cytokinin biosynthesis genes (*IPTs*) or cytokinin degradation genes (*CKXs*) demonstrate the impact of altered endogenous cytokinin levels. More importantly, the overproduction of endogenous cytokinin enhances drought stress tolerance in many plants. However, reduced cytokinin levels, on the other hand, have a positive impact on drought tolerance (Werner et al., 2010; Qin et al., 2011; Macková et al., 2013; Li et al., 2021). Additionally, the cytokinin signaling components play a significant role in abiotic stress tolerance. For example, Arabidopsis *AHK1*, a histidine kinase 1 involved in cytokinin signaling, acts as a positive regulator of salt and drought stress responses. Furthermore, the loss-of-function mutant phenotype in *ahk2*, *ahk3*, and *ahk2 ahk3* in Arabidopsis is associated with increased tolerance to salt and drought stress (Wohlbach et al., 2008; Kumar et al., 2013). Drought stress responses are negatively and redundantly regulated by AHPs (histidine phosphotransfer proteins) (Hwang et al., 2012; Nishiyama et al., 2013). However, in salt stress resistance phenotype in Arabidopsis was discovered while researching the quadruple loss-of-function mutant *arr3arr4arr5arr6* (Mason et al., 2010). These early finding indicated that cytokinin metabolism and signaling genes play an important role in responding to diverse environmental stress conditions.

Cytokinin, on the other hand, cannot reduce abiotic stress on its own; instead, it functions in conjunction with other signaling pathways (Antoniadi et al., 2020; Li et al., 2021). There is a wealth of information available on the function of cytokinin and its interactions with other phytohormones when plants are exposed to abiotic stress. So, this review exemplifies the regulatory role of cytokinin in abiotic stress tolerance and activation of possible novel crosstalk with other key stress phytohormones. In this review, we provide an inclusive overview of the advancement of genetic approaches in dissecting the function of cytokinin signaling components in regulating stress tolerance in plants under challenging environments stress, followed by brief insights into future approaches.

Plant mechanisms for sensing and response to abiotic stress

At the cellular level, several abiotic stressors such as drought, heat, cold, and salt may produce common cell disruptions and secondary stress such as membrane damage, reactive oxygen species (ROS) production and damage, protein denaturation, and osmotic stress (Figure 1). In general, the initial step in the stress response is perception, which is followed by the transmission of information through secondary messengers to regulators and, eventually, to effectors, which are responsible for the protective function. A sensor is a biological molecule that may detect an unfavourable change in its surrounding environment and immediately elicit a reaction by triggering the production of signal molecules inside the plant system. In general, receptors or membrane-associated proteins pick up on stresses, which causes an ionic imbalance across the membrane. Stresses caused by drought, heat, cold, and salt all induce an increase in the quantity of Ca^{2+} (signal molecules) that enters the cytoplasm of the cell from either its own reserves or an apoplastic source. One form of sensor for the stress signals is thought to be the passages that govern Ca^{2+} entrance (Aftab et al., 2021; Javaid et al., 2022; Paes de Melo et al., 2022). Other than Ca^{2+}, ROS and nitric oxide (NO) are other messenger molecules involved in inducing plant response to cold stress. Plants generate ROS such as superoxide (O_2), hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) in order to defend themselves against the diverse stress that they are exposed (He et al., 2018). In receptor-like kinases (RLKs), there is an extracellular domain, a transmembrane domain, and an intracellular kinase domain. The extracellular domain is where ligands bind, and the transmembrane domain is where protein-protein interactions take place (Ku et al., 2018). The histidine residue in the intracellular kinase domain is auto-phosphorylated when the ligand or signal binds to the extracellular domain, and the phosphoryl moiety is received by the aspartate receiver section of the sensor protein or a different protein (Yadav et al., 2021). After then, the activated sensor protein (or proteins) may either directly phosphorylate particular targets or trigger cellular responses that are unique to the signal that was received via the mitogen-activated protein kinase (MAPK) cascade. Protein phosphorylation and dephosphorylation, which are both forms of the intracellular signaling mode, govern a broad variety of cellular functions, including the activation of enzymes, assembly of macromolecules, localization of proteins, and their breakdown (Yadav et al., 2021). Plants are able to detect when they are being subjected to abiotic stress, which triggers a series of signaling cascades that activate ion channels, kinase cascades, the formation of ROS, and the accumulation of plant hormones, which ultimately leads to the induction of the expression of specific subsets of genes that are responsible combating the abiotic stress (Ku et al., 2018; Zandalinas et al., 2020). If the plant's stress-coping systems are ineffective in reducing the negative consequences of stress, mostly due to ROS accumulation, the cells activate environmental-triggered cell death processes, which include the plant's senescence (Zandalinas et al., 2020).

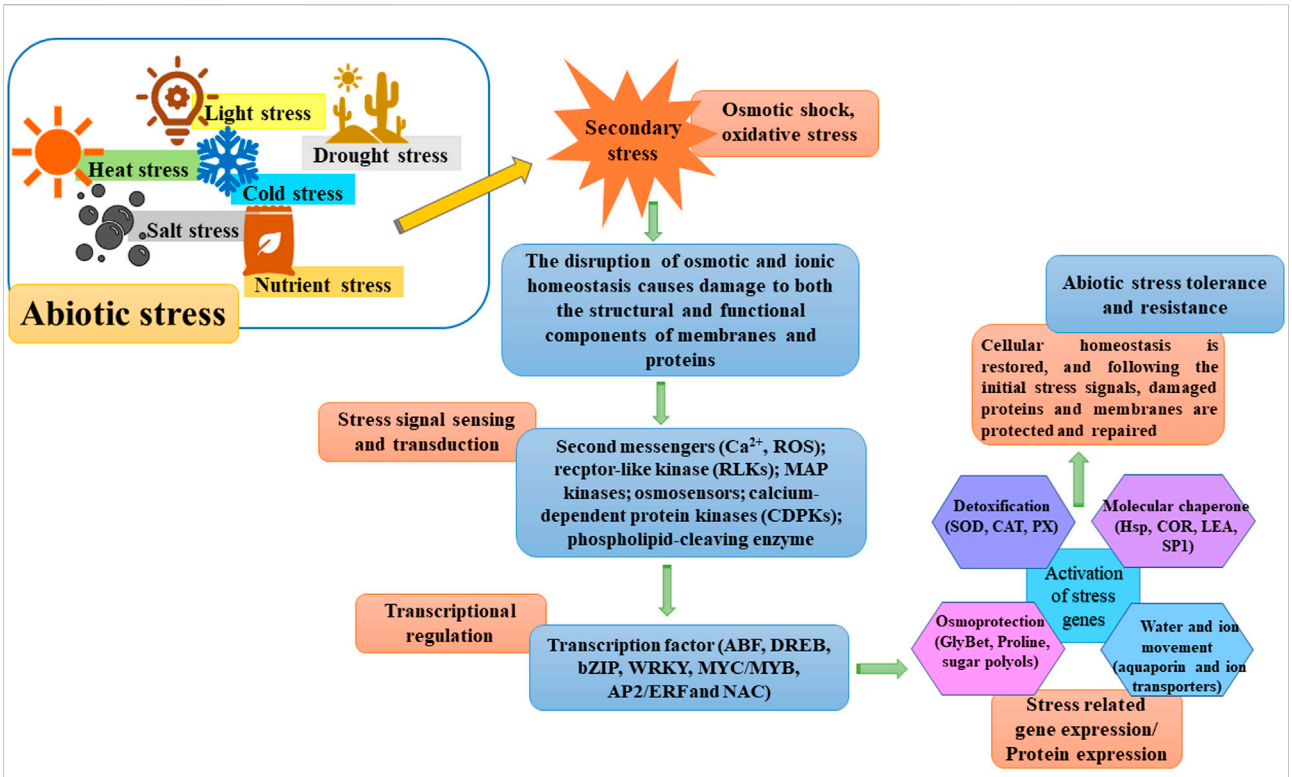

FIGURE 1
A simplified schematic representation of mechanism through which plant responses to diverse abiotic stress. The general signaling pathways in plants that are activated in response to abiotic stress, beginning with signal perception and leading to the stress responses. Cold, drought, light, nutrient and salinity frequently induce cellular damage and secondary osmotic and oxidative stress. Following upstream signaling processes and transcriptional controls activate stress-responsive mechanisms in order to restore cellular homeostasis and protect and repair damaged proteins and membranes after the initial stress signals (e.g., changes in temperature or osmotic and ionic effects or membrane fluidity). ABF, ABA responsive element (ABRE) binding factor; AP2/ERF, Apetala2/Ethylene Responsive Factor; bZIP, basic leucine zipper transcription factor; CAT, catalase; CBF/DREB, C-repeat-binding factor/dehydration-responsive-binding protein; CDPK, calcium-dependent protein kinase; COR, cold-responsive protein; HK1, histidine kinase-1; Hsp, heat-shock protein; ICE1, inducer of CBF expression 1; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; and SP1, stable protein 1.

Cytokinin in plant development and stress adaptation

Cytokinins are a family of plant hormones that are fundamental to a variety of growth and development processes (Pavlů et al., 2018). Cytokinins have been widely investigated for their metabolism, signal transduction pathway, chemical composition, and their role in plant growth and development, since their discovery in *Zea mays* (maize) seeds over 50 years ago (Figure 2). Inhibition of lateral root initiation, regulation of cell division, differentiation of metaxylem and phloem in roots, photomorphogenic cell differentiation in shoots and expanding leaves and inhibition or delaying of leaf senescence are all-important regulatory activity of cytokinins at the tissue and organ levels (Bielach et al., 2012; Chiang et al., 2012; Efroni et al., 2013; Zwack et al., 2015).

Adenine derivatives with aromatic side chains or isoprenoid make up the endogenous cytokinin. Isoprenoid cytokinin, which is abundant in nature, may be classified as trans-zeatin (tZ)-, isopentenyladenine (iP)-, cis-zeatin (cZ)-, or dihydrozeatin (DHZ)-type derivatives based on the side chain hydroxylation or reduction. In comparison, aromatic cytokinins, such as N6-(meta-hydroxybenzyl) adenine (BA), are less abundant in plants (Faiss et al., 1997). The isoprenoid cytokinins vary from one another in terms of their biological roles, metabolic conversions, biochemical characteristics, and transportability throughout the plant system (Pavlů et al., 2018). Cytokinin homeostasis is maintained by a number of enzymes engaged in cytokinin metabolism, including those involved in cytokinin production, inter-conversion between cytokinin types, and cytokinin degradation (Thu et al., 2017; Skalak et al., 2021). The role of a wide variety of genes and enzymes, along with the composed metabolic network controlled by cytokinins across the plant kingdom, has been thoroughly investigated (Zwack et al., 2015; Pavlů et al., 2018). The phospho-relay cascades of the two-component system (TCS) are established and lead to the expression regulation of specific genes involved in plant adaptation when the cytokinin signaling pathway is triggered by

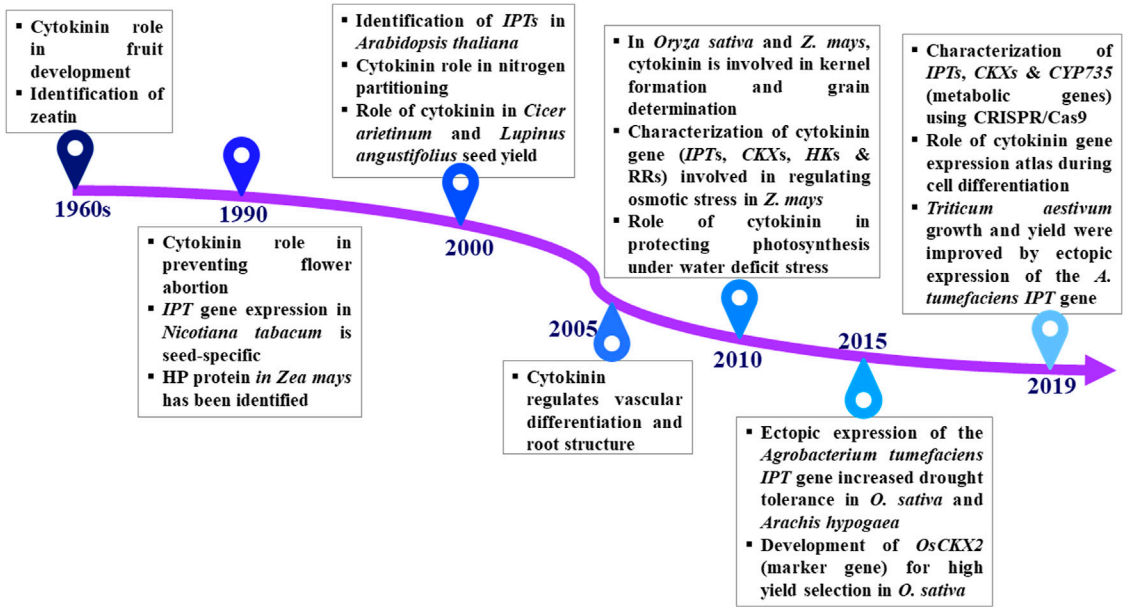

FIGURE 2
Cytokinin timeline: from discovery to crop plant application. A chronology of significant advancements in cytokinin research. Abbreviation: CKXs, CK oxidases/dehydrogenases; RRs, cytokinin response regulators; IPTs, isopentenyl transferases; HK, histidine kinas.

various environmental stimuli, like nutrition levels, changes in temperature, and osmotic conditions (Thu et al., 2017; Pavlů et al., 2018). Most recently, it was shown that cytokinin interacts with jasmonates (JAs), ethylene (ET), salicylic acid (SA), and abscisic acid (ABA), showing the presence of an interconnected coordinating network among the phytohormones involved in plant stress tolerance (Efroni et al., 2013; Thu et al., 2017; Artner and Benkova, 2019; Antoniadi et al., 2020; Skalak et al., 2021). Additionally, it is well established that cytokinin biosynthesis and signalling components operate as constitutive signals defining the plant response to drought stress and controlling drought acclimatization. Because of their spatiotemporal expression, rapid responses, and widely associated pathways, cytokinins are an ideal candidate for regulating complicated morphogenetic processes under water stress. We will highlight the importance of plant cytokinins and their regulation under abiotic stress in this review and will offer an approach to understanding the function and regulation of cytokinins in plants.

Role of cytokinin in plant response and regulation to abiotic stress

Multiple aspects of plant growth and development are regulated by the phytohormone cytokinin. A significant number of mutants have been created in the cytokinin signaling pathway, biosynthesis, and breakdown processes, which has resulted in the rapid advancement in the field of cytokinin (Li et al., 2021; Prasad, 2022). According to research, cytokinins are important signaling molecules that trigger a range of plant stress responses. Moreover, abiotic stress has a direct effect on cytokinin transport, responses, and concentrations. Table 1 summarizes the genes from cytokinin pathways investigated so far that react to various abiotic stresses, in addition to their involvement in stress tolerance. We focused on nutrient, light, heat, drought, cold, and salt stress in this review to highlight the function of cytokinin in abiotic stress response and it is potential to increase abiotic stress tolerance (Figure 3).

Nutrient deficiency stress

Stress caused by nutrient deficiency in the soil triggers a number of reactions, all of which include cytokinin at varying levels (Pavlů et al., 2018). Root system architecture (RSA) is modified by the nutrients availability, and cytokinin is one of the important components that regulate RSA in response to availability of nutritional signals (Kroevoets et al., 2016). Nutritional signals affect the transcript levels of cytokinin metabolism and signaling genes, which are both implicated in regulating RSA (Bielach et al., 2012; Ramireddy et al., 2014; Chang et al., 2015; Pavlů et al., 2018). Cytokinin modulates the

TABLE 1 A summary of genetic research aimed at elucidating the function of cytokinin in the response to abiotic stress.

S. No	Plant species	Target genes	Expression under stress conditions	Genetic approach	Significant outcome	Reference
Cytokinin biosynthesis						
1	*Solanum lycopersicum*	*SlIPT3*	Strongly repressed in roots under salt stress	*35S:SlIPT3*	Improved tolerance to salinity	Žižková et al. (2015)
2	*Arabidopsis thaliana*	*IPT3*		*ipt3*		Žižková et al. (2015)
3	*S. lycopersicum*	*SlIPT4*	Strongly repressed in roots under salt stress			Žižková et al. (2015)
4	*A. thaliana*	*IPT8*		*ER:IPT8*, estradiol-inducible	Reduce plant tolerance under salt and osmotic stress	Wang et al. (2015)
5	*A. thaliana*	*IPT1*; *IPT3 IPT5*; *IPT7*		*ipt1 ipt3 ipt5 ipt7*	Increased resistance to salt stress, drought stress	Nishiyama et al. (2011)
6	*A. thaliana*	*CKX1*		*bGLU:CKX1 in barley*	Increased resistance to drought stress	Pospíšilová et al. (2016)
7	*O. sativa*	*OsCKX2*		*OsCKX2-RNAi*	Increased resistance to salinity stress	Joshi et al. (2017)
8	*O. sativa*	*OsLOG*	Downregulated by cold, drought and salt stress			Tripathi et al. (2012)
Cytokinin homeostasis						
9	*A. thaliana*	*UGT76C2*	Downregulated by osmotic stress and drought stresses	*35S:UGT76C2*	Tolerant to drought stress as adult plants	Li et al. (2015)
10	*A. thaliana*	*UGT76C2*		*ugt76c2*	More sensitive to drought stress	Li et al. (2015)
Cytokinin signaling						
11	*A. thaliana*	*AHK1*	Induced by dehydration	AHK1 overexpressor	Tolerant to drought stress	Liu et al. (2008)
12	*A. thaliana*	*AHK2*	Downregulated by salt			Buer et al. (2004)
13	*A. thaliana*	*AHK2*	Induced by dehydration	*ahk2*	Increased survival to drought after rewatering, increased survival upon salt stress	Liu et al., 2008; Argyros et al., 2008
14	*A. thaliana*	*AHK2*		*ahk2-2*	Hypersensitive to salt stress in terms of root growth and fresh weight	Zürcher et al. (2016)
15	*A. thaliana*	*AHK3*	Induced by hydration, high salinity and cold stress (3-week-old plants)	*ahk3*	Drought and salinity tolerant	Liu et al. (2008)
16	*A. thaliana*	*AHK3*	Not responsive to cold (11-day-old seedlings)	*ahk3*	Enhanced drought tolerance	Argyros et al., 2008; Tran et al., 2007
17	*A. thaliana*	*AHK3*		*ahk3-3*	Increased root elongation after transfer to low water potential media	Zürcher et al. (2016)
18	*A. thaliana*	*AHK2 AHK3*		*ahk2 ahk3*	More tolerant to drought and salt than single	Liu et al. (2008)
19	*A. thaliana*	*AHK2 AHK3*		*ahk2 ahk3*	Enhanced cold tolerance	Argyros et al. (2008)
		AHK3 AHK4		*ahk3 ahk4*		
20	*A. thaliana*	*AHK4*	Induced by dehydration			Liu et al. (2008)
21	*O. sativa*	*OsAHP1*		OsAHP-RNAi	Hypersensitive to salt treatment but resistant to osmotic stress	Jeon et al. (2013)
22	*A. thaliana*	*AHP2*	Downregulated by dehydration	*ahp2 ahp3 ahp5*	Strong drought-tolerant phenotype	Jeon et al. (2010)
23	*O. sativa*	*OsAHP2*		OsAHP-RNAi	Hypersensitive to salt treatment but resistant to osmotic stress	Jeon et al. (2013)
24	*A. thaliana*	*AHP3*	Downregulated by dehydration	*ahp2 ahp3 ahp5*	Strong drought-tolerant phenotype	Jeon et al. (2010)

(Continued on following page)

TABLE 1 (*Continued*) A summary of genetic research aimed at elucidating the function of cytokinin in the response to abiotic stress.

S. No	Plant species	Target genes	Expression under stress conditions	Genetic approach	Significant outcome	Reference
25	*A. thaliana*	*AHP5*	Downregulated by dehydration	*ahp2 ahp3 ahp5*	Strong drought-tolerant phenotype	Jeon et al. (2013)
	A. thaliana			*ahp2 ahp3 ahp5*	Reduced type A *ARR* expression in response to cold	Sakai et al. (1998)
26	*A. thaliana*	*ARR1*		*35S:ARR1*	Hypersensitive cold response of type A *ARR*s as well as enhanced cold tolerance	Sakai et al. (1998)
27	*A. thaliana*	*ARR5*	Induced by cold, salinity and dehydration			Ha et al., 2013; Sakai et al., 2000
		ARR6				
28	*A. thaliana*	*ARR7*	Induced by cold, salinity and dehydration	*35S:ARR7*	Hypersensitive response to cold temperatures	Ha et al., 2013, Argyros et al., 2008
29	*A. thaliana*	*ARR9*	Weak and early induction by cold, downregulated by heat			Skalák et al., 2016; Ha et al., 2013
		ARR10				
30	*A. thaliana*	*ARR12*	Downregulated by heat stress	*arr1 arr12*	Less sensitive to salt stress	Skalák et al., 2016; Kang et al., 2013
31	*A. thaliana*	*ARR15*	Induced by cold, salinity and dehydration			Ha et al., 2013; Sakai et al., 2000
32	*A. thaliana*	*ARR22*	Weak and late induction by cold, induced by drought			Ha et al., 2013; Sakai et al., 2000
Cytokinin response						
33	*S. lycopersicum*	*SlCRF1*	Induced by cold in leaves and roots, repressed by heat in roots			Brenner et al. (2012)
34	*S. lycopersicum*	*SlCRF1*	Slightly reduced in leaves and strongly decreased in roots by drought			Brenner et al. (2012)
35	*S. lycopersicum*	*SlCRF2*	Induced by H_2O_2 treatment only in roots			Brenner et al. (2012)
36	*A. thaliana*	*CRF4*	Strongly induced in both root and shoot tissues by cold	*35S:CRF4*	Tolerant to cold treatment	Kang et al. (2012)
37	*A. thaliana*	*CRF4*		*crf4*	Sensitive to cold treatment	Kang et al. (2012)
38	*A. thaliana*	*CRF6*	Induced by heat shock, oxidative (H_2O_2) and salt stress			Reguera et al., 2013

expression of various transporter genes as well as the development of passage cells, which affects the plant's capacity to absorb nutrients (Werner et al., 2010; Andersen et al., 2018). As a result, cytokinin governs physiological and morphological adaptive responses to nutritional stress to survive. Furthermore, a study of the phosphate deprivation response in *Arabidopsis thaliana* mutants found that cytokinin signaling is essential for a significant response to decrease phosphate availability. This was the first study to demonstrate a specialized function for cytokinin signalling in nutritional sensing. (Franco-Zorrilla et al., 2002; Franco-Zorrilla, Martín et al., 2005). Sulfate transporter genes are suppressed by cytokinin, which is a negative regulator of sulphur acquisition (Maruyama-Nakashita et al., 2004). Cytokinin also inhibits genes involved in iron absorption and iron homeostasis (Séguéla et al., 2008). Cytokinin controls sodium (Na) build up through the sodium transporter gene *HKT1;1* as previously stated (Mason et al., 2010). Low potassium levels or an artificially reduced cytokinin status resulted in upregulation of the high-affinity K^+ transporter gene *HAK5*, promotes root hair development, and accelerates ROS accumulation, indicating that cytokinin plays a role in responding to low potassium availability (Nam et al., 2012). However, another micronutrient, boron (B), was reported in *Brassica napus* seedlings to be linked to enhanced cytokinin content, which was thought to be a prerequisite for a different growth response (Eggert and von Wirén, 2017). Cytokinin is a key regulator in plant arsenic (As) stress adaptation (Mohan et al., 2016). Reduced cytokinin status was shown to make *A. thaliana* plants more resistant to arsenate, which is the most prevalent form of arsenic (As). Cytokinin deficit boosted the expression of As (V)/phosphate transporter genes and arsenate stress tolerance machinery, resulting in the aggregation of complexing agents (Mohan et al., 2016).

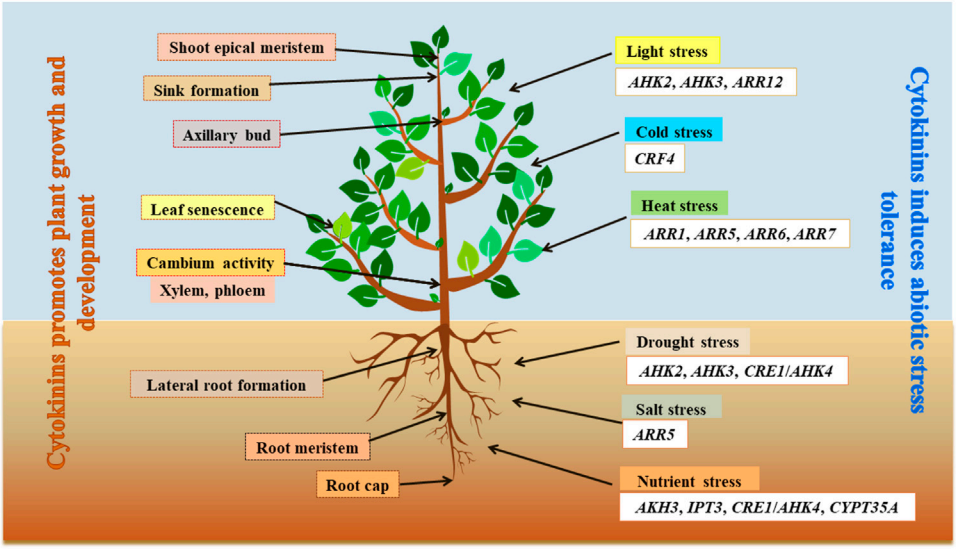

FIGURE 3
Cytokinins function in plant development and abiotic stress. Schematic illustration of cytokinin as a signaling molecule that regulates key plant developmental processes and its response to various abiotic stress.

Higher cytokinin levels, on the other hand, induced tolerance to another soil pollutant, selenium (Se) (Lehotai et al., 2012). Subsequently these findings imply that cytokinin has a role in the regulation of As and Se stress adaption. Cytokinins capacity to regulate plant development in response to nutrient stress was demonstrated in recent research on the role of cytokinin in signaling the availability of nitrogen (N) from the roots to the shoots (Landrein et al., 2018; Poitout et al., 2018). The expression of a GFP reporter gene under the control of WUS promoter, that governs the expression of a key regulator of shoot apical meristem (SAM) activity, was measured using quantitative microscopy (Landrein et al., 2018). The analysis of reporter gene activation in the context of cytokinin metabolism mutants indicated that IPTs were necessary for *de novo* synthesizes of cytokinin in response to changed N supply in the soil. Increases in soil N content consistently enhances the concentration of tZR, which is the primary cytokinin transport form. The reaction of the *pWUS::GFP* reporter gene to changing N availability happened quickly in the SAM, occurring within 24 h. Notably, grafting experiments between WT and cytokinin mutant plants demonstrated that while precursors to active cytokinins can be synthesized in the root (or elsewhere in the plant), the reporter gene response dependents solely on the ability of the shoot to form active cytokinins, presumably via LOG enzymes in the SAM (Chickarmane et al., 2012). Many studies have identified WUS as a primary target gene of RRBs (type-B response regulator proteins), suggesting a shorter signalling channel in the responsive meristem, which is consistent with cytokinin working *via* altering *WUS* activity (Dai et al., 2017). Poitout et al. (2018) determined that the ABCG14 transporter plays a critical role in the export of nitrate-induced cytokinin from roots, based on the fact that its mutation affected systemic N (nitrogen) signalling. Furthermore, they discovered a significant transcriptional reprogramming in shoots mediated by root-derived cytokinin, indicating that the hormone has functions beyond regulating SAM activity (Poitout et al., 2018). NLP transcription factors (TF), which act as positive regulators of *CYP735A* and *IPT* gene expression, are involved in the increase of cytokinin levels in the root in response to changes in nitrate availability. On the other hand, NIGT1 TF, which are also positively regulated by NLPs, act as negative regulators of *CYP735A* and *IPT* gene expression. (Maeda et al., 2018). The perennial grass *Lolium perenne* (Guo et al., 2017) produced results showing a role for cytokinin as a systemic N signal, implying that the function is evolutionarily conserved (Guo et al., 2017). Overall, it has been shown that cytokinin production in the root and translocation to the shoot contains significant information about soil conditions, particularly N availability, to the shoot, allowing the latter to control its own activity.

Light stress

In addition to supplying energy for photosynthesis, light transmits information about the time of day and season, and it has an impact on the direction in which plants are growing. Excessive or insufficient light stress plants and new research

TABLE 2 A list of CK signaling genes involved in the development of abiotic stress-tolerant plants.

S. No.	Gene	Response to stress	Host plant	Genetic engineering approach	References
1	*ARR1*	↑Heat tolerance	*Arabidopsis thaliana*	Constitutive overexpression	Karunadasa et al. (2022)
2	*TaIPT8*	↑Drought tolerance	Wheat (*Triticum aestivum*)	CRISPR/Cas9-based gene editing and constitutive overexpression	Wang et al. (2022)
3	*IPT*	↑Salt tolerance	*Nicotiana tabacum*	Stress-inducible senescence overexpression	Avni et al. (2020)
4	*OsERA1*	↑Drought tolerance	Rice (*Oryza sativa*)	CRISPR/Cas9	Ogata et al. (2020)
5	*IPT*	↑Drought tolerance	Creeping bentgrass (*Agrostis stolonifera*)	Stress-inducible overexpression	Xu et al. (2017)
6	*CKX1*	↑Drought tolerance	Barley (*Hordeum vulgare*)	Constitutive overexpression	Pospíšilová et al. (2016)
7	*CKX1*	↑Drought tolerance	*N. tabacum*	Constitutive overexpression	Lubovská et al. (2014)
8	*IPT*	↑Zinc tolerance, ↓leaf senescence	*N. tabacum*	Senescence-inducible overexpression	Pavlíková et al. (2014)
9	*AHP2, AHP3, AHP5*	↑Drought tolerance	*A. thaliana*	Knockout	Nishiyama et al. (2013)
10	*ARR22*	↑Drought, ↑cold tolerance	*A. thaliana*	Constitutive overexpression	Kang et al. (2013)
11	*IPT*	↑Drought tolerance, ↓leaf senescence	Cotton (*Gossypium hirsutum*)	Senescence-inducible overexpression	Kuppu et al. (2013)
12	*IPT*	↑Cold tolerance, ↓leaf senescence	*Saccharum* spp.	Stress-inducible overexpression	Belintani et al. (2012)
13	*IPT*	↑Salt tolerance, ↓leaf senescence	*N. tabacum*	Stress-inducible overexpression	Qiu et al. (2012)
14	*IPT*	↑Salt tolerance, ↓leaf senescence	Cotton (*G. hirsutum*)	Senescence-inducible overexpression	Liu et al. (2012)
15	*AHK2, AHK3*	↑Drought, ↑cold, ↑salt tolerance	*A. thaliana*	Knockout	Kang et al. (2012)
16	*CKX1, CKX2, CKX3, CKX4*	↑Drought, ↑salt tolerance	*A. thaliana*	Constitutive overexpression	Nishiyama et al. (2011)
17	*IPT1, 3, 5, 7*	↑Drought, ↑salt tolerance	*A. thaliana*	Knockout	Nishiyama et al. (2011)

↑: Increase, ↓: Decrease.

indicates that an altered day/night cycle may also stress plants (Bhaskar et al., 2021; Li et al., 2021). The response to these pressures involves cytokinin at several levels, which will be briefly summarized in the sections that follows. Even though photosynthesis needs light, too much light can harm the photosynthetic apparatus as well as other parts of the cell. To prevent being stressed by too much light (high light stress), plants have evolved a number of defensive mechanisms, including the cyclic electron transport, disposal of excess light energy as heat, and light avoidance motions of chloroplasts and leaves (Takahashi and Badger, 2011). However, even with these protective mechanisms, exposure to excessive levels of light may induce an over reduction of the photosynthetic electron transport chain, resulting in photoinhibition, which reduces the efficiency of photosynthesis (Yamamoto, 2016). In particular, protein D1, which is a component of the reaction centre of photosystem II (PSII), is one of the most often affected by UV radiation (Edelman and Mattoo, 2008). Reduced cytokinin status resulted in lower photoprotection and increased photoinhibition in plants, owing to a significant fall in the D1 level (Cortleven et al., 2014). Additionally, due to inadequate D1 repair, cytokinin-deficient plants had a reduced ability to recover from photoinhibition after high light stress. Plants antioxidant capacity was also lowered as a result of a lack of cytokinin. Thus, multiple photoprotective systems were disrupted in cytokinin-deficient plants, demonstrating that the hormone is required for plants to survive under conditions of extreme light stress. *Arabidopsis histidine kinases 3* (*AHK3*) and, to a lesser degree, *AHK2*, and the type-B RRs, *Arabidopsis Response Regulators1* (*ARR1*) and *ARR12* were shown to be involved in the regulation of cytokinin activity (Cortleven et al., 2014). Additional evidence supports a protective role for cytokinin in the photosynthetic machinery under conditions of intense light (Cortleven and Schmülling, 2015). For example, cytokinin increases the antioxidant-based

protection in chloroplasts, leading to an increase in the chloroplast's lifespan (Procházková et al., 2008). Furthermore, because of the activation of the *PSARK::IPT* gene, drought-stressed transgenic *N. tabacum* plants produced more CK and enhanced CO_2 respiration, indicating that photosynthetic activities were well protected (Rivero et al., 2009). In comparison, another study reported that inhibiting cytokinin signaling in *A. thaliana* by mutating the *AHK2* and *AHK3* receptor genes showed an increased photooxidative stress tolerance under water deficiency circumstances (Danilova et al., 2014).

In addition, plants that have evolved unique response systems may be severely affected by a lack of light. In *A. thaliana* and *N. tabacum*, cytokinin is a key xylem-borne signal for photosynthetic adaptation to canopy light gradients (Boonman et al., 2009). Shade avoidance response is induced by low light, and it is characterized by the stimulation of elongation growth toward the light and the halt the growth of leaf in response. The shade-dependent auxin-induced degradation of cytokinin in developing leaf primordia is reliant on the *CKX6* gene, which has been discovered to be crucial. An increase in cytokinin degradation slowed leaf primordia development, saving resources for hypocotyl extension growth (Carabelli et al., 2007).

Light has the ability to transmit information about the time of day and to control the circadian clock. A prolonged light period has recently been revealed to generate stress symptoms in *A. thaliana* plants during the following night, a condition known as photoperiod stress (Nitschke et al., 2016; Nitschke et al., 2017). Plants suffering from cytokinin deficiency were the first to exhibit this phenotype, which could be described as following a standard sequence of events. An extended light period resulted in the induction of stress marker genes such as *BAP1* and *ZAT12*. Moreover, there is a significant increase in JA level several hours after the start of the night; the next day, a significant reduction in PSII maximum quantum efficiency (Fv/Fm) resulted in visible lesion formation in the leaves. When compared to cytokinin-deficient plants, WT plants displayed a considerably milder stress response, demonstrating that cytokinin plays a protective role. Cytokinin works primarily through *AHK3, ARR2*, *ARR10*, and *ARR12* and RRBs. After photoperiodic stress, several clock mutants (e.g., *cca1 lhy*) exhibited a significant stress response. Furthermore, stress sensitive clock mutants and cytokinin deficient plants shared a decreased expression or dysfunction of LHY and CCA1, two critical regulators of the circadian clock, indicating that a working clock is required to deal with photoperiod stress. Despite the fact that this novel kind of abiotic stress is ceased in natural settings, it is instructive regarding linkages between cytokinin and stress pathways that have hitherto gone unreported in the scientific community. It remains to be discovered what these "mechanical linkages" natural functions are.

Cold stress

As a result of the membrane system hardening in low temperatures, cells become more vulnerable (Liu et al., 2016; Prerostova et al., 2021). Cold stress causes a number of physiological changes, including loss of membrane integrity, an imbalance between water and nutrients, and an increase in ion outflow. Cold stress triggers transcriptional and post-transcriptional regulatory processes that may be Abscisic acid (ABA) dependent or ABA independent (Prerostova et al., 2021). Low temperatures lead to the accumulation of ROS as the antioxidant enzyme activity is reduced which results in the failure of the proper functioning of the ROS scavenging system. As a result, an excessive accumulation of ROS will have harmful effects on the cell membrane, resulting in cell metabolism disorder and ion leakage (Sui, 2015). Additionally, low temperatures impair reproductive development. Cold stress during the flowering period of *O. sativa*, for example, will induce sterility and yield loss (Feng et al., 2014; Wang et al., 2017). Furthermore, when the temperature falls below 0°C, freezing stress develops, and the ice crystals formed leads to mechanical damage and metabolic dysfunction in plants (Liu et al., 2013; Cheng et al., 2014).

The effects of cold stress on energy generation and biochemical demand are profound (Koc et al., 2018). As a result, Zoysiagrass (*Zoysia japonica*) in high latitudes (relatively low-temperature regions) is more frost-resistant than Zoysia grass (*Z. japonica*) at low latitudes (relatively high-temperature areas). This might be because of the larger carbohydrate content employed as an energy store, as well as the involvement of phytohormones in controlling plant adaptation to cold temperatures (Li et al., 2018). *Carpobrotus edulis* produces more cytokinin under low-temperature stress, and an Arabidopsis mutant *amp1* with greater cytokinin concentrations displayed a higher relative growth rate and greater plant yield than WT (Xia et al., 2008; Khan et al., 2017; Fenollosa et al., 2018). Simultaneously, overexpression of *AtCOR15a:ipt* in *Saccharum officinarum* (sugarcane) increases cold tolerance by delaying leaf senescence and minimizing membrane damage, preventing significant production loss and freezing injury (Belintani et al., 2012).

Numerous studies have employed cytokinins to increase plant response to low-temperature stress. The multi-step phosphorylation mechanism is now the focus of research on the link between cytokinins and low-temperature resistance. According to some research, A-type *ARRs* such as *ARR5*, *ARR7*, and *ARR15* are positive regulators of *A. thaliana* cold tolerance (Shi et al., 2012). The C-type ARR *ARR22* contributes to low-temperature resistance in plants by keeping the membrane in a normal condition (Kang et al., 2013). When compared to the WT, B-type *arr1* mutant is more susceptible to low temperatures and has lower cold resistance, but *A. thaliana* with B-type ARR1 overexpression has higher cold resistance,

suggesting that *ARR1* is a positive effector of cold signal transmission (Jeon et al., 2013). An *AHK2* or *AHK3* sends low-temperature signals to *ARR1* through *Arabidopsis histidine phosphotransfer proteins 2* (*AHP2*), *AHP3*, or *AHP5*, which shows that *AHP2*, *AHP3*, and *AHP5* plays an important function in upregulating tolerance to low-temperature stress (Jeon et al., 2013).

Plants low-temperature tolerance is further influenced by other cytokinin response factors (CRFs), which are discovered downstream of the cytokinin signaling cascade. Recent studies by Zwack et al. (2016) showed that *CRF4* is a positive regulator for the increased cold tolerance in *A. thaliana* with a *CRF4* mutation under low-temperature stress. An adaptation mechanism under cold stress may be triggered by high levels of *CRF2* and *CRF3* expression, which may stimulate lateral root development and overcomes the inhibition of cold-induced root growth, thereby increasing the plant's ability to withstand low temperatures (Jeon et al., 2016). As a result, Arabidopsis with mutations in *AHK2*, *AHK3*, *AHK4* (cytokinin receptor histidine kinases) and *ARR7* (an A-type ARR) exhibits enhanced low-temperature tolerance, suggesting a function for these cytokinin receptor histidine kinases in cold stress signaling (Jeon et al., 2010). However, more experimental validation is required. Increased cytokinin levels, whether exogenous or endogenous, may enhance resilience to cold temperatures (Belintani et al., 2012; Jeon et al., 2016). Exogenous cytokinin pre-treatment may increase the cold tolerance of *Triticum aestivum* (wheat) seedlings exposed to cold stress by boosting endogenous cytokinin levels in the leaves (Veselova et al., 2005). Under cold stress, the administration of exogenous cytokinin in the *ahk* mutant is comparable to the higher-order mutation of AHK (a negative regulatory factor), which may increase plant cold tolerance. The molecular processes, on the other hand, remain a mystery Exogenous cytokinin treatment of wild-type plants has been shown to improve their ability to withstand cold stress. Furthermore, it has been claimed that certain A-type *ARRs* are positive regulators of the genes that control the response to cold stress. (Jeon et al., 2010). Type-A *ARRs* are extensively expressed in transgenic plants due to ARR protein stabilization (Shi et al., 2012).

When exposed to cold stress, the hormones ethylene (ETH) and cytokinin act antagonistically. The C-repeat binding factor/DRE binding factor (CBF/DREB) transcription regulation cascade is the most well-known cold signaling mechanism. An increase in cold sensitivity may be achieved by overexpressing CBF (Gilmour et al., 2000). ETH has been shown to negatively regulate the cold signal by regulating the expression of CBFs and A-type *ARRs* genes. Moreover, A-type ARRs are believed to be essential for integrating cytokinin and ethylene signals in regulating plant response to cold stress, and CBFs have been shown to negatively regulate the cold signal by modulating the expression of CBFs and A-type *ARRs* genes (Shi et al., 2012). The cytokinin signaling pathway or cytokinin-related transcription factors may be implicated in the response to cold, according to these studies.

Heat stress

Heat stress damage the biological components by the production of ROS and proteins denaturation. It also has a detrimental impact on photosynthetic capability, resulting in a metabolic imbalance. The accumulation of heat shock proteins (HSP), which operate as molecular chaperones to prevent protein denaturation and aggregation, is one of the plant's defence strategies against heat stress (Mittler et al., 2011). Heat stress decreases cytokinin levels, and exogenous cytokinin treatment usually improves heat stress resistance (Hare et al., 1997). In *Nicotiana tabacum* and *Agrostis stolonifera* (creeping bentgrass), for example, exogenous application of cytokinin and increased endogenous cytokinin concentrations reduced the inhibitory impact of heat stress on chloroplast and photosynthesis growth, increased antioxidant system activity, and upregulated heat shock proteins (Liu and Huang, 2002; Veerasamy et al., 2007; Xu et al., 2009; Xu et al., 2010). In *Oryza sativa*, *Zea mays*, and *Passiflora edulis* (passion fruit), cytokinin treatment improved thermotolerance of reproductive tissue and increase the yield of these plants, indicating that cytokinin is capable of priming heat stress defense (Sobol et al., 2014; Wu et al., 2016; Wu et al., 2017).

Photosynthetic capability is negatively impacted by heat stress, which affects chlorophyll concentration and photochemical efficiency (Fv/Fm) of leaves. Additionally, heat exposure increases ROS generation and protease activity, resulting in leaf senescence (Hu et al., 2020). Endogenous cytokinin levels in *A. thaliana* increase in response to heat stress, especially in the leaves, resulting in higher cytokinin concentration is essential for greater heat tolerance (Skalak et al., 2016). Furthermore, heat stress stimulates the generation of ROS, and higher cytokinin levels may activate the antioxidant system to eliminate ROS (Xu et al., 2009). Furthermore, hormone, proteome, and transcriptome study further reveal that cytokinin plays a critical role in plant tolerance to heat stress, with the majority of heat shock (HS) response proteins being elevated in response to higher cytokinin levels (Skalak et al., 2016). Under heat stress, however, when the cytokinin signaling pathway is disrupted and/or the concentration of endogenous cytokinin is decreased in Arabidopsis seedlings, the elongation of hypocotyls is significantly and continuously inhibited, both during the initial heat stress and during the subsequent seedling growth. The increase of endogenous cytokinins may preserve normal plant development under high-temperature stress and have a favourable effect on plants that have been treated with heat shock (Skalak et al., 2016). As a result,

elevated levels of endogenous cytokinin may increase plant heat stress tolerance.

Introducing *isopentenyl transferase* (*IPT*) into *A. thaliana* seedlings boost endogenous cytokinin levels and hence increase tolerance to high temperatures (Skalak et al., 2016). As a result, boosting the amount of endogenous cytokinin in plants may help them to better withstand heat stress conditions. According to Skalak et al. (2016), the duration of elevated cytokinin levels is crucial for plant heat tolerance. The overexpression of the *IPT* gene under the start of continuous induction of expression promoters, such as the *HSP18* promoter or the senescence-activated promoter (*SAG12*), may be used to maintain high amounts of cytokinin. According to Xu et al. (2009), under heat stress, the overexpression of *SAG12:ipt* in *A. stolonifera* maintained the development and elongation of root systems, reduced chlorophyll loss and delayed leaf senescence, boosting plant heat tolerance. Subsequently, Xu et al. (2010) demonstrated that the overexpression of *IPT* generated by two separate promoters (*HSP18:ipt* and *SAG12:ipt*) results in a considerable increase in heat stress proteins in plants, boosting the plants ability to resist high temperatures (Xu et al., 2010). The DEX (dexamethasone) promoter (transient expression promoter)-driven overexpression of the *IPT* gene in *A. thaliana* may results in opening of stomata and leaf transpiration stimulation, both of which are important in the early stages of the heat stress response (Skalak et al., 2016). Furthermore, plant transpiration may only reduce the immediate demand for cooling, but it cannot alleviate the long-term impacts of heat stress because of its low water content (Wu et al., 2017). Increasing transpiration may only reduce the immediate requirement for cooling and cannot relieve the long-term impacts of heat stress owing to plants limited water content.

Along with boosting endogenous cytokinin content by *IPT* overexpression, a high level of cytokinin may be maintained by inhibiting endogenous cytokinin breakdown. Endogenous cytokinin breakdown may be inhibited in two ways. One of the methods is the mutation of the cytokinin oxidase/dehydrogenase (CKX) gene, while the other is through the use of cytokinin degradation inhibitors spray to decrease the activity of cytokinin oxidase/dehydrogenase (a negative regulator of cytokinin production). The CKX gene mutation causes an increase in cytokinin levels and grain production in *O. sativa*. Furthermore, in heat-sensitive *O. sativa* cultivars, CKX activity rises dramatically, resulting in poor cytokinin levels and yield. But the heat-resistant *O. sativa* CKX enzyme activity remains consistent, and the plants heat resistance improves (Wu et al., 2017). The CKX inhibitor INCYDE (cytokinin degradation inhibitor) is quite effective. The application of INCYDE boosts *A. thaliana* roots active cytokinin levels when exposed to heat stress (Zatloukal et al., 2008). On the other hand, Prerostova et al. (2020) have successfully demonstrated the opposite finding, that a single INCYDE treatment under heat stress had a detrimental influence on plant heat tolerance. The use of INCYDE in conjunction with acclimating plants may partially enhance *A. thaliana* heat resistance. Cytokinin response factors (CRFs) are thought to be a transcription factor that is linked to cytokinin. Under heat stress, *CRF1* expression in *Solanum lycopersicum* (tomato) roots is drastically reduced (Shi et al., 2014). Accordingly, we conclude that CRFs play a significant part in the plants heat stress signal pathway and that more study is required to elucidate the underlying process.

Moreover, exogenous cytokinin performs a similar effect to endogenous cytokinin when exposed to heat stress. Exogenous cytokinin zeatin ribose (ZR) treatment improves *A. stolonifera* heat tolerance by reducing root mortality, increasing the antioxidant system activity, maintaining a higher chlorophyll content, and upregulating related heat shock proteins (Veerasamy et al., 2007; Xu et al., 2010; Hu et al., 2020). To further boost plant growth and production, exogenous cytokinin has been implemented to enhance the heat stress tolerance of reproductive organs in plants.

Salt stress

Salt stress affects numerous biochemical and physiological processes in plants. Accumulation of sodium ions (Na^+) in plants may disrupt ion homeostasis, imbalance the potassium ion (K^+)/Na^+ ratio, and Na^+ ion toxicity, all of which can result in secondary stress, including oxidative stress (Feng et al., 2015; Song et al., 2015; Liu et al., 2017; Guo et al., 2018). Moreover, ion leakage, cell membrane damage, and direct damage to proteins and other macromolecules are all caused by oxidative stress, which may result in cytotoxicity, membrane malfunction, and cell death in certain cases (Lin et al., 2018; Liu et al., 2018). Leaf senescence will be accelerated upon ion stress and oxidative stress, which will destroy the chlorophyll, limit photosynthesis and lower the yield (Han et al., 2011; Li et al., 2012; Liu et al., 2017). According to several research, the deleterious effects of salt stress on plants such as *Raphanus sativus* (radish) and *N. tabacum* are connected to cytokinins (Vankova et al., 2010). Under salinity stress, however, the alterations in endogenous cytokinins in various plants are not homogeneous. Due to the variability in cytokinin concentration under salt stress, there is no one-size-fits-all strategy for increasing plant salt tolerance via exogenous and endogenous cytokinin manipulation.

According to recent research, the cytokinin level of *Malus domestica* (apple) rootstock 'robusta' and *Solanum lycopersicum* seedlings remains high under salinity stress (Keshishian et al., 2018; Feng et al., 2019). Additionally, *A. thaliana*, *O. sativa*, and other plants exhibit an increase in cytokinin levels in response to salt stress (Prerostova et al., 2017; Joshi et al., 2018). The upregulation of cytokinin in certain plants may aid in the recovery from salt stress. Due to cytokinin accumulation, the *OsCKX2* knockout rice mutant exhibits a greater relative water content and yield under salinity stress than the WT, enhancing

salt tolerance (Joshi et al., 2018). Moreover, increased plant resistance to salt stress may be achieved by increasing the activity of antioxidant enzymes by spraying INCYDE on S. lycopersicum. (Aremu et al., 2014). Avalbaev et al. (2016) showed that *Triticum aestivum* pre-treated with methyl jasmonate (MeJA) can maintain a high concentration of cytokinin by lowering the level of CKX transcription that is caused by salt stress, thereby delaying the negative impact of salt on seedling growth, and enhance salt resistance (Avalbaev et al., 2016). In response to the deletion of 42 bp from the promoter region of *IPT5* gene, the expression level and cytokinin content of *M. domestica* rootstock "robusta" under salinaty stress were both increased, with the latter remaining at a high level and exhibiting greater tolerance to salt stress (Feng et al., 2019). *SlIPT3* overexpression makes *S. lycopersicum* more salt-tolerant by keeping their photosynthetic pigment and K^+/Na^+ ratio high, which means they can withstand more salt than the WT plants (Ghanem et al., 2011).

High cytokinin concentration, on the other hand, have been demonstrated in experiments to reduce plant salinity tolerance. Furthermore, overexpression of *AtIPT8* in *A. thaliana*, which has a high cytokinin level, results in a substantial reduction in the survival rate of plants under salt stress. This is due to the downregulation of stress-responsive genes, the inhibition of the antioxidant system, and the reduction of chlorophyll content in the plants (Wang et al., 2015). Furthermore, plants with lower cytokinin levels have been reported to be more resistant to abiotic challenges, such as salt stress, owing to decreased cytokinin production or increase degradation (Ghanem et al., 2011; Avalbaev et al., 2016; Zhang et al., 2018). In comparison to the wild type, salt tolerance is higher in cytokinin synthesis pathway mutants with loss-of-function mutations, such as *Atipt1*, *Atipt3*, *Atip5*, and *Atipt7* (Nishiyama et al., 2012; Zhang et al., 2018). In the moss *Physcomitrella patens*, overexpression of *PpCKX1* lowers cytokinin levels and increases salt tolerance (Hyoung et al., 2019). Salt tolerance in transgenic *Medicago sativa* (alfalfa) plants is improved by overexpression of *MsCKX*, which maintains a high K^+/Na^+ ratio and increases the activity of antioxidant enzymes to scavenge ROS (Li et al., 2019). These CKX-induced cytokinin-deficient plants are more valuable for deciphering the function of cytokinin than *ipt* mutants (Werner et al., 2003).

Plant salt tolerance is also influenced by components in the cytokinin signaling system. The cytokinin receptor *AHK1* is a positive regulator of salt stress response and plays an active regulatory function in osmotic stress signaling (Tran et al., 2007). Furthermore, plant tolerance to salt is improved by *ahk2*, *ahk3*, and *cre1* mutants, which upregulate the expression of homologous stress response genes, indicating that these members have a negative regulatory function in salt tolerance (Tran et al., 2007). By promoting the expression of *A. thaliana high-affinity K^+ transporter 1;1* (*AtHKT1;1*) in the roots, the *arr1* and *arr12* mutants decrease sodium accumulation in the aerial portions and improve salinity stress tolerance (Mason et al., 2010). Overexpression of *ARGONAUTE2* (*AGO2*) in *O. sativa* decreased cytokinin concentration in shoots and increase cytokinin level in roots, resulting in higher salt tolerance and grain length in *O. sativa* under salt stress, according to Yin et al. (2020). In addition, by boosting the production of *BIG3* (*GRAIN3*), which encodes a protein that may be involved in cytokinin transport, and AGO2, which changes the histone methylation level of BG3, AGO2 impacts the distribution of cytokinin. CRFs are thought to be downstream signaling molecules for RRs in certain instances (Hallmark et al., 2019). Consequently, under salt stress, RNAi silencing of *ThCRF1* reduced salt tolerance in *Tamarix chinensis* (halophyte: salt-tolerant plants), while overexpression of *ThCRF1* greatly increased salt tolerance in the halophyte through regulating osmotic potential and increasing antioxidant enzyme activity (Qin et al., 2017).

Depending on the plant species and the degree and duration of salt stress, increase or downregulation of cytokinin improves salt tolerance. Exogenous cytokinin treatment has a range of impacts on tolerance of salt stress in plants of different species. Although pre-treatment of legumes with exogenous cytokinins increases their susceptibility to salt, most research has shown that exogenous cytokinins improve plant salt tolerance, particularly in cereal crops like *O. sativa* and *T. aestivum* (Iqbal et al., 2006; Javid et al., 2011). By efficiently relieving salt-induced leaf senescence and other forms of physiological or developmental damage, foliar application of 6-Benzylaminopurine (6-BA; synthetic cytokinin) improves *Solanum melongena* (eggplant) and *Lolium perenne* (perennial ryegrass) salt resistance (Wu et al., 2014; Ma et al., 2016). These findings suggested that we can spray exogenous cytokinins onto plants to improve salt resistance.

Drought stress

Reduced photosynthesis, decreased crop yields, and accelerated senescence is some of the negative consequences that drought stress may have on plant physiological activities (Liu et al., 2012; Zheng et al., 2017; Hai et al., 2020). Subsequently to salt stress, the likelihood of increasing plant drought resistance via cytokinin regulation is dependent on the stress duration, plant dehydration rate and soil water potential, (Veselov et al., 2017). Furthermore, endogenous cytokinin upregulation and downregulation have both been reported to increase drought tolerance. (Werner et al., 2010; Zhang et al., 2018). According to several research, under drought stress, plant endogenous cytokinins accumulation is decreased, and this leads to improving plant drought tolerance through a variety of physiological responses such as early leaf senescence, leaf abscission and stomatal closure (Xu et al., 2016; Naidoo and Naidoo 2018; Calvo-Polanco et al., 2019). Moreover, cytokinin is a negative regulator of plant root development and branching,

enhancing cytokinin breakdown in the root may result in plants with an improved root-to-shoot ratio, larger root system, and long-term drought resistance (Pospisilova et al., 2016; Ramireddy et al., 2018).

Overexpression of CKX causes cytokinin to be downregulated, resulting in slower plant development and higher protective chemical content (proline, betaine, etc.) as well as drought resistance in *A. thaliana*, *N. tabacum*, *Cicer arietinum* (chickpea), and *Hordeum vulgare* (barley) (Werner et al., 2010; Nishiyama et al., 2011; Pospisilova et al., 2016; Ramireddy et al., 2018; Khandal et al., 2020). In *A. thaliana*, the *ipt1*, *3*, *5*, and *7* mutants had lower endogenous cytokinin content and improved drought tolerance (Nishiyama et al., 2011). Reduced levels of cytokinin cause roots to expand and shoot to have a higher root-to-shoot ratio, which improves root surface area available for water absorption. Smaller branches and leaf areas in comparison to roots may significantly reduce transpiration which further improves the tolerance (Werner et al., 2010; Lubovská et al., 2014; Prerostova et al., 2018). Accordingly, the whole plant can keep a relatively high relative water content and become more drought-resistant. Furthermore, by counteracting the effects of the oxidase system, downregulation of cytokinin may contribute to an improvement in drought tolerance (Lubovská et al., 2014). Conventional multi-step phosphorylation system in plants, including RRs, HKs, and HPs, is responsible for cytokinin signaling in the plant. Cytokinin signaling is also regarded to be a negative regulator of drought resistance because *ahk2*, *3*, *5*, *arr1*, *10*, and *12* display a significant drought-tolerance phenotype (Kang et al., 2012; Nguyen et al., 2016). Since the cytokinin signal suppresses stress response gene expression, it is hypothesized that decreasing the amount of cytokinin in the plant might enhance plant survival under challenging environmental conditions (Zhang et al., 2018). Moreover, it was shown that the expression of *SlCRF1*, *SlCRF2*, *SlCRF3*, and *SlCRF5* was regulated throughout the drought and recovery phase in *Solanum lycopersicum* plants, demonstrating that CRFs respond to drought stress and providing a novel idea for improving plant resistance to drought stress (Gupta et al., 2014; Shi et al., 2014).

Drought, salt, and cold stress all results in reduce water availability, resulting in physiological reactions that overlap. Different strategies have been developed in plants to optimize the use of water, including redirection of root development, change of cell membrane characteristics, and control of transpiration via stomata (Feng et al., 2016; Zhu, 2016). Moreover, drought response is tightly linked to the activity of abscisic acid (ABA), which increases in response to drought stress and binds to its corresponding receptor family PYRABACTIN RESISTANCE1 (PYR1)/PYR1LIKE (PYL)/ ABA RECEPTOR REGULATORY COMPONENTS (RCAR). An enzyme called PROTEIN PHOSPHATASES 2C (PP2c) is inhibited by the ABA-PYR/PYL complex. When ABA is absent PP2c dephosphorylate and keeps subclass III SUCROSE NONFERMENTING1 (SNF1)–RELATED PROTEIN KINASES2 (SnRK2s) inactive. Furthermore, SnRK2s that have been activated phosphorylate transcription factors known as ABRE BINDING FACTOR (ABFs)/ABSCISIC ACIDRESPONSIVE ELEMENT (ABRE) BINDING PROTEINS (AREBs), which control the expression of target genes to promote plant drought tolerance (Miyakawa et al., 2013; Joshi et al., 2016). Moreover, drought and osmotic stress activate another signaling pathway that is independent of ABA and includes GROWTH REGULATING FACTOR7 (Kim et al., 2012).

Drought reduces cytokinin levels in *A. thaliana* and *Glycine max* (soybean) by repressing *IPT* genes and upregulating *CKX* genes (Guo and Gan, 2011; Nishiyama et al., 2011; Le et al., 2012; Nishiyama et al., 2013; Ramireddy et al., 2014; Nguyen et al., 2016; Todaka et al., 2017). Moreover, genetic studies in *A. thaliana* have consistently shown that cytokinin functions as a negative regulator of drought stress tolerance, which is consistent with previous findings (Nishiyama et al., 2011; Nishiyama et al., 2013; Nguyen et al., 2016). Furthermore, lowered cytokinin levels and signaling resulted in at least two primary effects: greater sensitivity to ABA, establishing cytokinin as an ABA antagonist and a decrease in shoot growth, which is an adaptive response to drought (Werner et al., 2003; Riefler et al., 2006). Numerous components of the cytokinin signalling that are functionally significant in the tolerance to drought stress have been established (Li et al., 2016). Therefore, plants with mutant cytokinin receptor genes (*AHK2* and *AHK3*), HPT genes (*AHP2*, *AHP3*, and *AHP5*), or RRB genes (*ARR1*, *ARR10*, and *ARR12*) exhibited greater drought stress tolerance compared to control plants (Nishiyama et al., 2011; Nguyen et al., 2016). However, drought tolerance was connected with a variety of physiological changes, including an increase in cell membrane integrity, reduction in stomatal aperture, and an increase in ABA sensitivity (Nguyen et al., 2016). According to a transcriptomic study, cytokinin regulates a large number of dehydration/ drought and/or ABA-responsive genes involved in drought adaptation (Nguyen et al., 2016). Increasing evidence suggests that under drought and salt stress, the RRA genes *ARR5*, *ARR6*, *ARR7*, *ARR15*, and *ARR22*, which also react to cold stress in a cytokinin independent manner, were upregulated, showing a partial overlap of the response pathways (Kang et al., 2012; Jeon and Kim, 2013). Drought resistance is increased in plants that overexpress *ARR22* (Kang et al., 2013). *A. thaliana* has a *MYB2* gene, which is activated by the ABA, which suppresses the *IPT* genes and lowers the level of cytokinin (Guo and Gan, 2011). In turn, this reduces the output of the AHK/AHP/ARR signalling cascade that aids the plants adaptation to drought and osmotic stress.

The interaction between SnRK2s, RRAs, and RRBs is another connection. The RRBs *ARR1*, *ARR11*, and *ARR12* physically engage with SnRK2s in non-stress situations, repressing their

kinase activity and shutting off the drought response pathway. Furthermore, in drought-stressed plants, SnRK2s phosphorylate the *RRA ARR5*, inhibiting cytokinin signaling and limiting plant growth. These interactions demonstrate cytokinin regulates growth-trade-offs is well understood.

Interactions of cytokinin with other phytohormones under abiotic stresses

Plant responses to abiotic stress conditions are mostly based on interactions among hormone signals. Cytokinin participates in a complex signal network with other phytohormone signaling pathways rather than playing a separate regulatory function. Cytokinin does not have a regulatory function that is independent of other phytohormone signaling pathways; rather, it acts within a complex signal network that includes several pathways (Li et al., 2016). Furthermore, coordinating tissue expansion in response to environmental changes requires the interaction of other phytohormone. As a result, the plant is able to respond rapidly to its changing environment due to a network of tightly interlinked signaling systems.

Abscisic acid (ABA) regulates one of the earliest plant drought responses, stomata closure, which controls the trade-off between CO_2 intake and water loss via transpiration (Sah et al., 2016; Vishwakarma et al., 2017). Although ABA strongly interacts with cytokinins, it also regulates mid- and long-term plant responses to abiotic stress, including regulation of plant architecture. Accumulation of stress-induced ABA in turn downregulates cytokinin production by way of the MYB2 transcription factor (TF), alleviating the repression on multistep phosphorelay (MSP) and activating genes that are ABA- and stress-inducible (Li et al., 2016). Through the downregulation of shoot growth and the acceleration of root development, ABA-mediated suppression of cytokinin signaling starts the process of redesigning the plant body. This enables the plant to increase water intake from deeper soil layers while minimizing water loss (Li et al., 2016). Accordingly, ABA hypersensitivity and increased drought resistance are seen in MSP signaling mutants, such as those with defects in the cytokinin sensors AHK2, AHK3, and AHK4 and type-B ARRs ARR1, ARR10, and ARR16 (Tran et al., 2007; Tran et al., 2010; Nguyen et al., 2016). It has been demonstrated that ABA and drought may both downregulate the expression of *ARR1*, *ARR10*, and *ARR12* (Nguyen et al., 2016). According to Takatsuka and Umeda (2019), ABA also inhibits *ARR2* but not *AHK3* or *AHK4* and there may be a function for ABA in the regulation of AHP2's nucleocytoplasmic partitioning (Marchadier and Hetherington, 2014). Tran et al. (2007) found that the osmosensor AHK1 is not a negative regulator of the ABA-mediated stress response, but rather a positive regulator. This suggests that there may be some specificity at the level of signals that start the MSP-regulated (drought) stress response (Hai et al., 2020).

There are two ways in which ABA interferes with MSP activity: ABA-controlled downregulation of the generation of cytokines and the interaction between ABA signaling components and MSP. ABA-activated ABI4 binds promoters and downregulates *ARR6*, *ARR7*, and *ARR15*; arr4, arr6, arr7, and arr15 mutant lines are hypersensitive to ABA (Jeon et al., 2010; Wang et al., 2011). ARR5, a type-A ARR and negative regulator of MSP signaling, has numerous Ser residues that are phosphorylated by SnRK2.2, SnRK2.3, and SnRK2.6. By inhibiting cytokinin signaling, this results in stability of the ARR5 protein, which improves ABA responsiveness and drought tolerance.

Dautel et al. (2016) postulated an AHK2/AHK3-dependent phosphorylation of Thr6 and Tyr19 of KIN10, one of the two subunits of SnRK1, acting under energy stress in their phosphoproteomic analysis (Baena-González and Sheen, 2008). According to KIN10-based global gene regulation (Radchuk et al., 2006; Baena-González et al., 2007), SnRK1 down-regulation has previously been linked to cytokinin and auxin signaling. However, ethylene signaling was found to be negatively regulated by SnRK1 phosphorylation-mediated inactivation of EIN3 (Kim et al., 2017), leading to a growth. There is a bidirectional negative link between ABA and cytokinin levels/signaling. ABA insensitivity in seed germination was caused by the overexpression of cytokinin production via the upregulation of AtIPT8 (Wang et al., 2011). In the process of seed germination, ABA insensitivity was caused by an increased cytokinin production brought about by an overexpression of *AtIPT8*. Additionally, ABA was unable to inhibit the expression of the type-A ARRs ARR4, ARR5, and ARR6 that physically interact with ABI5 and reduce *ABI5* levels when endogenous cytokinin levels are increased (Wang et al., 2011). The cytokinin-responsive type-B ARRs ARR1, ARR11, and ARR12 that physically engage with SnRK2s and suppress the kinase activity of SnRK2.6 are most likely the cause of the decreased sensitivity to ABA under high endogenous cytokinin levels. The cytokinin-dependent control of ABA signaling may be regulated at the transcriptional level by ARR10, which was shown to bind the promoters of multiple ABA signaling genes (Zubo et al., 2017).

Cytokinins suppress expression of the Arabidopsis HIGH-AFFINITY K + TRANSPORTER 1; 1 (AtHKT1; 1), which is responsible for removing sodium ions from root xylem, in response to salt stress through ARR1 and ARR12. This transporter is responsible for removing sodium ions. Additionally, it was shown that cytokinins controlled the type A response regulator ARR5's expression in response to salt stress mostly *via* ARR1 and ARR12, demonstrating the role of specific MSP components in the roots in regulating sodium accumulation in the shoots (Mason et al., 2010).

The hormonal network underpinning the intricacy of plant responses to stress is also influenced by the ethylene (ET) pathway. ET has been investigated in the contexts of development and stress (Vanstraelen and Benková, 2012; Beguerisse-Díaz et al., 2013; Zhai et al., 2013), and it has most recently been shown to play a negative regulatory role in cold tolerance (Shi et al., 2012). Interestingly, cold stimulates the expression of *ARR5*, *ARR6*, *ARR7*, and *ARR15* in a manner similar to dehydration, most likely to inhibit cytokinin signal transduction and growth (Jeon et al., 2010; Kang et al., 2012). According to the findings of Shi et al. (2012), ethylene biosynthesis and signaling adversely affects the cold stress response in Arabidopsis. This is accomplished via the repression of cold-inducible *C-REPEAT BINDING FACTORS* (*CBFs*) (*CBF1*, *CBF2*, and *CBF3* genes), and the type-A ARR genes *ARR5*, *ARR7*, and *ARR15*. This ethylene-induced suppression was expected to be mediated by direct binding of EIN3 to the promoters of type-A ARRs, so possibly suggesting another mechanistic connection between classical ethylene signaling and MSP during plant desiccation.

Despite the fact that ET disrupts the cytokinin signaling pathway's output, the pathway itself is also influenced by cytokinin. In fact, cytokinin is responsible for the stabilization of the enzymes 1-aminocyclopropane-1-carboxylate synthase 5 (ACS5) and ACS9 (Chae et al., 2003; Hansen et al., 2009), which are responsible for the conversion of S-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid. This stability might result in an accumulation of ET, which would then have the potential to influence plant development processes such as root expansion (RůžiČka et al., 2007). According to Lehotai et al. (2012), the activation of both cytokinin and ET signaling in response to selenite-induced stress by means of the ARR5 and ACS8 markers and decrease in the levels of auxin suggests that the hormonal regulatory network that underlies stress responses is more complex than previously thought. Interestingly, there are tissue-specific characteristics in the cytokinin-ET and cytokinin-ABA interactions. Contrary to ET, which accumulates mostly in roots in response to high CK levels, CK treatments have been shown to enhance the accumulation of ABA in shoots but not in roots (Žďárská et al., 2013).

Priming as a strategy to develop abiotic stress tolerance

Cytokinin has been shown in the literature to be used as a priming agent to activate plant immune for biotic stress responses and biotrophic. Priming technology, on the other hand, is not widely used to protect plants from the negative effects of abiotic stressors or to prepare them to better withstand them. Moreover, it has been shown that priming the stress response pathways would be advantageous for the plant since it enables quicker and more robust responses with little energy expenditure. Although priming with several plant growth regulators (PGR) has been shown to be beneficial, evidence on employing cytokinins as priming agents is restricted to few experiments using kinetin or 6-benzylaminopurine (BAP). BAP has long been one of the cytokinins that is most often given to plants exogenously to dealyed senescence and decrease the impact of stress. Exogenous cytokinin treatment may reduce abiotic stressors on agricultural plants, resulting in enhanced growth, development, and yield. Similarly, cytokinin treatment lowers plant salinity stress (Ha et al., 2012) and promotes starch accumulation in salt-stressed rice plants (Javid et al., 2011).

Abiotic stress has been reduced in a variety of crop species by seed priming with cytokinins or a combination of cytokinins and other plant hormones. It is possible that genes associated to cytokinin play a significant role in the regulation of regeneration once a stress has been removed. Priming with cytokinins improves the production of chlorophyll (Chl) and the accumulation of biomass in plants. Additionally, it increases the photosynthetic rate, promotes membrane integrity, and keeps a stable ionic level. Wheat seeds primed with kinetin at concentrations of 100 mg L^{-1}, 150 mg L^{-1}, and 200 mg L^{-1} were shown to have improved germination and tolerance to salt. This was accomplished by lowering ABA concentrations and raising IAAs concentrations (Iqbal et al., 2006). Similar findings were made by Mangena (2020), who claimed that priming soybean seeds with cytokinins (Benzyl adenine; 4.87 mg L^{-1}) improved soybean root biomass, flowering, and fruiting under drought stress. *Arachis hypogaea* L. aged groundnut seeds were primed with cytokinins (150 ppm), which improved antioxidant enzyme activities and reduced oxidative damage to improve germination and seedling indices (Sepehri et al., 2016). In addition, the mode of action of these PGRs in enhancing seed and plant fitness through priming has not been investigated. Moreover, it has been reported that exogenous application of cytokinins reduces ABA-induced stomatal closure because this PGR, which are important for stomatal movement, are involved (Tanaka et al., 2006). However, the effects of seed priming with cytokinins on stomatal movement remain unknown.

Genetic engineering of cytokinin for improving or redesigning plant abiotic stress tolerance

Crop yield and production are threatened by abiotic factors such as severe temperatures, nutrient deficiency, low water levels, high salt concentrations and excessive light. Plants cytokinin mediated stress responses are highly dependent on the phytohormone concentrations (O'Brien and Benkova, 2013). Moreover, plants get acclimatized to stress as a result of both constitutive decrease and overproduction of cytokinins. As a result, precise manipulation will lead to altering its concentrations to achieve desired results (summarized in

Table 1) (Rivero et al., 2007; Werner et al., 2010; Nishiyama et al., 2011; Ha et al., 2012). *A. thaliana* the model plant, has been the subject of the most extensive study on cytokinin-mediated stress responses till date (Ha et al., 2012). The most common method for lowering cytokinin levels is to change the expression of the *CKX* or *IPT* genes. Subsequently, the overexpression of *CKX* genes, as well as the disruption of *IPT* genes, will result in a reduce cytokinin levels. Furthermore, plants with cytokinin deficiency show bushy root growth, diminished apical dominance, and stunted shoot developmental phenotype. Nishiyama et al. (2011) used gain and loss-of-function mutants to show that the Arabidopsis cytokinin-overexpressing plants (*35S:CKX1-35S:CKX4*) and *ipt1, 3, 5, 7* quadruple mutant were more salt and droughts -tolerant than WT plants. Furthermore, root growth assays and intracellular electrolyte leakage measurements suggested that cytokinin deficient plants were more resistant to salinity stress owing to increased primary root development and more tolerant to drought because of improved cell membrane integrity (Nishiyama et al., 2011). Additionally, the reduction in cytokinin levels shown in *AtCKX*-overexpressing Arabidopsis transgenic plants had a major influence on the growth and development of several tissues, including roots and shoots, reproductive organs, floral, and vascular development (Werner et al., 2003). Root-specific expression of *CKX* in *N. tabacum* using the *W6:CKX1* construct (expression of *CKX1* driven by a *WRKY6* promoter) and in *A. thaliana* using the *P10:CKX3* construct (expression of *CKX3* driven by a *PYK10* promoter) revealed an enlargement of the root system architecture (RSA) in the transgenic lines, which showed similarity to the root feature of plants grafted between *35S:CKX1* or *35S:CKX3* (Werner et al., 2010). Furthermore, the transgenic plants accumulated more minerals, such as calcium, phosphate, molybdenum, and magnesium when compared with the WT (Werner et al., 2010). These data collectively support that the root development improved plants tolerance to water stress and nutrient deficiency.

Constitutive overexpression of cytokinin gives rise to severe anomalies in biological processes such as organogenesis, cell division, meristematic activities, and gametophyte development (Kieber, 2002). Because of this, organ-specific and stress-inducible cytokinin synthesis is thought to be more desirable. Gain-of-function studies, for example, showed that using stress-inducible promoters alternative to constitutive promoters might prevent growth abnormalities associated with endogenous cytokinin overexpression, such as dwarf and limited root growth phenotypes, results in improved control of cytokinin biosynthesis (Xing et al., 2009; Peleg and Blumwald, 2011). Several promoters, such as *HSP* (*heat shock protein*), *PSARK* (*senescence-associated receptor-like kinase*), *PSAG12* (*senescence-associated genes12*), and *rd29A* (*response to dehydration 29A*), have been successfully used to drive conditional expression of the *Agrobacterium tumefaciens* mediated *IPT* gene in *A. thaliana* (Zhang et al., 2000), tobacco (*N. tabacum*) (Rivero et al., 2007; Rivero et al., 2010), creeping bentgrass (*Agrostis stolonifera*) (Merewitz et al., 2010), rice (*O. sativa*) (Peleg et al., 2011), peanut (*Arachis hypogaea*) (Qin et al., 2011), cotton (*Gossypium hirsutum*) (Kuppu et al., 2013), and cassava (*Manihot esculenta*) (Zhang P. et al., 2010), to increase tolerance to a variety of stresses, including waterlogging and drought (Figure 2). Transgenic plants with increased cytokinin exhibited better adaptive responses to numerous stresses with an improvement in photosynthesis capacity, transpiration rate, intracellular water content, and delayed leaf senescence, all of which shows the potential to be useful and economical in agriculture.

The promoter *PSAG12* deriving the expression of the *IPT* gene significantly delayed the onset of leaf senescence but resulted in an unexpected change in source-sink relationships, nitrogen (N) mobilization, reproduction and growth in response to water stress were also reported (Jordi et al., 2000). Thus, an alternate promoter, *PSARK*, has been frequently employed to overcome such a problem, since this promoter may activate *IPT* expression prior to the onset of leaf senescence (Rivero et al., 2007; Reguera et al., 2013). Similarly, *A. thaliana* stress-induced *rd29A* overexpressed the *IPT* gene to confer salinity stress tolerance in *N. tabacum* (Qiu et al., 2012). Salt tolerance in transgenic *G. hirsutum* was improved by overexpressing the *A. tumefaciens IPT* gene using the *Ghcysp* (*G. hirsutum* cysteine proteinase) promoter, which is from the same family of cysteine endopeptidase genes as *SAG12* (Liu et al., 2012). Furthermore, when drought stress occurs during the vegetative stage of the *G. hirsutum* plant, this strategy proved efficient in enhancing plant performance, but drought stress after the flowering stage failed to provide a yield advantage (Zhu et al., 2018). Early flowering and a later heading date were caused by *PSAG39:IPT* expression in *O. sativa*. Under drought conditions, this was expected to assist plants to deal with modest water restrictions and increase grain yields (Zou et al., 2007; Liu et al., 2010). Additionally, increased *IPT* expression assisted the plants survival ability under stressful events and at the recovery stage under waterlogging and submerging situations (Huynh et al., 2005).

Heavy metal stress (HMs) has a negative impact on plant metabolic processes, particularly when zinc concentrations are elevated (Gill, 2014). When compared to WT, *PSAG12:IPT* transgenic *N. tabacum* displayed stronger protection against high zinc contamination by reducing transpiration rates and net photosynthetic while retaining free amino acids synthesis, which is suggestive of appropriate nitrogen (N) metabolism (Pavlíková et al., 2014). Furthermore, a lack of nutrients, particularly important minerals like N, may cause massive self-destructive processes within plant cells. *N. tabacum* transformed with the *PSARK::IPT* construct might aid in the inhibition of ROS (reactive oxygen species) formation and prevent the detrimental effects on plants induced by decreasing the N concentration (Rubio-Wilhelmi et al., 2011).

Subsequently, another study employed the P*SAG12*:*IPT* construct to produce transgenic *A. stolonifera* that improved plant survival in the face of N or phosphate (P) deprivation (Zhang Y. et al., 2010).

Overexpression of *A. tumefaciens IPT* gene to increase endogenous cytokinins level as a countermeasure against adversarial temperature has also been implicated in many species, such as generating low temperature-tolerant sugarcane (*Saccharum* spp.) and cold-resistant tall fescue (*Festuca arundinacea*) using the Arabidopsis *COR15a* (cold-regulated gene15a) promoter and the maize ubiquitin promoter, respectively (Hu et al., 2005; Belintani et al., 2012). In response to heat stress, an effort was made on *A. stolonifera* utilizing HSP and PSAG12 promoters to overexpress the *IPT* gene from *A. tumefaciens* (Xing et al., 2009; Xu et al., 2009). Furthermore, Xing et al. (2009) have also established that *SAG12-ipt-* and *HSP18-ipt* bearing transgenic *A. stolonifera* exhibited significantly longer leaf life-span when compared to WT when subjected to dark and heat treatment, respectively. Another *A. thaliana* SAG family promoter, *SAG13*, may induce *IPT* expression in all mature leaves before senescence, similar to PSARK expression pattern, but with a more severe altered source-sink relationship (Swartzberg et al., 2006). Under salt stress, a modified strategy of employing root-specific cytokinin overproduction under the direction of a constitutive promoter might help *Solanum lycopersicum* (tomato) plants increase plant growth and yield (Ghanem et al., 2011). In salt-treated *S. lycopersicum* plants, root-to-shoot cytokinin transport was significantly boosted, resulting in better ion homeostasis, vegetative growth, delayed leaf senescence (in plants with root-specific *HSP70*:*IPT* expression), and increased fruit yield (Ghanem et al., 2011). Thus, this research proposed a unique effective technique to reduce salt-induced agricultural yield constraints.

Transiently increasing the cytokinin level *via* the crucial distance between the gene and its promoter is another application of the genetic engineering method. By utilizing the constitutive 35S promoter the *A. tumefaciens IPT* gene was fused to the downstream of other genes, like *AtGolS2* (Arabidopsis galactinol synthase) or *AOC* (*Bruguiera sexangula* allene oxide cyclase), which were involved in cold and salt stress tolerance, respectively (Guo et al., 2010). Subsequently, *A. thaliana* transgenic plants carrying the *pVKH35S-AOC-ipt* and *pVKH35S-AtGolS2-ipt* genes were able to achieve a small rise in cytokinin levels, resulting in improved plant development, higher chlorophyll production, and longer flowering (Guo et al., 2010). Another method of transiently increasing cytokinin activity is to modify the expression of O-glycosyltransferase moderately. In response to the successful isolation of the *ZOG1* (*Zeatin O-glucosyltransferase*) gene from the *Phaseolus lunatus* plant, which codes for the ZOG protein, transgenic *N. tabacum* expressing *35S:ZOG1* and *SAG12:ZOG1* transgenes were developed (Martin et al., 1999; Marie et al., 2008). Therefore, the stress-induced elevation in calcium levels aided the *SAG12:ZOG1*-transformed plants in establishing growth more quickly than the WT plants at the post-drought recovery stage, as compared to the WT plants. In contrast, transgenic plants that had the *35S:ZOG1* construct had a slower recovery rate, which suggests that having more cytokinin in the plant before a long and severe drought period could have a negative effect (Marie et al., 2008).

Conditionally or locally boosted cytokinin synthesis has been shown to improve plant growth and yield in *Brassica napus* (canola) and *A. stolonifera* utilizing various promoter and *IPT* gene combinations (Kant et al., 2015; Xu et al., 2016). In the field, yields of IPT transgenic *B. napus* were higher in both stressed and non-stressed conditions (Kant et al., 2015). The information that cytokinin is a negative regulator of elongation development of the main root and root branching has been used to develop another engineering strategy to increase tolerance to drought in plants (Werner et al., 2003). *A. thaliana*, *N. tabacum* and *Hordeum vulgare* roots had lowered cytokinin levels when *CKX* genes were only expressed in the roots. This led to a larger root system with mostly unaltered shoot growth and development (Werner et al., 2010; Macková et al., 2013; Ramireddy et al., 2018). A greater survival rate of *CKX* transgenic *N. tabacum* plants under drought stress in an intermixed planting revealed that these plants were more effective in competing for limited water resources than WT tobacco plants (Werner et al., 2010). Increased cytokinin degradation in the roots of transgenic *H. vulgare* (barley) resulted in a reduced stress response to long-term drought conditions which includes increased stomatal conductance and CO_2 assimilation rates, decreased activation of critical ABA metabolism genes, and decreased build-up of ABA (Ramireddy et al., 2018). While large soil volume explored by *CKX* transgenic plants has been linked to some of these favourable benefits, other mechanisms, such as interplay between the cytokinin and ABA signalling pathways, may also be responsible for the altered response to drought stress in these plants (Vojta et al., 2016; Ramireddy et al., 2018).

Cytokinin related gene applications in genetic engineering have shown considerable promise for stress tolerance, leading to sustainable agriculture, however, they are primarily overexpressed research. Therefore, genetic engineering and breeding strategies leads to increase abiotic stress resistance by manipulating these genes. Although these molecular players have been extensively examined in non-crop plants such as *A. thaliana* and *N. tabacum*, they have yet to be investigated in agronomically important crops. Thus, the mechanism governing cytokinin signaling in economically significant crops may be an intriguing topic to investigate for abiotic stress improvement. Novel technology breakthroughs in the previous decade have highlighted the potential of *de novo* domestication of wild plants as a realistic approach for

developing abiotic stress-tolerant crops while ensuring food safety and security. Several biotechnological strategies are being proposed to get a better knowledge of the cytokinin-related gene and various phytohormonal pathways involved in plant responses to abiotic stress. In order to generate stress-tolerant crops, new generation tools are now accessible that enable precise genome editing in specific genes. Genome editing is an excellent approach for speeding up the development of enhancing tolerance to abiotic stresses. However, there are only few studies that describe the use of CRISPR and cytokinin-related genes to enhance abiotic tolerance in commercially significant crops (Table 2) (Ogata et al., 2020; Wang et al., 2022). Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR associated (Cas) proteins, or CRISPR/Cas, when integrated with *de-novo* domestication becomes an ideal strategy for modifying plant genome that has a potential to be one of the most promising possibilities to enhance stress tolerance.

Future prospects

There is mounting evidence that cytokinin plays a number of roles in plant responses to various stress. Stress-related cytokinin signaling pathways and several genes that encode cytokinin metabolism enzymes have been identified as functionally significant, although little is known about their downstream components. Remarkably, the majority of the same components engaged in cytokinin regulated development are also implicated in the stress response. This suggests that there has been no distinct evolution of stress response modules, but rather the response to stress is intricately associated with the control of development. In this regard, a better understanding of cytokinins interactions with known stress response pathways would improve our understanding of the hormone's function in regulating growth-defense trade-offs. To get a better understanding of the downstream events of the cytokinin signaling pathway and to identify linkages to traditional stress response pathways, refined genetic techniques and system analysis will be critical.

Many of the cytokinin responsive genes are engaged in signaling, metabolic, and transport systems that affect plant growth and development, and next-generation technologies have revealed insights into global transcriptome alterations in connection to cytokinin responsive genes (cytokinin response factors (CRFs), *CKXs, ARR1, ARR10,* and *ARR12*) (Shi et al., 2013; Abdelrahman et al., 2021). At the transcriptome and proteome levels, genome-wide investigations may reveal interaction protein–protein networks that regulates biological development processes in growing crop plants. Global insights into molecular mechanisms and genes involved in abiotic stress tolerance are viable targets for the development of novel strategies for crop improvements. Furthermore, many studies on the influence of cytokinins on stress are conducted in controlled conditions, suggesting that cytokinins are stress modulators that function through a growth-defense trade-off (Cortleven et al., 2019). Influences of cytokinins on genotype–environment interactions are still poorly understood in natural and/or in-field agricultural settings, and future studies should focus more rigorously on testing cytokinin stress modulation in natural environments for agricultural ecosystem management, especially in the context of climate change. Understanding the functions of cytokinin, a key growth-regulating hormone in stress defense is particularly vital for understanding the influence of a changing environment on plant development and ensuring the sustainability of food supply.

Concluding remarks

The significance of cytokinins in the regulation of key developmental processes has become more apparent in recent years, providing new insights. While significant progress has been achieved, the true challenge remains in deciphering the molecular mechanisms by which cytokinins govern these developmental processes. In general, cytokinin signaling and metabolism are important for abiotic stress tolerance, and manipulating genes in the signaling pathway in major crops might be advantageous for long-term agricultural sustainability. Furthermore, the growing amount of molecular data adds to our understanding of cytokinin interplay in its developmental aspects while also adding a new degree of complexity. In addition, there are several crosstalk pathways with other plant hormones that aids to the cytokinin large pleiotropy in stress-induced growth regulation.

Furthermore, the genetic mechanism behind abiotic stress has been unravelled with the recent advancement in genomics and transcriptomics technologies. Stress tolerance genes in CWRs and other cultivated crops may be mapped using transcriptome profiling, a powerful tool. Because of recent developments in next-generation sequencing (NGS) technology, it is now possible to create high-quality pangenomes for a wide range of crops, giving researchers a full picture of genetic diversity within each species as well as the total gene pool for each specific crop. It is now feasible to implement next-generation breeding effectively for a complex trait like abiotic stress tolerance by combining *de novo* domestication with genome editing tools like CRISPR/Cas system.

Author contributions

SM, MG, and UA: conceptualization, review structure, literature survey, and writing major original draft preparation. DS, NK, TM, MR, NJ, SJ, ML, RT, MK, R, DP, AM, AG, PB, and JP: writing-reviewing and editing, tables and figures preparation,

revision, data curing, suggestions, response, and guidance. AD: conceptualization, planned and designed review structure, critically revised the manuscript, overall guidance, supervision, suggestions, and final draft. All authors contributed to the writing or revision of the final manuscript. All authors have read and approved the final version of the manuscript for submission to this journal.

Funding

The Article Publishing Charge (APC) is received from the Wrocław University of Environmental and Life Sciences, Wrocław, Poland. We acknowledge the financial support provided by Wrocław University of Environmental and Life Sciences, Poland.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Genetics

ORIGINAL RESEARCH
published: 19 August 2022
doi: 10.3389/fgene.2022.931659

Genome-Wide Analysis and Evolutionary Perspective of the Cytokinin Dehydrogenase Gene Family in Wheat (*Triticum aestivum* L.)

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Cytokinin dehydrogenase (*CKX*; EC.1.5.99.12) regulates the level of cytokinin (CK) in plants and is involved in CK regulatory activities. In different plants, a small gene family encodes *CKX* proteins with varied numbers of members. These genes are expanded in the genome mainly due to segmental duplication events. Despite their biological importance, *CKX* genes in *Triticum aestivum* have yet to be studied in depth. A total of 11 *CKX* subfamilies were identified with similar gene structures, motifs, domains, cis-acting elements, and an average signal peptide of 25 amino acid length was found. Introns, ranging from one to four, were present in the coding regions at a similar interval in major *CKX* genes. Putative cis-elements such as abscisic acid, auxin, salicylic acid, and low-temperature-, drought-, and light-responsive cis-regulatory elements were found in the promoter region of majority *CKX* genes. Variation in the expression pattern of *CKX* genes were identified across different tissues in *Triticum*. Phylogenetic analysis shows that the same subfamily of *CKX* clustered into a similar clade that reflects their evolutionary relationship. We performed a genome-wide identification of *CKX* family members in the *Triticum aestivum* genome to get their chromosomal location, gene structure, cis-element, phylogeny, synteny, and tissue- and stage-specific expression along with gene ontology. This study has also elaborately described the tissue- and stage-specific expression and is the resource for further analysis of *CKX* in the regulation of biotic and abiotic stress resistance, growth, and development in *Triticum* and other cereals to endeavor for higher production and proper management.

Keywords: cytokinin, gene ontology, phylogenetics, synteny, wheat

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Edited by:
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Specialty section:
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

Received: *29 April 2022*
Accepted: *21 June 2022*
Published: *19 August 2022*

Citation:
Jain P, Singh A, Iquebal MA, Jaiswal S, Kumar S, Kumar D and Rai A (2022) Genome-Wide Analysis and Evolutionary Perspective of the Cytokinin Dehydrogenase Gene Family in Wheat (Triticum aestivum L.). Front. Genet. 13:931659. doi: 10.3389/fgene.2022.931659

INTRODUCTION

Wheat (*Triticum aestivum L.*) is a widely cultivated cereal grain that provides approximately 20% of the protein in human diet. It ranks second to corn and rice as the most important staple food, with global production of around 778.6 million tonnes of grain (USDA, 2022). Looking at the increasing human population, its consumption is estimated to rise to 900 million tonnes by 2050 (FAO, 2022). Cytokinins, the plant hormone family, affect crop yield in terms of grain size and number, and are involved in the regulation of biotic and abiotic stress (Cortleven et al., 2019). They are reported to

control both shoot and root architecture as well as crown formation (Chen L. et al., 2020). Cytokinin dehydrogenase (*CKX*) gene family members encode the enzyme *CKX*, and play an important role in the cell cycle, developmental processes, and senescence process of plants such as seed germination, shoot/root growth, photosynthesis, chloroplast formation, and crop productivity (Mok and Mok 2001; Takei et al., 2001; Werner et al., 2001). Recent research suggests that a majority of plants had cytokinin (CK) metabolic genes closely linked to them and, therefore, strongly regulate cytokinin content in different organs of plants. (Ashikari et al., 2005; Bartrina et al., 2011; Yuldashev et al., 2012). Further, CKs are adenine derivatives with a side chain at the N6 position, which causes the N6 substituent to be classified as an isoprenoid or aromatic side chain (Galuszka et al., 2000; Frebort et al., 2011). Since numerous changes have been observed in the CK content during grain development in crops like wheat and barley, genetic manipulation of the genes involved in CK homeostasis is frequently utilized for yield improvement in these crops (Schmuelling et al., 2003).

CKX (EC: 1.5.99.12) is the only known enzyme that permanently degrades CKs by cleaving the N6-unsaturated side chain of the CK to adenine and adenosine in a single step. The catalytic activity of *CKX* is catalyzed by two conserved domains, *namely,* a FAD-binding domain at the N terminus and a CK-binding domain at the C terminus of the protein. *CKX* activity was first discovered in crude extracts from tobacco plants (Paces et al., 1971). Subsequently, *CKX* was recognized as a member of a small gene family and several *CKX* genes were duplicated and identified in different plants. Cytokinin dehydrogenase has been identified as a significant enzyme present in different cereal crops. It has been reported in 13 gene families in *Zea mays* (Gu et al., 2010; Houba-Hérin et al., 1999; Brugière et al., 2003), 11 gene families in rice (Ashikari et al., 2005), 9 gene families in Arabidopsis (Galuszka et al., 2007), 13 gene families in *Brachypodium distachyon* (Mameaux et al., 2012), four gene families in *Hordeum vulgare* (Mameaux et al., 2012), and 11–14 gene families in wheat (Ogonowska et al., 2019; Shoaib et al., 2019; Song et al., 2012). In rice, an increase in CK is a product of low Os*CKX*2 expression which promotes the total yield by increasing the number of reproductive organs using short hairpin RNA-mediated silencing technology. This hinders the expression of Os*CKX*2 in rice, resulting in an increased number of tillers, thus increasing the grain weight (Ashikari et al., 2005; Yeh et al., 2015). Similarly, *Arabidopsis CKX* genes showed diverse countenance, which supports that differential expression of *CKX* genes can play a vital role in regulating CK levels (Bartrina et al., 2011).

The CKX gene family has been reported to show natural variations in the form of SNPs which were used for the potential marker-assisted selection for higher yield as in soybean (Nguyen et al., 2021). Besides, CKs are widely used for crop yield improvement/management because of their broader effects on plant growth/development and physiology. Applications of CRISPR-based gene-editing tools in manipulating cytokinin metabolism at the genetic level for yield improvement has been reported in literature (Mandal et al., 2022). Genome editing occurs via natural process of random mutagenesis, mostly through SNPs to improve yield (Songstad et al., 2017).

Although > 20 different cytokinins are reported in wheat (Sýkorová et al., 2008), the complete analysis on the early development time frame has not yet been reported for wheat. The hexaploidy, large genome size (~17 GB), and complicated interactions between the three genomes of wheat (*Triticum aestivum* L.) have led to limited information on the CKX gene families in this cereal crop.

This study aims at studying the genome-wide identification and distribution of *CKX* genes in wheat, motif characteristics, distribution and evolutionary relationships along with the presence of cis-elements in the promoter regions across 11 subfamily members of CKX. This study also aims at comparing motifs and domains present among the CKX family members at the protein level along with tissue- and stage-specific expression. This analysis will lead to useful information for further functional dissection of *CKX* genes in plants.

MATERIALS AND METHODS

Identification of *CKX* Family Members in *Triticum*

The *CKX* genes were identified from the *Triticum aestivum* (bread wheat) genome (IWGSC RefSeq), available at Ensemble plant database (https://plants.ensembl.org/index.html). The cytokinin dehydrogenase (*CKX*), FAD- and cytokinin-binding (cytokinin-bind PF09265) protein domain, family was obtained from the PFAM database (https://pfam.xfam.org/). HMMER 3.0 program was used to identify the *CKX* protein domain-containing putative protein in bread wheat. Blastp was performed on these proteins with parameters e < 1e− 5 and 50% identity, keeping the query as *Oryza sativa, Arabidopsis thaliana, Hordeum vulgare, and Zea mays* to find the putative *CKX* protein from the bread wheat protein sequence. The NCBI-CDD web server (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was used to confirm the candidate *CKX* genes in *Triticum aestivum*. ProtComp was used to predict the subcellular localization (Kamper et al., 2006) followed by the identification of the signal peptides and the location of their cleavage sites in *CKX* protein using SignalP (Armenteros et al., 2019). TargetP was used to identify the presence of N-terminal presequences: signal peptide (SP), mitochondrial transit peptide (MTP), chloroplast transit peptide (cTP), or thylakoid luminal transit peptide (lTP) in *CKX* proteins. Other protein parameters like molecular weight (MW) and theoretical isoelectric point (PI) of *CKX* proteins was found using the ExPASy server (Gasteiger et al., 2003). The subcellular localization predictions of the analyzed proteins were also performed using WoLF PSORT (https://wolfpsort.hgc.jp/).

Chromosomal Distribution, Gene Structure, and Conserved Motif of *CKX* Gene Family

The distribution of *CKX* genes was observed with its chromosomal location using Plant ensemble database (Bolser

TABLE 1 | Detailed information of the cytokinin dehydrogenase gene family (*Triticum aestivum*) with respect to their chromosomal location and general protein parameters.

Suggested Nomenclature	Gene ID	Protein Stable ID	Chromosome	Gene Start (bp)	Gene End (bp)	Gene Length	Protein Length	No. of Exons	Theoretical pI	Molecular Weight (Average) Dalton (Da)
CKX1_3A	TraesCS3A02G109500	TraesCS3A02G109500.1	3A	75545535	75547971	2436	524	3	5.59	56150.7
CKX1_3B	TraesCS3B02G128700	TraesCS3B02G128700.1	3B	107958655	107961101	2446	524	3	5.75	56149.79
CKX1_3D	TraesCS3D02G111300	TraesCS3D02G111300.1	3D	64761714	64764211	2497	524	3	5.78	56112.69
CKX2.1_3A	TraesCS3A02G311000	TraesCS3A02G311000.1	3A	549902478	549907314	4836	567	3	7.16	61590.05
CKX2.1_3B	TraesCS3B02G161100	TraesCS3B02G161100.1	3B	157763715	157768151	4436	578	3	5.81	62557.13
CKX2.1_3D	TraesCS3D02G143600	TraesCS3D02G143600.1	3D	107041806	107046044	4238	551	3	6.17	59559.72
CKX2.2.1_3A	TraesCS3A02G311100	TraesCS3A02G311100.1	3A	550047133	550050899	3766	552	3	5.56	59371.38
CKX2.2.1_3B	TraesCS3B02G161000	TraesCS3B02G161000.1	3B	157660250	157664488	4238	547	3	5.56	59014.96
CKX2.2.1_3D	TraesCS3D02G143500	TraesCS3D02G143500.1	3D	106736568	106740664	4096	547	3	5.57	59158.15
CKX2.2.2_3D	TraesCS3D02G143300	TraesCS3D02G143300.1	3D	105891267	105895512	4245	547	3	5.65	59195.27
CKX2.2.3_3D	TraesCS3D02G143200	TraesCS3D02G143200.1	3D	105704626	105709481	4855	523	3	5.24	56059.49
CKX3_1A	TraesCS1A02G159600	TraesCS1A02G159600.1	1A	286006369	286010711	4342	522	5	6.09	57687.03
CKX3_1B	TraesCS1B02G176000	TraesCS1B02G176000.2	1B	317439232	317444264	5032	522	5	6.28	57740.1
CKX3_1D	TraesCS1D02G157000	TraesCS1D02G157000.1	1D	221120218	221124390	4172	522	5	6.17	57673.02
CKX4_3B	TraesCS3B02G525300	TraesCS3B02G525300.1	3B	766978875	766981579	2704	525	5	6.57	57557.89
CKX4_3D	TraesCS3D02G475800	TraesCS3D02G475800.1	3D	576755407	576758217	2810	525	5	6.66	57540.78
CKX5_3A	TraesCS3A02G321100	TraesCS3A02G321100.1	3A	564437512	564442094	4582	530	5	5.94	57743.55
CKX5_3B	TraesCS3B02G344600	TraesCS3B02G344600.1	3B	554115957	554120243	4286	531	5	6.15	57876.68
CKX5_3D	TraesCS3D02G310200	TraesCS3D02G310200.1	3D	424349539	424353886	4347	531	5	6.03	57823.56
CKX7_6B	TraesCS6B02G214700	TraesCS6B02G214700.1	6B	290213802	290215403	1,601	533	1	8.23	58047.33
CKX7_6D	TraesCS6D02G172900	TraesCS6D02G172900.1	6D	159787916	159792494	4578	467	2	6.08	50655.82
CKX8_2A	TraesCS2A02G378300	TraesCS2A02G378300.1	2A	621182540	621186230	3690	528	5	5.62	57186.26
CKX8_2B	TraesCS2B02G395200	TraesCS2B02G395200.1	2B	560261181	560265014	3833	523	5	6.32	56987.2
CKX9_1B	TraesCS1B02G248700	TraesCS1B02G248700.1	1B	439845400	439847579	2179	521	5	6.68	58242.54
CKX9_1D	TraesCS1D02G237200	TraesCS1D02G237200.1	1D	326285642	326287883	2241	521	5	6.86	58326.58
CKX10_7A	TraesCS7A02G363400	TraesCS7A02G363400.1	7A	538150224	538152325	2101	551	2	6.22	59913.79
CKX10_7B	TraesCS7B02G264400	TraesCS7B02G264400.1	7B	484892227	484894256	2029	540	2	6.19	59024.94
CKX10_7D	TraesCS7D02G359700	TraesCS7D02G359700.1	7D	461956360	461958099	1739	532	2	6.15	57595.18
CKX11_7A	TraesCS7A02G536900	TraesCS7A02G536900.1	7A	714340482	714342889	2407	516	4	5.77	55390.7
CKX11_7B	TraesCS7B02G455000	TraesCS7B02G455000.1	7B	715713483	715716700	3217	516	4	5.93	55654.04
CKX11_7D	TraesCSU02G106300	TraesCSU02G106300.1	Un	93131788	93134766	2978	517	4	5.99	55628.01

et al., 2016). Also, their gene structures were displayed in the Gene Structure Display Server (GSDS: http://gsds.gao-lab.org/; Guo et al., 2007). Motif identification was performed using the MEME suite. The multiple expectation maximization for the motif elicitation with MEME v4.9. (Bailey and Elkan, 1994) utility program (http://meme-suite.org/tools/meme) was used to display motifs in *CKX* proteins.

Phylogenetic and Synteny Analysis of the *CKX* Gene Family

Multiple sequence alignment was performed through ClustalW (Thompson et al., 1994) based on the full sequence of the proteins with default parameters from *Triticum aestivum*, *Oryza sativa, Arabidopsis thaliana, Hordeum vulgare, and Zea mays.* The phylogenetic tree was constructed by maximum likelihood method using the MEGA11 (Tamura et al., 2021) software with 1,000 replicates as bootstrap values. iTOL (https://itol.embl.de/) was used to visualize the phylogenetic tree and motifs in the *CKX* proteins (Letunic and Bork, 2019).

TBtool (Chen et al., 2020b) (https://github.com/CJ-Chen/TBtools/releases) was used for synteny analysis of *CKX* genes in *Triticum aestivum* with *Aegilops tauschii* via the MCScanX function. MCScanX is a toolkit for the detection and evolutionary analysis of gene collinearity (Wang et al., 2012).

Promoter and Gene Ontology Analysis of *CKX* Gene Family With Expression Across Different Tissue and Stages

A 1.5-kb region upstream from the start of the gene was retrieved for the promoter analysis from the *CKX* gene. The PlantCARE (Lescot et al., 2002) database was used for the identification of cis-regulatory elements in the promoter region. *CKX* genes in developmental and tissue time course expression in *Triticum* varieties were obtained from the wheat expression browser (http://www.wheat-expression.com/download) to detect the expression profile of the *CKX* gene. Gene ontology analysis was performed using the PlantRegMap tool (Tian et al., 2020).

RESULTS AND DISCUSSION

Identification of the *CKX* Gene Family in *Triticum aestivum*

CKX proteins were identified from the whole genome of *Triticum aestivum* using blastp and HMM. The putative *CKX* proteins were confirmed using Pfam and a conserved domain database. In earlier published literature, 11–14 gene family members have been reported in wheat (Ogonowska et al., 2019; Shoaib et al., 2019). In rice, 11 *CKX* homologs have been identified and in *Arabidopsis thaliana*, 7 homologs of the *CKX* gene, in *Hordeum vulgare* 15, and in *Zea mays* 34 homologs of *CKX* were identified.

Earlier studies on *CKX* have given different nomenclatures to the different gene families of *CKX*. We have renamed *CKX* based on recent nomenclature in IWGSC taking into consideration the earlier reports and true orthologs in rice and maize. Finally, 11 *CKX* families were identified and their genomic location was extracted from Plant Ensemble. The average molecular weight (MW) and theoretical isoelectric point (PI) of *CKX* proteins ranged from 50,000 to 60,000 Da and from 5-8, respectively (**Table 1**).

To determine whether the analyzed proteins are directed to the secretory pathway and secreted out of the cell, predictions of the presence of signal peptides at the N terminus of the sequence were performed. For this purpose, program SignalP-6.0 and TargetP were used. Using the SignalP, the signal peptides were found in 21 of the analyzed proteins. The average length of sequences provided as a signal peptide was 25 aa. Similar results were obtained using the TargetP tool—the signal peptides were found in 25 of the investigated sequences (**Figure 1A**; **Supplementary Tables S1A,B**). The most likely location of the *CKX* protein was predicted as extracellular using the ProtComp tool. The subcellular localization prediction program, WoLF PSORT helps to trace the protein in the intracellular compartments. Out of the total analyzed proteins, 13 sequences were classified as chloroplast, 7 as the vacuole membrane protein, 4 as extracellular, 3 as endoplasmic reticulum, 2 as cytoplasmic, 1 as the mitochondria, and 1 as the nuclear pathway (**Figure 1B**).

Chromosomal Distribution, Gene Structure, and Conserved Motif of *CKX* Genes

Motif diversity and gene structure promote evolution in gene families (Zhu et al., 2020). We identified 31 Ta*CKX* gene family members that were present on chromosomes, linked with the A, B, and D subgenomes of *Triticum*. Among the 11 gene families, Ta*CKX*2 has gene duplication on chromosome 3D, that is further subdivided, based on homology, into different groups (Mameaux et al., 2012; Lu et al., 2015). *CKX* gene families identified different homologs in the subgenome A, B, and D. All *CKX* gene family members were found on chromosomes 1 to 7, except chromosome 6 (**Figure 2**). One of the gene families, *i.e.,* the *CKX1*1 subfamily was not present on any of the 21 chromosomes (of A, B, and D subgenomes) in IWGSC Refseq. In the *CKX* genes, a number of exons varied from 1 to 5. Only *CKX7*_6B identified one exon and no intron while 11 *CKX* genes and 13 *CKX* genes had 3 exons and 5 exons in their gene structures, respectively (**Figure 3**). The gene transfer format (gff) was used to extract the 5′ UTR, 3′ UTR, exonic, intronic, start, and end position of the gene. It was found that the 5' UTR position was absent for *CKX2.1_3B, CKX2.2.3_3D, CKX7_6B, CKX7_6D, CKX10_7A, CKX11_7A, CKX10_7B,* and *CKX*10_7D gene family members in the gff file. Intronic region regulated region expression via small and long noncoding RNA in genome (Jain et al., 2021). *CKX* gene structures showed that a majority of the genes had the presence of more than one intron. However, the length of the intron kept varying between the subgroups. The longest intron was found in *CKX*7_6D. Previous studies show that introns present in β-tubulin might regulate the expression and evolution of genes in *Fusarium graminearum* (Li et al., 2019).

Comparison of 31 *CKX* family members showed the presence of the PLN02441 domain family. The predicted *CKX* proteins had typical FAD- and CK-binding domains, which were specific to

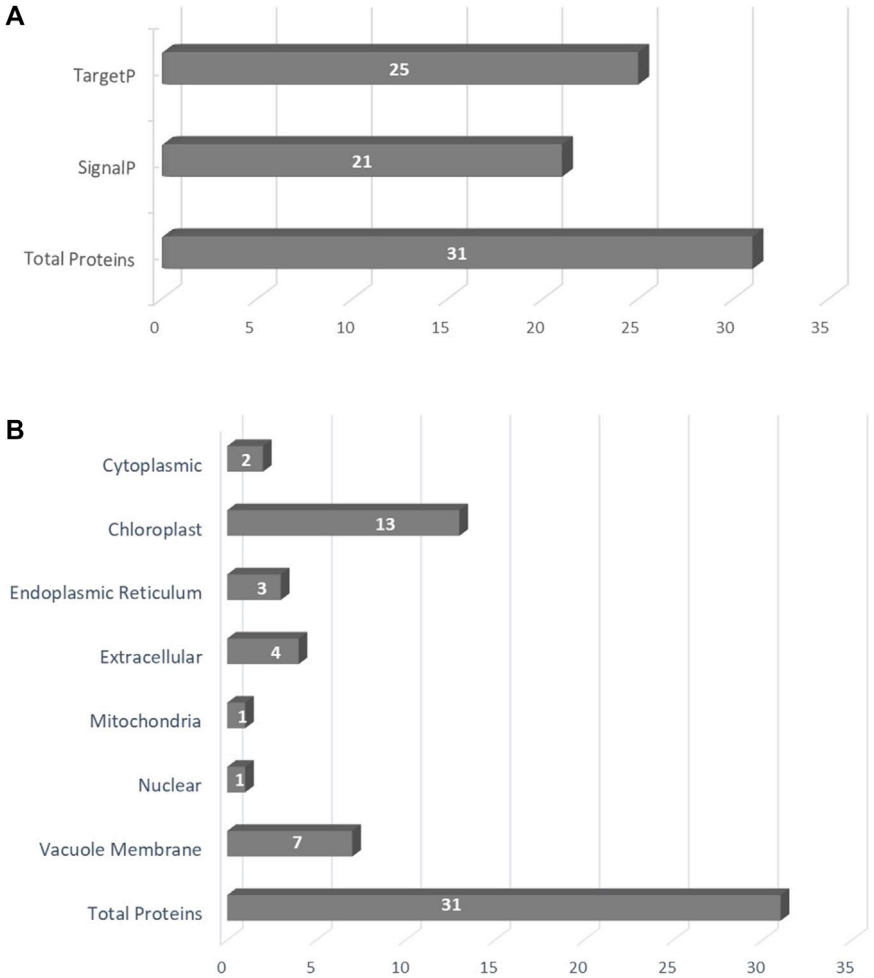

FIGURE 1 | (A) The subcellular localization of predicted proteins; **(B)** The presence of signal peptides in the analyzed sequences using SignalP and TargetP.

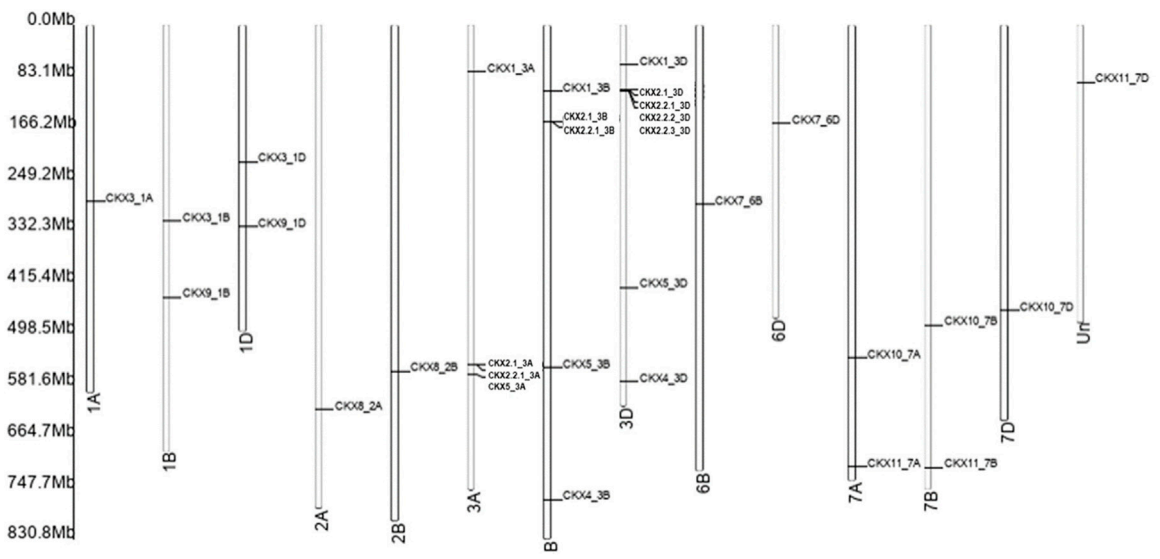

FIGURE 2 | Chromosomal locations of the CKX gene family in *Triticum aestivum*.

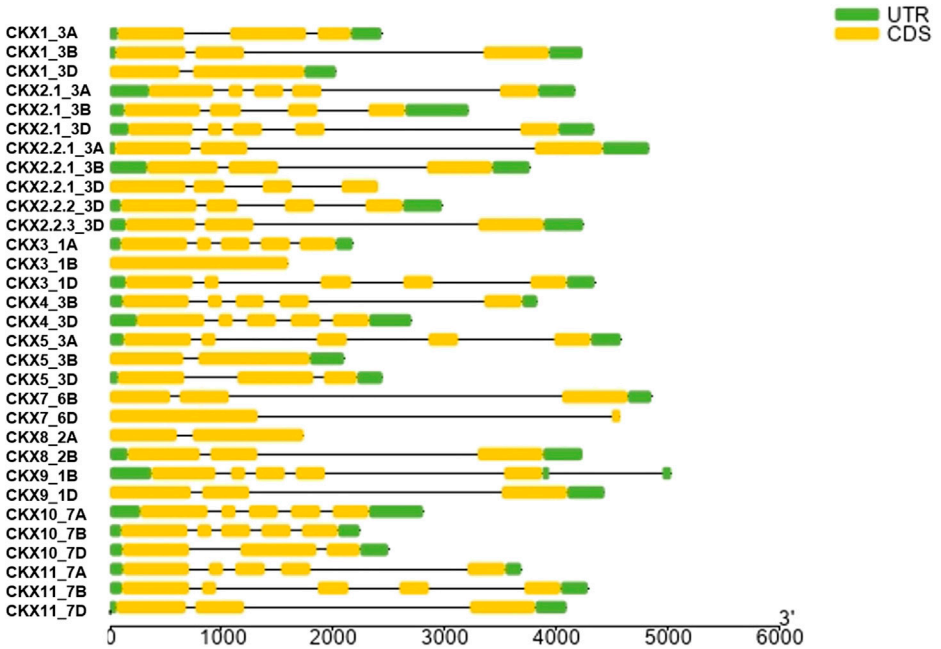

FIGURE 3 | Gene structures of the CKX gene family in *Triticum aestivum*.

CKX family members as shown in **Supplementary Figure S1**. Similar results have been obtained in genome-wide analysis and identification of cytokinin oxidase/dehydrogenase (*CKX*) gene family in foxtail millet (Liu et al., 2018).

Phylogenetic and Synteny Analysis of the *CKX* Gene Family

Highly conserved motif regions in *CKX* proteins were identified using MEGA 11 in classic mode with the maximum number of motifs set at ten. Ten-motif consensus sequences were identified in all 31 *CKX* proteins as shown in **Figure 4A**. To uncover the evolutionary relationships among *Triticum aestivum*, *Oryza sativa, Arabidopsis thaliana, Hordeum vulgare,* and *Zea mays CKX*s, the amino acid sequences of the *CKX* genes were aligned using ClustalW. This was followed by the maximum likelihood analysis method to construct a phylogenetic tree (**Figures 4A,B**). All the proteins fell into four major clusters (I, II, III, and IV). Cluster I contained 39 members (with 16 of *Triticum aestivum*, 1 *Oryza sativa*,1 *Hordeum vulgare*, 3 *Zea mays,* and 3 *Arabidopsis thaliana,* respectively). This could be further divided into the following subclusters: IA,IB,1C,1D, 1E, and a diverge member (*CKX*). Cluster II included 18 members (with five of *Triticum aestivum*, two *Oryza sativa*, two *Hordeum vulgare,* and nine *Zea mays*) and could be separated into subclusters IIA and IIB. Cluster III contained 11 members (three *Triticum aestivum*, one *Oryza sativa*, one *Hordeum vulgare*, and five *Zea mays*) that could be separated into subclusters IIIA and IIIB. Cluster IV had 30 members (with seven *Triticum aestivum*, three *Oryza sativa*, five *Hordeum vulgare,* six *Zea mays,* and two *Arabidopsis thaliana,* respectively) which could be further divided into subclusters IVA and IVB.

The paralogs have been separated into two subgene families (*CKX2.1* and *CKX2.2*) as shown in the phylogenetic tree (**Figure 4B**). Notably, KAE8806376.1 and LOC_Os01g10110.1 from *Hordeum vulgare* and *Oryza sativa*, respectively grouped with the *CKX2.1* and *CKX2.2, CKX1* was grouped with AAK51495.1 and KAE8807039.1 (*Hordeum vulgare*) and *CKX7* with KAE8776326.1 and KAE8768522.1 (*Hordeum vulgare*) subclusters, respectively. Similarly, Cluster II *CKX3* 1A was grouped with KAE8771049.1 and LOC_Os10g34230.1 (*Oryza sativa*) and *CKX8* 2A were grouped with KAE8805484.1 (*Hordeum vulgare*) while *CKX8* 2B was grouped with LOC_Os04g44230.1 (*Oryza sativa*). In Cluster III, *CKX11* was clustered with KAE8774039.1 (*Hordeum vulgare*) and in Cluster IV, *CKX5* 3D was clustered with KAE8804960.1 and *CKX7* 3D LOC_Os01g71310.1. Functional investigation of these new members is critical as they could be of considerable importance for genetic improvement.

Gene duplication, an indispensable mechanism, can expand new genes that share similar or different functions (Ohno., 1970). Therefore, we analyzed the duplication events that occurred in the gene family through synteny and collinearity of the *CKX*S gene. Three *CKX2* have undergone gene duplication suggesting that the *CKX2s* on chromosome 3D could be subdivided into two groups based on their homology. Collinearity is identified between *Triticum aestivum* and *Aegilops tauschii* as shown in (**Figure 5**; **Supplementary Table S2**). Similar results for synteny has been identified in *Brassica oleracea* and *Arabidopsis thaliana CKX* genes (Zhu et al., 2022)

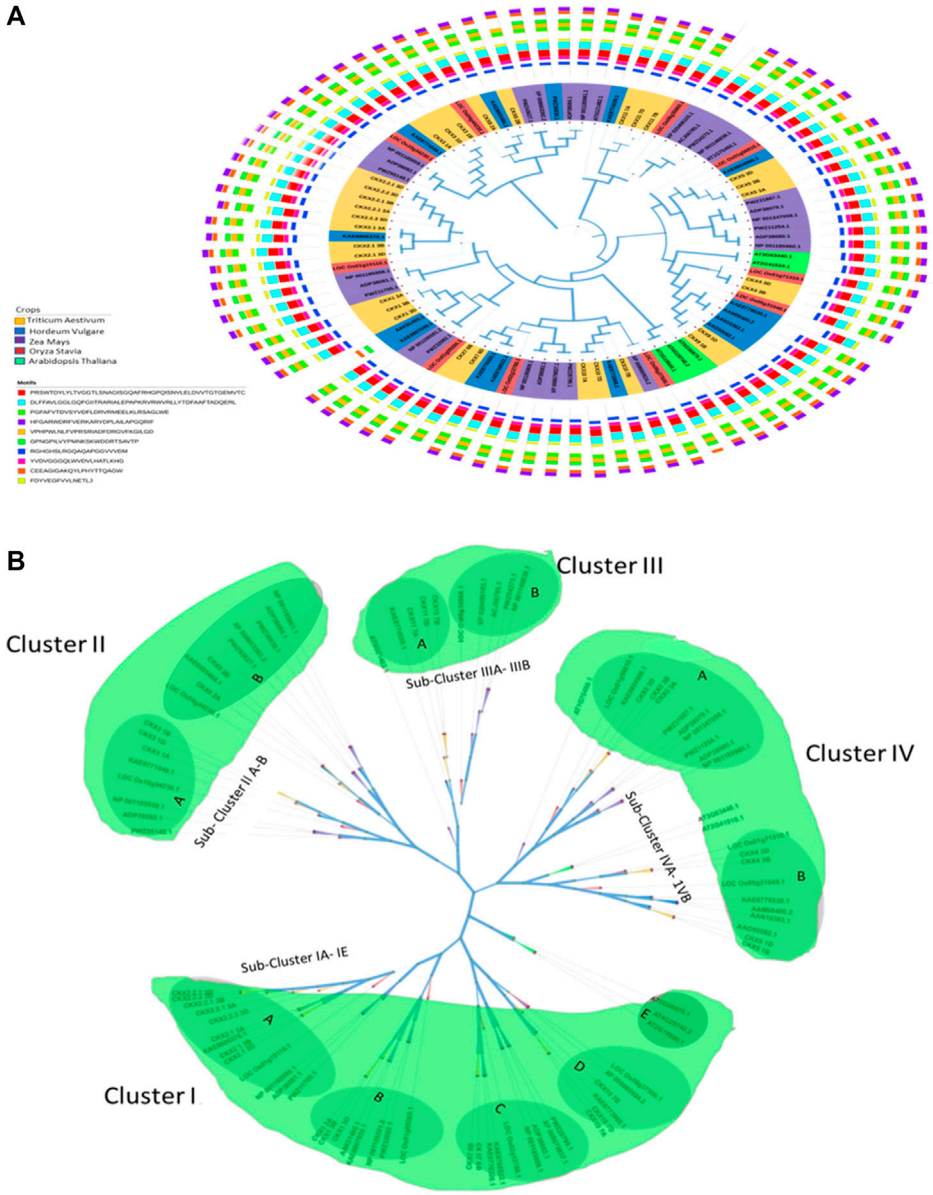

FIGURE 4 | (A) Phylogenetic tree for CKX proteins of *Triticum aestivum*, *Oryza sativa, Arabidopsis thaliana, Hordeum vulgare,* and *Zea mays*. **(B)**: Four major clusters of CKX proteins of *Triticum aestivum, Oryza sativa, Arabidopsis thaliana, Hordeum vulgare*, and *Zea mays* using the maximum likelihood method.

FIGURE 5 | Synteny and collinearity analysis between *Triticum aestivum* and *Aegilops tauschii*.

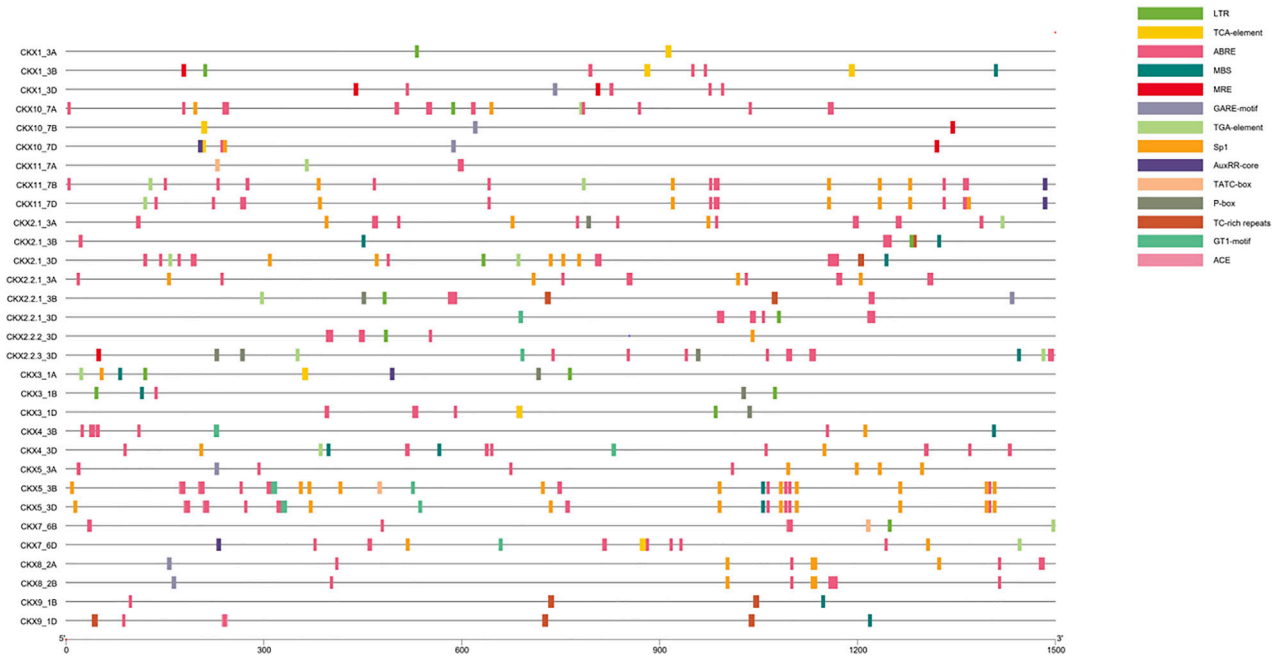

FIGURE 6 | Promoter analysis of CKX gene family in *Triticum aestivum*.

Promoter and Gene Ontology Analysis of *CKX* Gene Family

Cis-regulatory elements present in the promoter region play an important role in regulating gene expression of metabolic pathway-related genes (Lescot et al., 2002). In a 1.5-kb upstream region of *CKX* family members, the cis-regulatory elements predicted were mainly phytohormone responsive (salicylic acid, abscisic acid, gibberellin, auxin-responsive element), drought, light, defense, and stress responsive (**Figure 6**; **Supplementary Table S3**). Cis-acting element involved in low-temperature responsiveness (LTR) was found in *CKX1_3A, CKX1_3B, CKX2.1_3B, CKX2.1_3D, CKX2.2.1_3B, CKX2.2.1_3D, CKX2.2.2_3D, CKX3_1A,CKX3_1B,CKX3_1D, CKX7_6B,* and *CKX1-_7A*. Transcription factor MYB-binding site involved in drought inducibility (MBS) was found in *CKX1_3B, CKX2.1_3B, CKX2.1_3D, CKX2.2.3_3D, CKX3_1A, CKX3_1B, CKX4_3B, CKX4_3D, CKX5_3B, CKX5_3D, CKX9_1B,* and *CKX9_1D*. The MYB-binding site involved in light responsiveness (MRE) was found in *CKX1_3B, CKX1_3D, CKX2.2.3_3D, CKX1-_7B,* and *CKX1-_7D*. Light responsive element (*Sp1*) was found in *CKX2.1_3A, CKX2.1_3D, CKX2.2.1_3A, CKX2.2.2_3D, CKX3_1A, CKX4_3B, CKX4_3D, CKX5_3A, CKX5_3B, CKX5_3D, CKX7_6D, CKX8_2A, CKX8_2B, CKX1-_7A, CKX1-_7D, CKX11_7B,* and *CKX11_7D*. Light responsive element (GT1-motif) was found in *CKX2.2.1_3D, CKX2.2.3_3D, CKX4_3B, CKX4_3D, CKX5_3B, CKX5_3D,* and *CKX7*_6D. Cis-acting element involved in light responsiveness (ACE) was observed in *CKX5*_3B, *CKX5_3D, CKX7_6B,* and *CKX9_1D*. Cis-acting element involved in defense and stress responsiveness (TC-rich repeat) was found in *CKX2.1_3B, CKX2.1_3D, CKX2.2.1_3B, CKX9_1B,* and *CKX9_1D*. In tomato TC-rich repeats were found in seven promoters of protein disulfide isomerases (PDI) and high expression of PDI was found in response to abiotic stress in tomato (Wai et al., 2021). Cis-acting element involved in salicylic acid responsiveness (TCA element) was found in *CKX1_3A, CKX1_3B, CKX2.1_3A, CKX3_1A, CKX3_1D, CKX7_6D, CKX1_7B,* and *CKX1-_7D*. Cis-acting element involved in the abscisic acid responsiveness (ABRE) was found in *CKX1_3B, CKX1_3D, CKX2.1_3A, CKX2.1_3B, CKX2.1_3D, CKX2.2.1_3A, CKX2.2.1_3B, CKX2.2.1_3D, CKX2.2.2_3D, CKX2.2.3_3D, CKX3_1B, CKX3_1D, CKX4_3B, CKX4_3D, CKX5_3A, CKX5_3B, CKX5_3D, CKX7_6B, CKX7_6D, CKX8_2A, CKX8_2B, CKX9_1B, CKX9_1D, CKX1-_7A, CKX1-_7D, CKX11_7B,* and *CKX11_7D*. Gibberellin-responsive element (GARE-motif) was found in *CKX1_3D, CKX2.2.1_3B, CKX5_3A, CKX8_2A, CKX8_2B, CKX1-_7B, and CKX1-_7D*. Auxin-responsive element (TGA-element) was *found in CKX2.1_3D, CKX2.2.1_3B, CKX2.2.3_3D, CKX3_1A, CKX4_3D, CKX7_6B, CKX7_6D, CKX1-_7A, CKX11_7B,* and *CKX11_7D*. Cis-acting regulatory element involved in auxin responsiveness (AuxRR-core) was found in *CKX3_1A, CKX7_6D, CKX1-_7D, CKX11_7B,* and *CKX11_7D*. Cis-acting element involved in gibberellin responsiveness (*TATC-box*) was found in *CKX5_3B* and *CKX7_6B*. Gibberellin-responsive element (*P-box*) was found in *CKX2.1_3A, CKX2.2.1_3B, CKX2.2.3_3D, CKX3_1A, CKX3_1B,* and *CKX3_1D*. In *Brassica napus, the CKX* gene family also has abscisic acid-responsive elements (ABRE), auxin-responsive elements

FIGURE 7 | Heatmap for gene expression trend of CKX gene family in different tissue and stages of *Triticum aestivum*.

(TGA-element), gibberellin-responsive elements (GARE-motif, P-box, and TATC-box), and salicylic acid-responsive elements (TCA-element)(Liu et al., 2018). Phytohormone-responsive cis-elements were found in majority of the promoter region of *CKX* genes, reflecting that cytokinins are involved in the development and growth of the plant. Cis-acting element involved in the abscisic acid responsiveness (ABRE) was found in the highest number (27) among all responsive elements. Literature supports the cross-talk between cytokinin and abscisic acid (El-Showk et al., 2013).

Validation of CKX Gene Family's Expression Across Different Tissues of Wheat

Cytokinin receptor kinases (AHK2 and AHK3) inhibit ABA signaling for regulating cold stress response (Jeon et al., 2010). Hence, *CKX* and ABA crosstalk to regulate the stress responses. It has been validated earlier using qRT-PCR that ABA downregulates *CKX* genes (Werner et al., 2006). *CKX5_3B, a* family member of *CKX,* has the highest number of cis-elements.

The different regulatory elements predicted responsive to abiotic and biotic stress show that the *CKX* family is stabilizing CK content under both abiotic and biotic stresses. Gene expression pattern helps to identify the key genes playing a role in an important biological function (Jain et al., 2017; Jain et al., 2019). *CKX* gene family members in our study show differential tissue-specific expression (**Figure 7**). Expression of GmCKX03, 05, 10, and 11 was reported to be low while GmCKX13, 15, and 16 were highly expressed in soybean (Le et al., 2012). From the comparative expression analysis of PtCKXs, OsCKXs, ZmCKXs, and AtCKXs, it was found that tissue-specific gene expression pattern was conserved in each phylogenetic group (Gu et al., 2010). The expression data for *CKX* genes for different studies in *Triticum* were studied from a wheat expression browser. In reproductive stages grain tissue shows expression of *CKX1, CKX2, CKX3, CKX4, CKX5, CKX8, CKX9,* and *CKX11* while *CKX7* And *CKX10* show very low-level expression in the majority of the studies. In leaf and shoot tissue under all three stages, namely, reproductive, seedling, and vegetative, we found a good level of expression of *CKX3, CKX4, CKX5, CKX8, CKX9,* and *CKX11* while *CKX1* and *CKX2* had a lower level of expression. *CKX7* and *CKX 10* were found to have the least expression in shoot and leaf tissues in all three developmental stages for the majority of the studies. In root tissues, *CKX1, CKX3, CKX4, CKX5, CKX8,* and *CKX11* showed a good level of expression while *CKX9* and *CKX7* had a low level of expression while *CKX2* and *CKX10* have the lowest level of expression in reproductive, seedling, and vegetative stages for the majority of the samples for studies reported in wheat expression data. In spike tissue at the reproductive stage, *CKX7* and *CKX10* have very low expression while the rest of *CKX*

have a good level of expression. Chen et al., 2020a reported the higher expression of *CKX1* and *CKX2* during grain development similar to our results. Overall, CKX7 expression was at lower levels in the leaf, inflorescence, and spike tissue. CKX4 and CKX5 had a better level of expression in the leaf tissue. In all the tissues studied, CKX3 and CKX11 were expressed at various levels while the expression of CKX10 was found at lower level in all tissues except leaves. These expression trends across tissues found in our study are in concordance with the qRT-PCR results of wheat (Ogonowska et al., 2019). CKX genes functionally diverged and expanded after duplication in angiosperm having tissue/organ-specific patterns that show abiotic stimulus response of expression (Wang et al., 2020).

Gene ontology analysis shows the enrichment of the biological processes, cytokinin metabolic process, cellular hormone metabolic process, hormone metabolic process, and regulation of hormone levels (**Supplementary Figure S2**). The molecular functions enriched were cytokinin dehydrogenase activity, oxidoreductase activity, acting on the CH-NH group of donors, and flavin adenine dinucleotide binding (**Supplementary Figure S3**). Gene ontology analysis shows the enrichment of cellular hormone metabolic process, regulation of hormone levels, and molecular functions like oxidoreductase activity and flavin adenine dinucleotide binding were enriched which shows the role of *CKX* in regulating developmental events, enhancing grain yield in rice and enhancing drought and heat stress tolerance in tobacco (Mackova et al.,2013; Werner et al., 2010).

CONCLUSION

In this study, we identified 31 members of the *CKX* family in *Triticum* and analyzed their distribution on chromosome, subcellular localization, molecular weight, theoretical isoelectric point, signal peptide, motifs, domains, gene structure, cis-elements, and phylogenetic relationship among *CKX* members. The highest number of phytohormone-responsive cis-elements was found in the *CKX* gene, so, the protomer region has elements involved in the development and growth of *Triticum*. *CKX7* and *CKX10* were found to have very low gene expression compared to other *CKX* subgene family members in both reproductive and vegetative stages of *Triticum*. The *CKX* expression from the wheat express database and the analysis at vegetative and reproductive stages in different tissues showed that *CKX* has tissue-specific expression. Gene ontology analysis showed the enrichment of cellular hormone metabolic process, regulation of hormone levels, and molecular functions like oxidoreductase activity and flavin adenine dinucleotide binding that obviates the role of *CKX* in regulating developmental events, and enhancing grain yield and stress tolerance. This study gives a platform for the further identification of the *CKX* family and increases our understanding about the role of *CKX* in the regulation of biotic and abiotic stress resistance, growth, and development in *Triticum* to endeavor for higher production and proper management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MI and SJ conceived the theme of the study. PJ and AS performed the computational analysis and drafted the manuscript. MI, SJ, SK, and DK, and AR edited the manuscript. All co-authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Biotechnology, Government of India (BT/Ag/Network/Wheat/2019-20), and Indian Council of Agricultural Research (ICAR), Ministry of Agriculture and Farmers' Welfare, Government of India, for the use of the Advanced Super Computing Hub for Omics Knowledge in Agriculture (ASHOKA) facility at ICAR-IASRI, New Delhi, India, created under the National Agricultural Innovation Project. The authors further acknowledge the supportive role of the Director of ICAR-IASRI, New Delhi, and Director, ICAR-NBPGR, New Delhi, India. The authors are thankful to the Department of Biotechnology, Government of India, for financial support in the form of research project "Germplasm Characterization and Trait Discovery in Wheat using Genomics Approaches and its Integration for Improving Climate Resilience, Productivity and Nutritional quality" (BT/Ag/Network/Wheat/2019-20).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.931659/full#supplementary-material

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frontiers | Frontiers in Genetics

TYPE Original Research
PUBLISHED 26 August 2022
DOI 10.3389/fgene.2022.957360

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Genomics,
a section of the journal
Frontiers in Genetics

RECEIVED 31 May 2022
ACCEPTED 08 July 2022
PUBLISHED 26 August 2022

CITATION
Mandal NK, Kumari K, Kundu A, Arora A, Bhowmick PK, Iquebal MA, Jaiswal S, Behera TK, Munshi AD and Dey SS (2022), Cross-talk between the cytokinin, auxin, and gibberellin regulatory networks in determining parthenocarpy in cucumber.
Front. Genet. 13:957360.
doi: 10.3389/fgene.2022.957360

Cross-talk between the cytokinin, auxin, and gibberellin regulatory networks in determining parthenocarpy in cucumber

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Cucumber is a model plant for studying parthenocarpy with abundant slicing- and pickling-type germplasm. This study was undertaken to understand the role of the important cytokines (CKs), auxin (AUX) and gibberellin (GA) biosynthesis and degradation genes for the induction of parthenocarpy in slicing and pickling germplasm. Two genotypes of gynoecious parthenocarpic cucumber, PPC-6 and DG-8, along with an MABC-derived gynoecious non-parthenocarpic line, IMPU-1, were evaluated in this study. The slicing and pickling cucumber genotypes PPC-6 and DG-8 were strongly parthenocarpic in nature and set fruit normally without pollination. Endogenous auxin and gibberellin were significantly higher in parthenocarpic than non-parthenocarpic genotypes, whereas the concentration of cytokinins varied among the genotypes at different developmental stages. However, the exogenous application of Zeatin and IAA + Zeatin was effective in inducing parthenocarpic fruit in IMPU-1. Expression analysis with important CK, AUX, and GA biosynthesis-related genes was conducted in IMPU-1, PPC-6, and DG-8. The expression of the CK synthase, *IPT, IPT3, PaO, LOG1, LOG2, CYP735A1,* and *CYP735A2* was up-regulated in the parthenocarpic genotypes. Among the transcription factor response regulators (RRs), positive regulation of *CSRR8/9b, CSRR8/9d, CSRR8/9e*, and *CSRR16/17* and negative feedback of the CK signalling genes, such as *CsRR3/4a, CsRR3/4b, CsRR8/9a*, and *CsRR8/9c*, were recorded in the parthenocarpic lines. Homeostasis between cytokinin biosynthesis and degradation genes such as CK oxidases (*CKXs*) and CK dehydrogenase resulted in a non-significant difference in the endogenous CK concentration in the parthenocarpic and non-parthenocarpic genotypes. In addition, up-regulation of the key auxin-inducing proteins and GA biosynthesis genes indicated their crucial role in the parthenocarpic fruit set of cucumber. This study establishes the critical role of the CKs, AUX, and GA regulatory networks

and their cross-talk in determining parthenocarpy in slicing and pickling cucumber genotypes.

KEYWORDS

cucumber, parthenocarpy, slicing and pickling cucumber, cytokinin, gene expression, endogenous level, exogenous spray

Introduction

Successful pollination and fertilization in flowering plants are integral to fruit setting and development. However, a few plant species exhibit a typical mechanism for successful fruit setting and development without fertilization—termed parthenocarpy (Zinn et al., 2010; Knapp et al., 2017). Elongation of the pollen tubes and development of seeds result in the release of several phytohormones which facilitate the fruit set and enlargement of ovaries (Crane, 1964; Ozga et al., 2003). Cucumber (*Cucumis sativus* L.) is a model plant species for genetic and genomic study (Li et al., 2014); some of the genotypes of this important vegetable crop have the ability of parthenocarpic fruit development. Cucumber is the fourth most important vegetable crop and is cultivated in more than 150 counties around the world. It has emerged as the top crop for protected cultivation because of its ability to set fruit without pollination. The productivity of parthenocarpic cucumber cultivars is much higher than that of non-parthenocarpic monoecious genotypes which require successful cross-pollination for fertilization and fruit development. Cucumber has emerged as a model organism for understanding parthenocarpy because of its abundant parthenocarpic germplasm. Fruit set without pollination in a gynoecious genotype with only female flowers has enabled a manifold increase in cucumber productivity, giving it high potential as an economic crop for protected cultivation (Sharif et al., 2022).

Parthenocarpy in cucumber is a complex phenomenon controlled by a series of genetic mechanisms and the endogenous concentration of phytohormones (Gou et al., 2022; Sharif et al., 2022). The interest in parthenocarpic traits in horticultural crops is increasing because of their importance in improving fruit quality, tolerance to biotic and abiotic stresses, and reduced fruit drop. The development of seedless fruit results in greater flesh content as seeds and seed cavities are replaced by edible pulp or expended mesocarp (Liu et al., 2018). Endogenous levels of important plant hormones in the ovaries are reported to be closely associated with the parthenocarpic development of fruit pulp (Li et al., 2014; Su et al., 2021). Cross-talk between auxins (AUX), cytokinin (CKs) and gibberellins (GAs) in fruit set and parthenocarpic fruit development have been reviewed by Kumar et al. (2014) and Sharif et al. (2022) in important horticultural crops.

CKs play a major role in fruit set and their further development. It is observed that many plants accumulate high concentrations of endogenous CKs during the development of fruit; its exogenous application promotes parthenocarpic fruit development (Lu et al., 2016; Qian et al., 2018; Chai et al., 2019). CK stimulates cell division during fruit development and promotes cell proliferation in the ovarian tissues. CK also increases initial fruit set and delays fruit abscission in the case of parthenocarpic fruit. In the absence of pollination and fertilization, where the process of fruit set is about to fail, high concentrations of CKs can promote the quick proliferation of ovarian tissue and the retention of ovaries to develop into fully grown fruits (Kim et al., 1992). Homeostasis between CK synthesis and catabolism determines spatial and temporal biosynthesis (Frébort et al., 2011). CKs are reported to promote parthenocarpy in many fruits and vegetables such as tomato, cucumber, watermelon, eggplant, grape, and fig. In the case of tomato, concentrations of cytokinin ribosides and isopentenyladenine, and transcript levels of CK biosynthetic genes such as *SlPT3*, *SlPT4,* and *SlLOG6* were high during anthesis (Ren et al., 2011; Matsuo et al., 2012). Concentrations of trans-zeatin and transcript levels of *SlPT1*, *SlPT1*, *SlLOG2,* etc. were increased after anthesis (Matsuo et al., 2012). Indole-3-acetic acid (IAA) is reported as having the potential of inducing parthenocarpy in important vegetable crops like tomato, cucumber and zucchini (Martinelli et al., 2009; Pomares-Viciana et al., 2017). In addition, a single gene such as a transcriptional factor or a receptor in phytohormone signalling pathways can also control parthenocarpy (Martí et al., 2007; Serrani et al., 2010; Fuentes et al., 2012). The auxin response factor (ARF) and AUX/IAA are two important auxin-responsive gene families reported to be related to parthenocarpic fruit development in *Arabidopsis* and tomato (Kumar et al., 2014). Among the different AUX biosynthesis pathways, the role of *Trp-IPyA* (tryptophan-indole-3-pyruvic acid) has been established in the development of parthenocarpic fruits. The role of the *YUCCA10*, *PavYUCCA10*, *SlTAR1*, *ToFZY2*, *ToFZY3,* and *PARENTAL ADVICE-1 (PAD-1)* genes in the parthenocarpic fruit development of loquat, tomato and eggplants have been reviewed in detail by Sharif et al. (2022). The cultivation of GA signalling in ovules and valves was reported to be because of fertilization-triggered AUX signalling in *Arabidopsis* (Dorcey et al., 2009). Interaction among AUX and GA signalling pathways is also reported to be essential for fruit set and development (Srivastava and Handa, 2005; de; Jong et al., 2011; Carrera et al., 2012; Ruan et al., 2012). Auxin biosynthesis genes encoding proteins such as *YUCCA5*, *YUCCA11*, and tryptophan aminotransferase-related 1 and

GA biosynthesis genes encoding enzymes such as *GA 20-oxidase3* and *GA 3-oxidase3, 4, 5*, and *6* are reported to play important an role in fruit set and development in strawberry (Kang et al., 2013).

The mechanisms of parthenocarpy in cucumber have been the subject of a number of studies (Fu et al., 2008; Li et al., 2014; Su et al., 2021; Gou et al., 2022). In cucumber, the nature of parthenocarpy is classified as facultative with the ability of fruit set without pollination. However, the parthenocarpic genotypes can successfully produce seeds when pollinated to effect fertilization. The inheritance of parthenocarpy in cucumber is reported to be governed by single dominant genes to complex polygenes (Pike and Peterson, 1969; Ponti and Garretsen, 1976; Kim et al., 1992). Most of the recent studies indicated that a large number of QTLs are associated with parthenocarpic fruit development of in cucumber. In the case of European greenhouse slicing cucumber, parthenocarpy was reported to be controlled by seven QTLs, including one major QTL on chromosome 2 (Wu et al., 2015). In North American pickling-type cucumber 2A, seven QTLs were detected for parthenocarpy and one QTL each on chromosomes 5 and 7 (parth5.1 and parth7.1), and two on chromosome 6 (parth6.1 and parth6.2) (Lietzow et al., 2016). Recently, four novel QTLs associated with parthenocarpy were detected in South China ecotype cucumber (Niu et al., 2020). These studies depict the complex genetic mechanisms associated with parthenocarpy in cucumber. Different ecotypes in cucumber, including slicing and pickling types, also had a different genetic architecture determining parthenocarpy.

There is a consensus based on earlier reports that parthenocarpy is a complex trait. Different genomic regions and QTLs were identified as determining parthenocarpy in slicing and pickling/processing-type cucumbers (Wu et al., 2015; Lietzow et al., 2016; Niu et al., 2020). However, there have been no reports regarding the comparative analysis of parthenocarpy in two different groups of cucumbers: slicing and pickling. These two groups have a different evolutionary lineage and, therefore, understanding the role of important phytohormones and genetic mechanisms of parthenocarpy need to be studied further. The present study involved two parthenocarpic gynoecious lines and one non-parthenocarpic gynoecious line for better insight into parthenocarpy in cucumber genotypes. The study aimed to determine the role of the important phytohormones' CK, AUX and GA biosynthesis and degradation-related genes in determining parthenocarpy in slicing- and pickling-type cucumber genotypes.

Materials and methods

Plant materials and growing conditions

The present experiment was undertaken using two gynoecious parthenocarpic genotypes and one gynoecious non-parthenocarpic genotype. The genotype Pusa Parthenocarpic Cucumber-6 (PPC-6) is a commercially cultivated slicing type suitable for cultivation under protected conditions. Pusa Pickling Cucumber-8 (DG-8) was the second gynoecious parthenocarpic genotype which is a speciality genotype suitable for pickling and cultivation under protected conditions. The non-parthenocarpic genotype, IMPU-1, was developed through introgression of *F* locus-determining gynoecy into a commercially cultivated elite monoecious genotype, Pusa Uday, through marker assisted breeding (MABC) (Behera et al., 2022). All the genotypes under investigation were gynoecious in nature, thus enabling a precise analysis of important phytohormones and determining the role of different CK, AUX and GA biosynthesis and degradation genes in inducing parthenocarpy in different groups of cucumber. All the genotypes were grown under protected conditions with standard recommended practice for the protected cultivation of cucumber.

Exogenous application of auxin, cytokinin, and gibberellin for parthenocarpic fruit development

The parthenocarpic and non-parthenocarpic genotypes PPC-6, DG-8, and IMPU-1 were grown in three replications with five plants per genotype for recording parthenocarpic fruit development under control with no exogenous application of phytohormones. Fruit set was recorded for the fifth node onwards for 15 fruits in each plant. An average of five plants in each replication was taken for analysis. In an unpollinated condition, the length of the fruit was measured from ten fruits in each plant. The exogenous application of IAA, Zeatin and GA_3 was done in the genotype IMPU-1 in 15 fruits in each plant to reach 20 nodes, starting from the sixth node. For exogenous application, seven treatments—IAA, GA_3, Zeatin, IAA + GA_3, IAA + Zeatin, Zeatin + GA_3, and IAA + GA_3+Zeatin—were applied. Exogenous application was done on the day of flower anthesis before opening the flowers in the early morning between 6:00 and 7:00 a.m. Flower buds sprayed with different combinations of phytohormone spray were covered with butter paper to avoid any chance of pollination. Three growth hormones were initially applied alone at four different doses—25, 50, 100, and 150 mg/lit—before the start of the experiment in the genotype IMPU-1; it was observed that a concentration of 100 mg/lit exhibited better response in parthenocarpic fruit set. Therefore, each of the phytohormones were sprayed with a concentration of 100 mg/lit to observe parthenocarpic fruit development. Fruit set was observed to ten days after anthesis and application of the phytohormones. As there was sequential flowering in all the lines, three to five fruits were sprayed in a single day in each plant and 15 female buds in each plant were taken for data recording. The experiment was conducted in three

replications with five plants in each replication. The fruits in each plant were removed after recording of the data on 10 DAA to allow other fruits in the plant to develop. Average data of each replication analysed using STAR software (http://bbi.irri.org › products). Tukey's honest significant difference (HSD) at $p = 0.05$ was used to determine the test of significance.

Estimation of endogenous IAA content

The frozen fruit samples at different developmental stages (5 g) were powdered in liquid nitrogen. The protocol described by Kim et al. (2006) was followed to extract IAA from the fruit samples. These were ground with liquid nitrogen and extracted with 100% methanol (2.5 ml g^{-1} fresh weight). The prepared extract was centrifuged at 16,000 g for 10 min. at 4°C. A vacuum concentrator was used to prepare a concentrate of the resulting supernatant. The conditions for HPLC were optimised and used for IAA quantification (Sharma et al., 2018) with an injection volume of 20 µL for each sample. A standard IAA sample was obtained from Sigma-Aldrich and final concentration was represented as µg IAA g^{-1} FW.

Estimation of trans-zeatin and dihydrozeatin

Trans-Zeatin and dihydrozeatin were extracted using a modified method suggested by Arteca et al. (1980). Fruit samples (10 g each) were submerged in 80% (v/v) methanol in water and extracted using a ultrasonicator (VCX-750, Sonics, Sonics and Materials Inc., Newtown, United States) at 50 kHz for 20 min. Aqueous methanolic extracts were centrifuged at 25000 g for 20 min separately and the supernatant was concentrated in a vacuum using a rotary evaporator (Heidolph, Germany) at 40°C. The remaining solution was partitioned with 10 ml of acetonitrile (0.1% TFA). Acetonitrile soluble fraction was subjected to UPLC-QTOF-ESIMS analysis in a Acquity Ultra Performance Liquid Chromatograph, coupled to a Quadrupole-Time of Flight mass spectrometer (QToF-MS, Synapt G2 HDMS, Waters Corporation, Manchester, United Kingdom). Reference standards of trans-zeatin [(purity 99.5%)] and dihydrozeatin (purity 99%) were used to prepare respective calibration curves for estimation. The QToF-ESI-MS was operated with electrospray ionization (ESI) at a nominal mass resolution of 20,000 and controlled by MassLynx 4.1 software. The data acquisition was done with the MSE function in continuum mode in the range of m/z 50–1000. The MSE mode provides full-scan MS data (low energy, 4 V) and MS/MS data (high energy, 10–60 V ramping) simultaneously. The source parameters were set as follows: capillary 6 kV, sampling cone 30 V, extraction cone 5 V, source temperature 100°C, desolvation temperature 500°C, desolvation gas flow 1000 L h^{-1} and cone gas flow 50 L h^{-1}. For mass spectrometer calibration, 0.1 mM sodium formate was used. The lock spray, the reference mass leucine enkephalin (m/z 556.2771 in positive and m/z 554.2670 in negative polarity) at 1 µg ml^{-1} concentration was used for mass correction with a flow rate of 20 µL min^{-1} in every ten desolvation gas flow 1000 L h-1s.

Estimation of gibberellic acid

The fine powder of the ground fruits at different developmental stages were placed into screw cap tubes filled with 30 ml methanol 70% (v/v) and kept overnight at 4°C. Supernatant was prepared by centrifugation and evaporation of methanol using a vacuum. The aqueous phase was partitioned with ethyl acetate after adjusting the pH at 8.5. The pH of the aqueous phase was again adjusted to 2.5 after removal of the ethyl acetate phase. The solution was partitioned with diethyl ether, and then passed through sodium sulfate. Diethyl ether was evaporated under vacuum, and dry residue containing GA_3 was dissolved in 2.0 ml of absolute methanol. The GA_3 analysis was performed using high performance liquid chromatography (HPLC) (Waters) equipped with reversed-phase column Crestpak C18 (150 mm × 4.6 mm i.d.; 5 µm) maintained at 30 ± 1°C. The mobile phase of acetonitrile-water (30:70%; v/v) was used with pH-4.5 and a flow rate of 1 ml/min. An injection volume of 10 µL was used for each analysis, and the wavelength of 208 nm was used for analysis.

RNA isolation from the fruit at different developmental stages

RNA isolation was performed for each genotype at three developmental stages: on the day of anthesis (0 DAA), two days after anthesis (2 DAA) and four days after anthesis (4 DAA). Both pollinated and unpollinated ovaries from all three genotypes were collected for gene expression analysis. The flower buds were covered with a butter paper bag one day before anthesis and pollination on the next day morning with the male flowers from each genotype. Fresh pollen from the plants sprayed with silver thiosulphate to induce male flowers was used for pollinating the female buds of each genotype. After pollination, the buds were again covered with a butter paper bag to avoid any chance pollination. The fruit collected at different developmental stages was immediately immersed in liquid nitrogen and kept in −80°C until isolation of RNA. Tissues from three fruits at similar developmental stages were homogenised for RNA isolation. Total RNA was isolated using 100 mg of cucumber fruit from three genotypes at three different developmental stages using TRIZOL reagent.

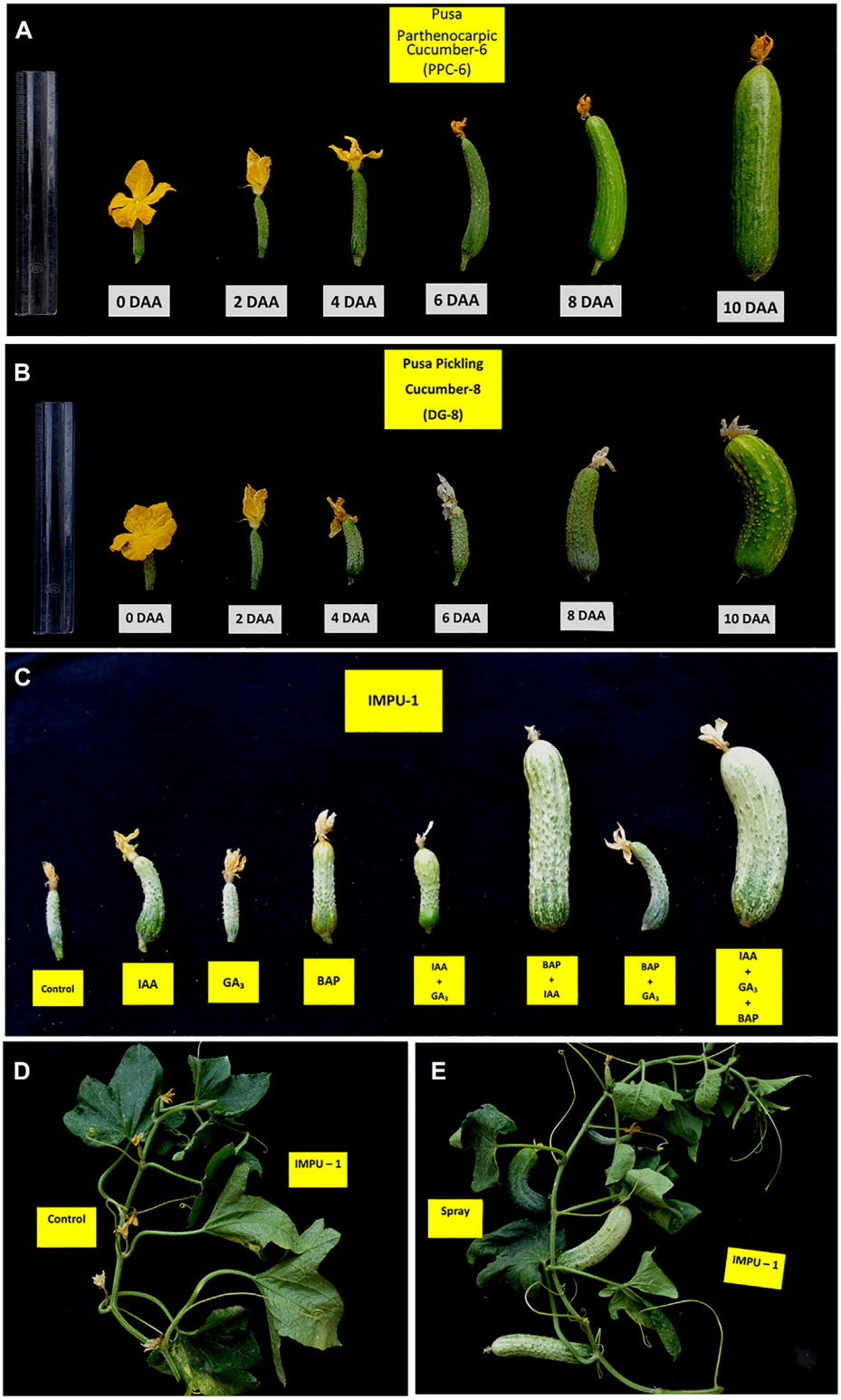

FIGURE 1
Parthenocarpic fruit development and the effects of the exogenous application of CK, AUX, and GA in the induction of parthenocarpy in cucumber. **(A)** Parthenocarpic fruit development in the slicing cucumber genotype PPC-6. **(B)** Parthenocarpic fruit development in the pickling cucumber genotype DG-8. **(C)** Effects of IAA, GA3, and Zeatin and their combination in fruit set and development in the non-parthenocarpic genotype IMPU-1. **(D)** Shrivelling of the flower buds of the non-parthenocarpic genotype, IMPU-1 under control with no pollination and exogenous application of phytohormones. **(E)** Normal fruit set and development in the genotype IMPU-1 with exogenous application of IAA + Zeatin in an unpollinated state.

Primer designing

The Cucurbits Genomic Database (http://www.icugi.org/) was searched for important CK, AUX and GA biosynthesis genes by using the predicted amino acid sequences of *Arabidopsis* homologues as queries. Primers were made using the Primer3 Input Version 4.0 (https://primer3.ut.ee/) tool. The primers were synthesized for expression analysis (Integrated DNA Technologies, United States). The primer sequence of the important genes associated with biosynthesis of CK, AUX and GA are provided in the Supplementary Table S1.

Phylogenetic study of the genes associated with cytokinin, auxin, and gibberellin biosynthesis

A total of 35 sequences of AUX, CK, and GA biosynthesis and degradation-related gene family of cucumber were extracted from the Cucurbits Genomic Database (http://www.icugi.org/). These were subjected to pairwise/multiple sequence alignment at default parameters for gap opening and gap extension penalty (i.e., 15 and 6.66, respectively in MEGA (Tamura et al., 2021)). The evolutionary history was inferred using the maximum likelihood method and the Tamura-Nei model (Tamura and Nei, 1993)]. An initial tree for the heuristic search was automatically obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A bootstrap method with 500 replications was employed at uniform rates among sites. The tree generated was scanned in Newick format to visualize and annotate in iTOL (Letunic and Bork, 2021).

RT-PCR analysis for expression analysis of the important phytohormone biosynthesis and degradation genes

The cDNA was synthesized using a cDNA synthesis kit (GoScript™ Reverse Transcriptase) from 1 µg of isolated RNA. cDNA (200 ng) from all three genotypes of cucumber were used in a 10 µL Real-Time PCR machine (Bio-Rad, United States) using SYBR Green Master Mix (Promega, United States). Three biological replicates of cucumber fruit were taken to carry out qPCR reactions; a reference gene (Actin-mRNA) was used for the normalisation of gene expression. The relative level of gene expression was calculated by ΔCt method.

Results

Effects of exogenous application of auxin, cytokinin, and gibberellins in parthenocarpic fruit set

It was found that the fruits of the parthenocarpic genotypes PPC-6 and DG-8 developed normally and attained a length of 15.5 cm and 8.9 cm, respectively at 10DAA, whereas more than 60% of the fruit of the unpollinated ovaries fell off the plant within 10 DAA in the genotype, IMPU-1. The retained fruits were shrivelled and did not develop beyond a certain length (Supplementary Figure S1). Fruit set in the non-parthenocarpic genotype IMPU-1 was observed through application of AUX, CK and GA and their combinations along with two parthenocarpic genotypes under control with no exogenous application. In the non-parthenocarpic genotype IMPU-1, the fruit set under control was very low (13.3%) and the initially retained fruits were shrivelled and fell down at a later stage. The highest parthenocarpic fruit set was recorded in the genotype PPC-6 (89.97%), followed by DG-8 (85.03%) under control with no exogenous application (Figures 1A,B). Exogenous application of the phytohormones had pronounced effects in the parthenocarpic development of fruit in the non-parthenocarpic genotype IMPU-1. The development of the fruit with the exogenous spray of CK, GA and AUX in the non-partheocarpic genotype IMPU-1 is presented in Figure 1C. The highest parthenocarpic fruit set (89.3%) was recorded with the combined application of IAA + Zeatin (Figures 1D,E) followed by Zeatin alone (80.8%) and IAA + Zeatin + GA_3 (78.6%). Parthenocarpic fruit set in the non-parthenocarpic genotype with the exogenous application of IAA + Zeatin was on par with the parthenocarpic fruit set in the genotypes PPC-6 and DG-8 (Table 1)

Estimation of endogenous AUX, CK, and GA in different developmental stages

The estimation of IAA and trans-zeatin and dihydrozeatin was undertaken in six development stages from the day of anthesis (0DAA) to ten days after anthesis (10DAA) in alternate days (Figure 2; Supplementary Table S2). The trans-zeatin concentration was initially higher in the non-parthenocarpic genotype IMPU-1. However, its concentration increased in the parthenocarpic genotypes with advancement in the developmental stages. At 8DAA, trans-zeatin was highest in the pickling-type cucumber genotype DG-8, followed by slicing-type PPC-6 (Figure 2A). In the genotype, DG-8 concentration of dihydrozeatin was highest in the initial stages to 4DAA and then declined. However, in the genotype PPC-6, its concentration was consistently lower across the developmental stages (Figure 2B). A significant difference in IAA concentration

TABLE 1 Effects of exogenous application of auxin, cytokinin, and gibberellins in the induction of parthenocarpic fruit set in cucumber.

Sl. no.	Genotype	Nature of the genotype	Treatments	Percentage fruit set	Development of fruit
1	Pusa Parthenocarpic Cucumber-6 (PPC-6)	Gynoecious Parthenocarpic	Control	89.97 a	Well developed
2	Pusa Pickling Cucumber-8 (DG-8)	Gynoecious Parthenocarpic	Control	85.03 ab	Well developed
3	IMPU-1 (F locus introgressed Pusa Uday)	Gynoecious non-parthenocarpic	Control	13.30 f	Shrivelled and fallen down at later stage
			IAA	67.77 d	Underdeveloped
			GA3	34.33 e	Underdeveloped
			BAP	80.80 bc	Developed
			IAA + GA3	68.97 d	Underdeveloped
			IAA + BAP	89.30 a	Well developed
			BAP + GA3	76.33 c	Developed
			IAA + GA3+BAP	78.60 c	Developed

Test of significance was done through Tukey's honest significant difference (HSD) test at $p = 0.05$. The same letter in the column indicates no significant difference, and different letters indicate a significant difference among them.

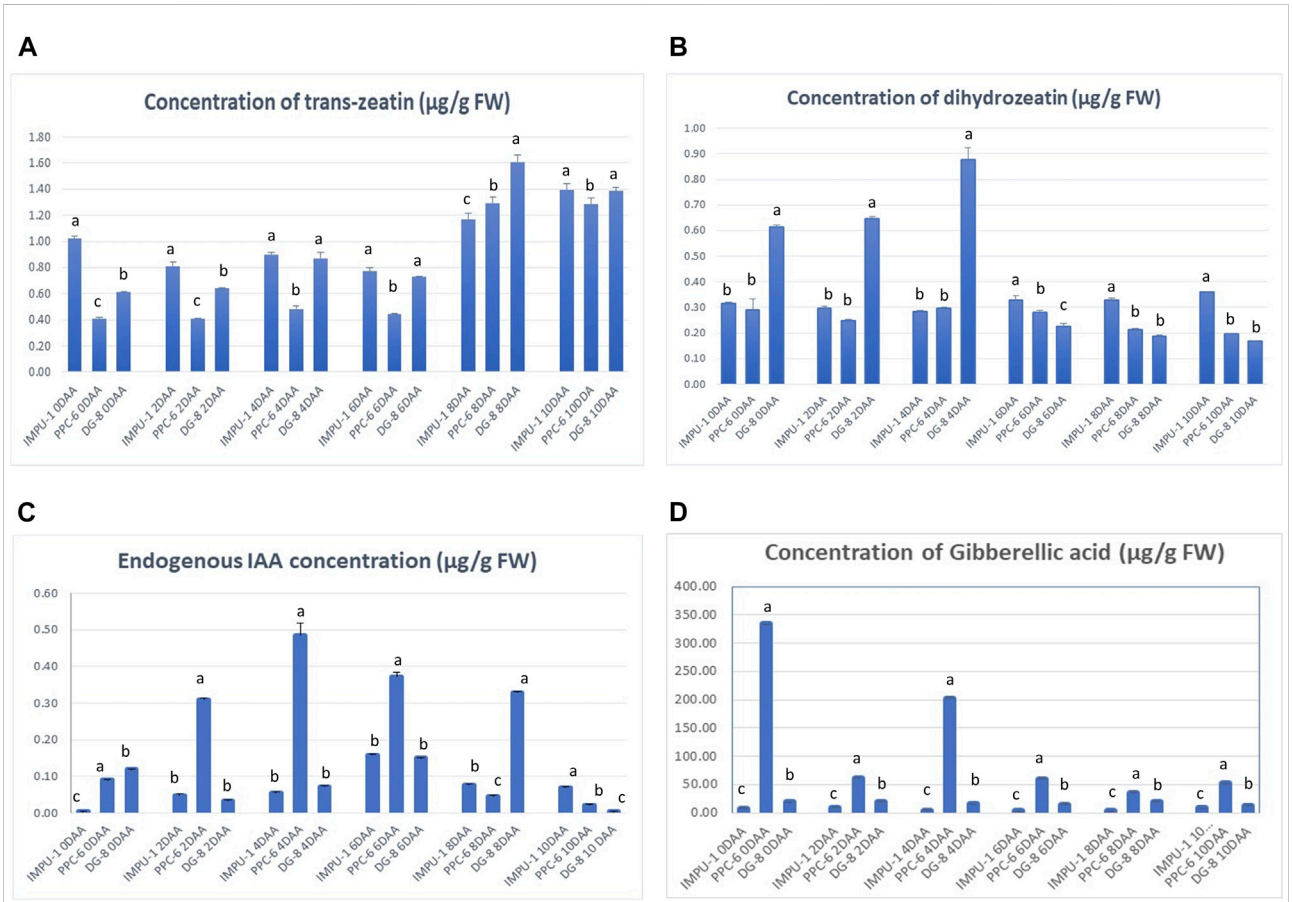

FIGURE 2
Endogenous concentration of **(A)** trans-zeatin, **(B)** dihydrozeatin **(C)** indole acetic acid (IAA) and **(D)** gibberellic acid (GA_3) in the genotypes IMPU-1, PPC-6, and DG-8 at six different developmental stages in an unpollinated state. Test of significance performed through Tukey's honest significant difference (HSD) test at $p = 0.05$. The same letter in the bar indicates no significant difference while different letters indicate significant difference among them.

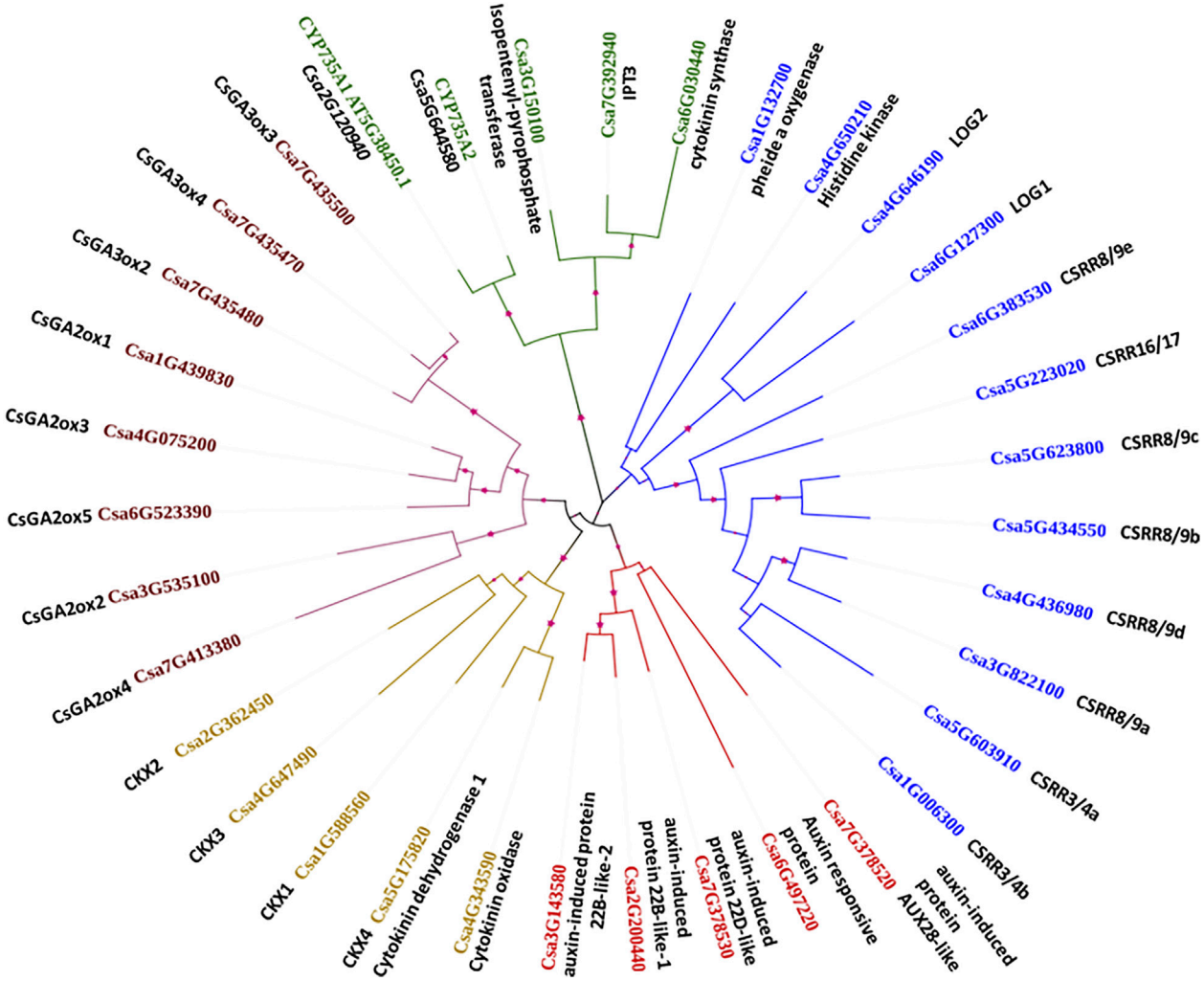

FIGURE 3
Phylogenetic analysis of the important cytokinin, auxin and gibberellin biosynthesis and degradation genes and their grouping into five major clusters.

was recorded among the genotypes when comparing the parthenocarpic genotypes with the non-parthenocarpic genotype (Figure 2C). In addition, the concentration of IAA significantly varied among the slicing- and pickling-type parthenocarpic genotypes. On the day of anthesis, the highest concentration of IAA was recorded in the genotype DG-8, followed by PPC-6; it was lowest in the non-parthenocarpic genotype IMPU-1. While studying the concentration of IAA in the slicing cucumber genotype PPC-6, its concentration was recorded as increasing till 4DAA from the day of anthesis, and declined thereafter. In the pickling-type parthenocarpic genotype, the concentration of IAA was initially lower than PPC-6 but kept increasing till 8DAA before its decline. The IAA concentration was lower in the non-parthenocarpic genotype IMPU compared to the two parthenocarpic genotypes in all developmental stages. The concentration of GA_3 varied significantly in different developmental stages among the genotypes studied (Figure 2D). The parthenocarpic genotype PPC-6 had the highest concentration of GA_3 in all the developmental stages, whereas the non-parthenocarpic genotype IMPU-1 had the lowest. The concentration of GA_3 in pickling cucumber genotype DG-8 was intermediate between PPC-6 and IMPU-1. In the parthenocarpic genotype PPC-6, concentration of GA_3 was higher in the initial developmental stages and declined with the advancement of fruit development. In contrast, the processing parthenocarpic genotype DG-8 did not express such drastic changes in the endogenous GA_3 level in different fruit developmental stages. The lowest concentration of GA_3 was recorded in the non-parthenocarpic genotype IMPU-1 and was significantly lower than PPC-6 and DG-8 in all the six developmental stages (Figure 2D).

Phylogenetic studies involving the CK, AUX, and GA biosynthesis-related genes

Five major clusters were generated for the 35 CK, AUX and GA biosynthesis and degradation gene family sequences, as indicated by the different colours in Figure 3. The branch lengths are shown for each clade. Cluster I contains crucial genes for CK biosynthesis such as pheophorbide a oxygenase (Csa1G132700), CK signalling rich gene (*Csa4G650210*), *LOG1* (*Csa6G127300*), and *LOG2* (*Csa4G646190*), along with transcription factor-response regulators *CSRR8*/9e (*Csa6G383530*), *CSRR16*/17 (*Csa5G223020*), *CSRR8/9c* (*Csa5G623800*), *CSRR8/9b* (*Csa5G434550*), *CSRR8/9d* (*Csa4G436980*), *CSRR8/9a* (*Csa3G822100*), *CSRR3/4a* (*Csa5G603910*), and *CSRR3/4b* (*Csa1G006300*). Cluster II is represented by another group of CK biosynthesis genes such as cytokinin synthase (*Csa6G030440*), *IPT3* (*Csa7G392940*), *IPT* (*Csa3G150100*), *CYP735A1* (*Csa2G120940*), and *CYP735A2* (*Csa5G644580*). All the GA biosynthesis and degradation genes such as *CsGA3ox3* (*Csa7G435500*), *CsGA3ox4* (*Csa7G435470*), *CsGA3ox2* (*Csa7G435480*), *CsGA2ox1* (*Csa1G439830*), *CsGA2ox3* (*Csa4G075200*), *CsGA2ox5* (*Csa6G523390*), *CsGA2ox2* (*Csa3G535100*), and *CsGA2ox4* (*Csa7G413380*) were grouped together in Cluster III. Cluster IV consists of CK degradation genes *CKX2* (*Csa2G362450*), *CKX3* (*Csa4G647490*), *CKX1* (*Csa1G588560*), CKX4/cytokinin dehydrogenase 1 (*Csa5G175820*), and cytokinin oxidase (*Csa4G343590*); cluster V contained auxin-induced protein 22B-like-2 (*Csa3G143580*), auxin-induced protein 22B-like-1 (*Csa2G200440*), auxin-induced protein 22D-like (*Csa7G378530*), auxin-induced protein AUX28-like (*Csa7G378520*), and auxin-responsive protein (*Csa6G497220*).

Relative expression of genes associated with CK biosynthesis

A total of 22 CK biosynthesis and degradation-related genes and transcripts were investigated at three different developmental stages using both pollinated and unpollinated flower buds. The relative expression of the parthenocarpic genotypes were calculated in comparison with the non-parthenocarpic genotype IMPU-1. Cytokinin synthase was significantly up-regulated in the parthenocarpic genotypes PPC-6 and DG-8, both pollinated and unpollinated. At 2DAA, the relative expression was higher in both parthenocarpic genotypes; its expression later declined. It was also significant that a higher expression of cytokinin synthase was recorded at a pollinated state compared to the unpollinated state in both the parthenocarpic and non-parthenocarpic genotypes. While studying the relative expression of *IPT*, its expression was found to be higher in the initial stage (0DAA) and then declined at 2DAA. However, the expression of *IPT* again peaked at 4DAA, particularly in the pollinated ovaries of the parthenocarpic genotypes. Similarly, consistent up-regulation of *IPT3* was recorded from the anthesis to 4DAA in both the pollinated and unpollinated ovaries of the genotypes PPC-6 and DG-8. The expression of cytokinin synthase, *IPT* and *IPT3* were higher in the pollinated flower buds of the non-parthenocarpic genotype IMPU-1 than in the unpollinated ovaries. Up-regulation of the *PaO, LOG1*, and *LOG2* were observed in the parthenocarpic genotypes PPC-6 and DG-8 in both pollinated and unpollinated states. The extent of up-regulation was higher in the case of *LOG1* from 2DAA onwards (Figure 4). Eight CK response regulator genes were studied for their relative expression in parthenocarpic and non-parthenocarpic genotypes (Figure 5). Among them, *CsRR8/9b, CsRR8/9d*, and *CsRR8*/9e were significantly up-regulated in the parthenocarpic genotypes and *CSRR8/9a*, *CSRR3/4a*, and *CSRR3/4b* were down-regulated in the parthenocarpic genotypes, with the development of the ovaries from anthesis onwards. The relative expression of the cytokinin oxidase/dehydrogenase (*CKXs*) was also investigated in the three genotypes in both pollinated and unpollinated states. It was found that *CKX2 and CKX3* were down-regulated in the parthenocarpic genotypes at a later stage after initial up-regulation. Cytokinin oxidase and cytokinin dehydrogenase were up-regulated in the parthenocarpic genotypes: the extent of the up-regulation was stronger in the genotype PPC-6 than DG-8. In the genotypes PPC-6 and DG-8, stronger expressions of *CKX1 and CKX4*, respectively, were recorded at 2DAA, before declining thereafter. However, no significant difference in the expression of *CKXs* was recorded in the pollinated and unpollinated flowers of the non-parthenocarpic genotype IMPU-1 (Figure 6).

Relative expression of genes associated with AUX and GA biosynthesis and degradation

The relative expression of the four AUX-induced proteins and eight gibberellin oxidases were also investigated in the three sets of genotypes at different time intervals. Among the AUX induced proteins, Auxin-induced protein 22B-like-2 and Auxin-induced protein AUX28-like were significantly up-regulated in the parthenocarpic genotypes at 2DAA. However, Auxin-induced protein 22D-like and Auxin-induced protein 22B-like-2 had significantly higher expression from the day of the anthesis to 4DAA (Figure 7). It was interesting that the level of expression of the auxin-induced proteins in the slicing-type parthenocarpic genotype PPC-6 was relatively lower in the unpollinated state than the pollinated state in the parthenocarpic pickling-type genotype DG-8. Among the eight gibberellin biosynthesis pathway-related genes taken for the study, six were either down-regulated or did not differ significantly from the non-parthenocarpic genotype IMPU-1 under both pollinated and unpollinated states. However, the expression of *CsGA3ox2* was higher in the parthenocarpic genotypes in all the developmental stages;

FIGURE 4
Relative expression of the cytokinin biosynthesis genes in the genotypes IMPU-1, PPC-6, and DG-8 under three different developmental stages of pollinated and unpollinated ovaries.

FIGURE 5
Relative expression of the cytokinin transcription factor-response regulators (RRs) in the genotypes IMPU-1, PPC-6, and DG-8 under three different developmental stages of pollinated and unpollinated ovaries.

FIGURE 6
Relative expression of the cytokinin degradation genes in the genotypes IMPU-1, PPC-6, and DG-8 under three different developmental stages of pollinated and unpollinated ovaries.

CsGA2ox4 was up-regulated in the initial development stages at 0DAA and 2DAA and was later down-regulated at 4DAA (Figure 8). There was no significant difference in the pollinated and unpollinated ovaries of the non-parthenocarpic genotype IMPU-1 at different stages for pollinated and unpollinated ovaries for both AUX and GA biosynthesis-related genes.

Discussion

Successful completion of pollination and a unique double fertilization is the prerequisite for initial fruit setting and further development of fruits in most of the angiosperms (Raghavan, 2003). However, a few selected organisms can produce fruit without requiring

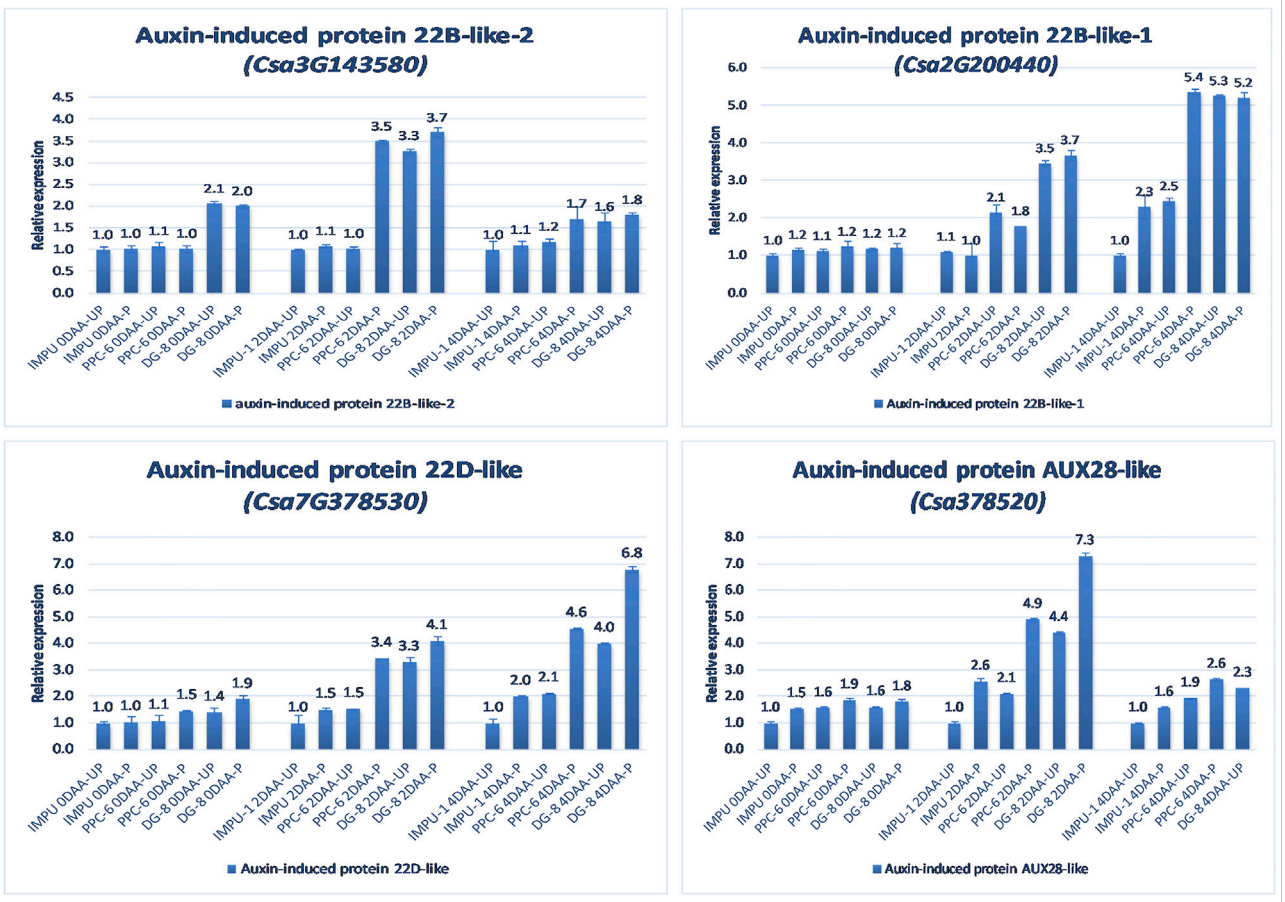

FIGURE 7
Relative expression of the important auxin-induced proteins in the genotypes IMPU-1, PPC-6, and DG-8 under three different developmental stages of pollinated and unpollinated ovaries.

pollination and fertilization—termed 'parthenocarpy". Cucumber is one such model organism with abundant germplasm which has the typical phenomenon of parthenocarpy. In case of non-parthenocapic genotypes, successful pollination and fertilization results in formation of seed which promotes synchronized cell division and fruit growth. A number of studies have indicated the role of three important phytohormones—AUX, GAs, and CKs—in the regulation of fruit set. These three hormones are required in combinations for fruit set and further development because individually they can only set fruit to a certain extent and cannot support the development of fully-grown fruit (Mariotti et al., 2011; Sharif et al., 2022). Cross-talk between the phytohormones is well established in the regulation of different biological processes in plants (Coenen and Lomax 1997). Induction of parthenocarpy in various plant species is mainly regulated by AUX, GAs, and CKs (Fu et al., 2008; Serrani et al., 2008; Sharif et al., 2022).

The non-partheocapic gynoecious genotype IMPU-1 did not set fruits parthenocarpically, although a few ovaries were enlarged in the initial stage and then shrivelled and fell off later. However, the parthenocarpic lines PPC-6 and DG-8 successfully developed fruit without pollination and expressed a very high degree of parthenocarpy. Exogenous application of AUX, GA, and CK individually and in combination had a very significant role in fruit set and development in the non-parthenocarpic genotype. The highest percentage of fruit set and the normal development of fruit were observed when AUX and CK were applied in combination. Individual application of CK was also effective in fruit set and their enlargement. However, individual application of AUX and GA were less effective as their application enabled fruit set but could not carry the fruit to full development. A better suitability of CK and AUX in the development of parthenocarpic fruit in cucumber has been demonstrated by Su et al. (2021). The exogenous application of growth hormones like CKs and GAs were found to be effective in developing parthenocarpic fruits in non-parthenocarpic genotypes of tomato (Matsuo et al., 2012), eggplant (Donzella et al., 2000), and pear (Niu et al., 2014).

Varied concentrations of trans-zeatin and dihydrozeatin were recorded in different developmental stages; at the initial stage, a higher concentration of trans-zeatin was recorded in the genotype IMPU-1 in an unpollinated state. In contrast, dihydrozeatin was present in higher concentrations in the genotype DG-8 in the initial stages,

FIGURE 8
Relative expression of the important gibberellin biosynthesis and degradation genes in the genotypes IMPU-1, PPC-6, and DG-8 under three different developmental stages of pollinated and unpollinated ovaries.

indicating a different role for the CKs in regulating the parthenocarpy of slicing and pickling cucumber. A lower concentration of *trans*-zeatin in the parthenocarpic genotypes in initial stages of development might be due to a stronger expression of some CK degradation enzymes such as cytokinin oxidase, *CKX2*, and *CKX4* in the initial stages. The expression level of the CK biosynthesis and degradation genes were different even among the parthenocarpic lines. The endogenous concentration of the CKs was ultimately determined by the homeostasis between CK biosynthesis and catalytic genes and regulators (Frébort et al., 2011). The relatively weaker expression of the *CKXs* in the pollinated ovules was due to the negative regulation of these enzymes when there was successful fertilization and seed development. However, the concentration of AUX was higher in the unpollinated ovaries of the parthenocarpic genotypes at all the developmental stages. However, the concentration of IAA was highest in the slicing-type genotype at 4DAA and then started to decline. On the other hand, it increased in the pickling-type genotype to 8DAA before declining. This trend indicated potentially different genetic mechanisms that control parthenocarpy in slicing- and pickling-type cucumbers. Different genomic locations determining parthenocarpy in these types of cucumber were reported by Wu et al. (2015), Lietzow et al. (2016) and Niu et al. (2020). Higher concentrations of AUX in the ovules of the parthenocarpic cucumber genotypes were earlier reported by Su et al. (2021) and Qian et al. (2018).

CKs are important regulators for the development of fruit. CK-regulated cell division and development of fruit have been reported in tomato (Matsuo et al., 2012). The findings of the present study also revealed a higher concentration of CK in the ovaries of the parthenocarpic genotypes PPC-6 and DG-8, thus establishing the role of CK in parthenocarpic fruit development in cucumber genotypes. A higher expression of the genes cytokinin synthase, isopentenyl pyrophosphate transferase 3, and *LOG2* was recorded in the parthenocarpic genotypes in different developmental stages. It was also notable that the expression level of these important genes associated with cytokinin biosynthesis was higher in the pollinated flower buds than in unpollinated ones. In the non-parthenocarpic genotype IMPU-1, higher expression of the CK synthase and *IPT3* was recorded in the pollinated flower buds. Homeostasis between CK synthesis and catabolism determined spatial and temporal biosynthesis in different parts of the plant (Frébort et al., 2011). The production of isopentenyladenine nucleotides catalysed by adenosine phosphate-isopentenyl transferase (*IPT*) involves the first step of CK biosynthesis (Sakakibara, 2005; Sakakibara, 2005). The hydroxylation of the prenyl side-chain of isopentenyl adenosine phosphates is mediated by a cytochrome P450 mono-oxygenase (*CYP735A*) to produce trans-zeatin-type species. The nucleotide precursors are converted into their active forms by the *LONELY GUY (LOG)* while degradation of CK is catalysed by CK oxidases (*CKXs*) (Sakakibara, 2005; Sakakibara, 2006; Schäfer et al., 2015). Up-regulation of the genes associated with CK biosynthesis such as *CYP735A1*, *CYP735A2*, and *LOG1* and down-regulation of the CK dehydrogenase genes *CKX1* and *CKX3* was reported to be the reason behind parthenocarpic fruit development in cucumber (Su et al., 2021). Our study also revealed the enhanced expression of the CK biosynthesis genes such as *IPT*, *IPT1*, *IPT3*, *LOG1*, *LOG2*, *CYP735A1,* and *CYP735A2* and reduced expression of *CKX1, CKX2,* and *CKX3* in the parthenocarpic genotypes PPC-6 and DG-8. Therefore, the role of the CK in parthenocarpic fruit set of both slicing and pickling cucumber is established. However, the extent of up-regulation and down-regulation of CK biosynthesis genes varied among the slicing and pickling parthenocarpic genotypes. In addition, a higher expression of the CK biosynthesis-related genes was recorded in the pollinated ovules compared to the unpollinated buds. Enhanced expression of CK biosynthesis genes (*SlIPT3*, *SlIPT4*, *SlLOG6*, and *SlLOG8*) was associated with CPPU-induced parthenocarpy in tomato (Matsuo et al., 2012). Transcription factor-response regulators (RRs) are vital for the interaction of CK with an array of other hormones via the multistep phosphorelay system (MSP) (Arkhipov et al., 2019). CSRR8/9a was down-regulated in the parthenocarpic genotypes with the advancement of developmental stages in both unpollinated and pollinated states. Type-A RR genes are classified as negative feedback regulators of CK signalling (Osugi and Sakakibara. 2015; Kieber and Schaller, 2018). CK signalling genes such as *CsRR3/4a*, *CsRR3/4b, CsRR8/9a*, and *CsRR8*/9c are reported to be strongly expressed in the non-parthenocarpic cucumber genotype, thus elucidating their negative regulation in parthenocarpic fruit development (Su et al., 2021). The present study also revealed the negative feedback of the *CsRR3/4a, CsRR3/4b, CsRR8/9a,* and *CsRR8*/9c genes in parthenocarpic fruit development. However, positive regulation of the *CSRR8/9b, CSRR8/9d, CSRR8/9e,* and CSRR16/17 genes were also revealed, which was not known earlier in the parthenocarpic fruit development of cucumber. The role of the CK signal transduction in the induction of parthenocarpy in cucumber is established from the finding of the study. Significant up-regulation of the *IPTs* in the parthenocarpic genotypes explained CK regulated parthenocarpy in cucumber. The results of this study indicated the role of Ck biosynthesis genes in parthenocarpic fruit set through their up-regulation in the parthenocarpic genotypes. However, the AUX and GA metabolisms were also found to be intermingled with CK pathways in determining parthenocarpy.

AUX signalling genes are responsible for the dynamic role of AUX-regulated growth activities (Sharif et al., 2022). The roles of AUX signal transduction genes in parthenocarpic fruit formation have been more intensively studied than the AUX biosynthesis and transportation genes. Auxin-induced protein 22B-like-1, auxin-induced protein 22B-like-2, auxin-induced protein 22D-like, and auxin-induced protein AUX28-like were strongly expressed in the parthenocarpic genotypes at different developmental and pollinated stages of the ovaries. However, their relative expression varied among the slicing and pickling parthenocarpic cucumber genotypes. Their expression was also enhanced in the pollinated state in the non-parthenocarpic genotype IMPU-1 and slicing cucumber genotype PPC-6. Su et al. (2021) also reported the positive expression of AUX signal transduction genes such as *AUX 22A-like-1*, *AUX22B-like-2,* and *AUX 28-like* in the parthenocarpic cucumber genotype DDX. Auxin is well known for its role in the development of fleshy fruits

and reported to be integral to the initial signal for fertilisation and increased fruit size through its influence in cell division and expansion (Godoy et al., 2021). Once the fruits set parthenocarpically, their further development is influenced by auxins, evidenced by up-regulation of the auxin biosynthesis-related genes in the later stages of fruit development in the parthenocarpic genotypes.

Gibberellin is another crucial hormone for fruit set and development (Wang et al., 2020). GA13-oxidase (*GA13ox*), GA 20-oxidase (*GA20ox*), and GA 3-oxidase (*GA3ox*) enzymes are involved in the biosynthesis of GA_1 and GA_4 (Hedden, 2020). The primary GA deactivation enzyme GA2-oxidase determines the concentrations of these active GAs (Hedden, 2020). On the other hand, the degradation of GA_{1+4} (active GA) is catalysed by a major GA degradation gene, *GA2ox*, which is crucial for GA homeostasis in plants (Martínez-Bello et al., 2015). The role of the GA biosynthesis and degradation genes in relation to parthenocarpic fruit development in cucumber is unknown. In our study, *CsGA3ox2* was strongly expressed in the parthenocarpic genotypes PPC-6 and DG-8 whereas *CsGA3ox4* was strongly expressed in the parthenocarpic genotypes at the initial stage of ovary development (2DAA); its expression later declined, indicating the stage-specific role of this enzyme in induction of parthenocarpy. The enzymes *CsGA3ox3*, *CsGA3ox4, CsGA2ox1, CsGA2ox2, CsGA2ox3,* and *CsGA2ox5* were weakly expressed in the parthenocarpic genotypes, the pollinated state indicating their negative regulation in the induction of parthenocarpic fruit in cucumber. In pear, the dominant expression of the GA biosynthesis gene *GA20ox* was reported in the pollinated fruit (Wang et al., 2020). Increased GA_4 levels in tomato and pear through overexpression of *SlGA20ox* and *PbGA20ox* genes, respectively, resulted in parthenocarpic fruit development (Wang et al., 2020). In tomato, fruit setting was associated with the up-regulation of GA biosynthesis genes (*SlGA20ox1*, *SlGA20ox2*, and *SlGA20ox3*) and the down-regulation of the GA deactivation gene *SlGA2ox1* (Okabe et al., 2019). Varied concentrations of endogenous CKs and AUX and their differential expression at different developmental stages established the potentially different molecular mechanisms and regulatory networks that determine parthenocarpy in slicing and pickling cucumbers. The findings of the present study reveal the cross-talk between the important plant hormones in relation to parthenocarpic fruit development in cucumber.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Author contributions

Conceiving the theme of the study and design of the experiment: SD. Data curation: SD, MI, SJ, SD, and NM. Investigation: NM, SD, and KK. Resources: SD, TB, and AM. Supervision: SD, AM, TB, and AA. Visualization: SD, TB, and AM. Writing original draft: NM and SD. Review and editing: SD, TB, and PB. All authors read and approved the final manuscript.

Funding

This research work was supported by CRP on Molecular Breeding (Cucumber; 12-143 H) and NSHEP-CAAST programme of Indian Council of Agricultural Research (ICAR).

Acknowledgments

The authors are thankful to the ICAR-Indian Agricultural Research Institute, New Delhi, for providing financial support and the conduct of the research programme of the M.Sc student NM.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.957360/full#supplementary-material

SUPPLEMENTARY TABLE S1
List of the genes, respective gene IDs and primer sequence used for RT-PCR analysis.

SUPPLEMENTARY TABLE S2
Concentration of the different phytohormones at six developmental stages in three cucumber genotypes.

SUPPLEMENTARY FIGURE S1
Sequential development of the unpollinated fruits in the genotypes IMPU-1, PPC-6 and DG-8 at different developmental stages.

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frontiers | Frontiers in Genetics

TYPE Review
PUBLISHED 09 September 2022
DOI 10.3389/fgene.2022.933226

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

RECEIVED 30 April 2022
ACCEPTED 25 July 2022
PUBLISHED 09 September 2022

CITATION
Uniyal S, Bhandari M, Singh P, Singh RK and Tiwari SP (2022), Cytokinin biosynthesis in cyanobacteria: Insights for crop improvement.
Front. Genet. 13:933226.
doi: 10.3389/fgene.2022.933226

Cytokinin biosynthesis in cyanobacteria: Insights for crop improvement

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Cytokinins, a type of phytohormones that induce division of cytoplasm, have considerable value in agriculture due to their influences on several physiological processes of plants such as morphogenesis, development of chloroplast, seed dormancy, leaf senescence, etc. Previously, it was assumed that plants obtain cytokinin from the soil produced by microbes as these hormones were first discovered in soil-inhabiting bacteria i.e., *Agrobacterium tumefaciens*. Later, the cytokinin biosynthesis gene, i.e., ipt gene, has been reported in plants too. Though plants synthesize cytokinins, several studies have reported that the exogenous application of cytokinins has numerous beneficial effects including the acceleration of plant growth and boosting economic yield. Cyanobacteria may be employed in the soil not only as the source of cytokinins but also as the source of other plant growth-promoting metabolites. These organisms biosynthesize the cytokinins using the enzyme isopentenyl transferases (IPTs) in a fashion similar to the plants; however, there are few differences in the biosynthesis mechanism of cytokinins in cyanobacteria and plants. Cytokinins are important for the establishment of interaction between plants and cyanobacteria as evidenced by gene knockout experiments. These hormones are also helpful in alleviating the adverse effects of abiotic stresses on plant development. Cyanobacterial supplements in the field result in the induction of adventitious roots and shoots on petiolar as well as internodal segments. The leaf, root, and stem explants of certain plants exhibited successful regeneration when treated with cyanobacterial extract/cell suspension. These successful regeneration practices mark the way of cyanobacterial deployment in the field as a great move toward the goal of sustainable agriculture.

KEYWORDS

cyanobacteria, cytokinins, cytokinin biosynthesis, crop improvement, sustainable agriculture

1 Introduction

Cytokinins (CKs) are N^6-substituted adenine derivative phytohormones responsible for nutrient uptake, cell division, embryonic development, and the overall growth of plants (Mishra et al., 2022). The word "cytokinin" has been derived from the role of these compounds in cytokinesis i.e., cell division. The cytokinins were discovered in the 1950s by Skoog et al., and play critical roles in the interaction of plants with different biotic and abiotic factors (Han et al., 2022). The first cytokinin, kinetin (6-furfural-aminopurine), was discovered as a degradation product of herring sperm DNA (Le Bris, 2017). However, the first natural cytokinin, zeatin, was purified from immature maize kernels. These hormones are abundantly found in shoot and root apexes, as well as in immature seeds. CKs are functionally active in plants at every minute i.e., micro to nano molar concentration (Žižková et al., 2017). The side chains available in the cytokinin hormones have a great influence on its biological activity depending on its configuration and conformation.

Based on the available side chains, isoprenoid and aromatic cytokinins are the two broad groups of CKs. Isoprenoid CKs are the more abundant category as compared to the aromatic ones (Raspor et al., 2020). Isoprenoid CKs include N^6-(Δ^2-isopentenyl adenine (iP), dihydrozeatin (DHZ), trans- as well as cis-zeatin, while aromatic CKs include N^6-Benzyladenine (BA) and its hydroxyl derivatives such as ortho- and meta-topolin (Žižková et al., 2017). Natural CKs undergo a certain kind of modifications in which amino acids or sugar moieties like alanine, glucose, or ribose get attached to the basic cytokinin structure (Hošek et al., 2020). In plants, cytokinin occurs as nucleotides, nucleosides (ribosides), glycosides (O- and N-glycosides) and free bases depending on the molecule conjugated to the basic cytokinin structure (Ashihara et al., 2020). For example, when a ribose sugar gets attached to the purine ring at the N^9 position, it is termed as a riboside. If a phosphate group is also present along with the ribose sugar, it is called a ribotide (Kalra and Bhatla, 2018). Similarly, O- and N-glycosides are formed by the addition of sugar moiety, commonly glucose, at O (at hydroxyl group of N^6 side chain) and N (N^3, N^7 and N^9) sites, respectively (Pokorna et al., 2020). Based on the physiological activity, CKs can be grouped under two different categories. The first category comprises of highly active forms and includes free bases, nucleosides, and nucleotides, whereas another category encompasses moderately active CK-N and CK-O glycosides (Žižková et al., 2017). Ribosides are the form with lower activities while free bases are forms with higher activities. The higher abundance of inactive forms as compared to the active forms suggests that the enzymes involved in the synthesis, modification, and degradation of cytokinins are regulated in a controlled and coordinated manner (Kroll and Brenner, 2020).

The biosynthesis of cytokinins is catalyzed by the enzyme isopentenyl transferase encoded by the *ipt* gene. The gene was initially discovered in the soil-inhabiting bacterium i.e., *Agrobacterium tumefaciens* (Nester et al., 1984). Plants were known to obtain cytokinin from soil. Later, the gene was discovered in plants as well which led to the consideration that plants are able to produce cytokinin (Kakimoto, 2001). The fact has raised a question that if plants themselves are capable of producing cytokinin, what is role of cytokinin-producing microorganisms in the soil? Cytokinin-producing microbes further enhance the level of cytokinin in plants and soil as well (Akhtar et al., 2020).

Cytokinins are known to alleviate the damage engendered by different abiotic stresses (Bryksová et al., 2020) and it also has wide application in plant tissue culture due to its stimulatory impact over the regeneration of plants (Grzegorczyk-Karolak et al., 2021). Further genetic manipulations in the cytokinin metabolism genes can lead to enhancements in crop growth parameters (Liu et al., 2020). Due to its tremendous applications in agriculture and possibilities for improvement in its metabolic enzyme pathways to achieve even better results, cytokinin has become a subject requiring more concern and attention. Hence, the present article attempts to discuss the different applications of cytokinins in agriculture, cyanobacteria as exogenous sources of cytokinins, cytokinin metabolism in cyanobacteria, and genetic manipulation strategies to improve its production.

2 Applications of cytokinins in agriculture

As mentioned in the earlier section, cytokinins have a wide range of applications in agriculture as they promote overall plant growth. These hormones along with auxins are responsible for maintaining root and shoot growth. Also, an establishment of a balance between cytokinins and salicylic acid is very critical during the early development of the plant i.e., during the initial stage of seed germination and root elongation (Toribio et al., 2020). The specific application of cytokinins in agriculture is discussed below.

2.1 Regulation of leaf development

Leaves are the major organ of a plant that helps in perceiving light and responding to external environmental conditions. Leaves and other aerial organs of plants are generated from three functional zones, i.e., CZ (Central Zone), RB (Rib Zone), and PZ (Peripheral Zone) of the shoot apical meristem (SAM) (Wu et al., 2021). SAM is a highly organized tissue located at the shoot apex and consists of pluripotent cells (Armenta-Medina and Gillmor, 2019). Cytokinins are considered to be important for the proper care of SAM. The size as well as activities of SAM are controlled by cytokinins as a decline in cytokinin level, either by mutation in the *ipt* gene or by an overexpression of the *CKX* gene, may lead to the reduction in the activity and size of SAM

(Wu et al., 2021). In addition, cytokinins have a key contribution in the organization of the leaf layout. Marginal blastozone (MB), a region at the leaf margin, gives rise to lobes or leaflets in a leaf (Wu et al., 2021). For this, the stem cell or meristematic activity requires to be maintained for a longer duration for the formation of leaf and this is achieved through cytokinins (Wu et al., 2021). Also, the structure of a developing leaf can be altered by modifying the endogenous cytokinin level (Shamsi et al., 2019). Thus, it can be summarized that cytokinins play critical roles such as the regulation and modulation of genes as well as signaling during leaf development (Wu et al., 2021). Apart from this, CKs are helpful in maintaining leaf health as these hormones not only postpone the leaf senescence but also increase the size of stomatal apertures and rate of transpiration in numerous plants (Guo et al., 2021).

2.2 Roles of cytokinins in seed enhancement

Seed enhancement is a process or a technique through which the germination efficiency of a seed is improved and growth of the seedling is enhanced. Cytokinins play roles in seed enhancement as the cytokinin-primed wheat seeds are known to have better germination, growth, and yield under salt stress condition (Rhaman et al., 2020). A study to evaluate the impact of seed priming with two different cytokinins, kinetin and benzylaminopurine (BAP), was conducted by Iqbal et al. (2006). They observed that the germination rate and early seedling growth rate of both, salt-intolerant and salt-tolerant cultivars under salt stress were enhanced after priming with kinetin.

2.3 Regulation of plant response to stress

Cytokinins not only play their role in normal growth, but also help in the redistribution of essential compounds like nucleic acids, inorganic salts, and hormones throughout the plant body (Ullah et al., 2018). This re-distribution inhibits the degradation of chlorophyll, proteins, and nucleic acids and thus restricts the senescence of plants. Further, it can be inferred from recent research studies that cytokinin has the potential to ameliorate the impairments caused by different stress conditions, such as heat, cold, drought, and salt (Prerostova et al., 2018). Under a heat stress situation, the production of reactive oxygen species (ROS) is geared up (Hu et al., 2020). Cytokinins mitigate the effect of ROS and impart the heat-tolerance ability to plants by stimulation of the antioxidant system as well as upregulation of heat shock response proteins (Li et al., 2021). Similarly, the cold-tolerant capacity of plants can be enhanced by enhancing the cytokinin level either exogenously or endogenously (Liu et al., 2020).

Crop production is frequently impacted by a variety of abiotic challenges; hence, crop protection against abiotic stress is critical to their survival. Water shortage, heat stress, drought, high and low salt concentration in soil, as well as high and low temperature are example of abiotic stressors (Ronga et al., 2018). These stressors induce phenotypic, genotypic, and metabolic variations in plants that have a negative impact on their growth and productivity (Ronga et al., 2018). Cyanobacteria might be considered a prospective source of new biostimulants as these organisms may help to ameliorate the negative consequences of abiotic stress and promote plant growth by producing a variety of chemicals (Santini et al., 2021). Auxins, cytokinins, betaines, amino acids, vitamins, and polyamines are such chemicals produced by cyanobacteria with very low effective concentrations (Ronga et al., 2019). Among these chemicals, CKs are significant regulators of plant activities under harsh ecological situations (Castander-Olarieta et al., 2021). Thus, cyanobacteria, such as *Nostoc entophytum* and cyanobacterial filtrates, containing high levels of cytokinins and auxins (IAA), could be a new avenue for applying exogenous phytohormones in agriculture (Ronga et al., 2019; Kollmen and Strieth, 2022).

Exogenous and endogenous cytokinins have different effects on stress tolerance by plants (Liu et al., 2020). Exogenously supplied cytokinin can not only improve salt tolerance, but can also lead to a phenotypic variety more vulnerable to salt treatment (Quamruzzaman et al., 2021). All drought-stressed plants showed growth inhibition, which was linked to an enhanced level of abscisic acid and a reduced levels of auxins and active cytokinins (Prerostova et al., 2020).

Apart from its role in drought resistance, CKs also play a role in temperature detection and heat signaling in *Arabidopsis* (Castander-Olarieta et al., 2021). The impact of exogenous CK administration on modulating stress tolerance has been investigated with the help of a wide range of hormone-treatment options. Cyanobacterium, *Synechocystis* sp., can tolerate salt levels up to 1.2 M NaCl due to cytokinin production (Yang et al., 2020). On cytokinin-supplemented media bean plants, sprouting potato tubers and wheat seedlings showed better salt tolerance (Naqvi et al., 1982; Abdullah and Ahmad, 1990). Spraying with cytokinin before a drought boosted the capacity of bean plants to tolerate stress but had a negative impact on maize and sugar beet (Zwack and Rashotte, 2015). More so, *Arabidopsis* growing on a cytokinin-supplemented medium had resulted in a higher yield. Cytokinin-supplemented plants had shown a higher survival rate than non-supplemented plants in conditions of cold or dehydration stress also (Zwack and Rashotte, 2015). Another example is heat tolerance in tobacco and radiata pine plants, as well as improved osmotic defense, recovery, regulated photosynthesis, and antioxidants (Prerostova et al., 2020; Castander-Olarieta et al., 2021).

Furthermore, CKs has also been shown to have a favorable influence on photosynthesis. Benzyladenine (BA) is a naturally occurring cytokinin that increases CO_2 fixation, resulting in increased sugar production and preserving the chloroplast under stress conditions. CKs may potentially act as non-enzymatic ROS scavengers. For example, zeatin riboside is known to protect the viability of seeds by scavenging the superoxide anions. These findings suggest that a variety of factors such as geographical, temporal, and environmental factors determine how cytokinin therapy affects stress signaling.

2.4 Impact of cytokinins on root nodulation

Root nodules in leguminous plants are induced by rhizobia infection and controlled by nodulin gene expressions. Based on the expression timing, the nodulin genes can be categorized into early and late nodulin genes. Infection and nodule organogenesis are the concern of early nodulin genes while the nodule function, maintenance of the oxygen level by leghaemoglobin, etc., are regulated by the products of late nodulin genes (Lebedeva et al., 2021). Cytokinin is responsible for inducing the expression of early nodulin genes in legumes, cortical cell division, and ultimately, the formation of a nodulin-like structure (Yang et al., 2022). Mens et al. (2018) studied the role of cytokinin in the nodulation of soybean plants. They concluded that, cytokinin plays a critical role in initiating cortical cell division and thereby in expression of early nodulation transcription factors. Furthermore, nodule development was favored by an exogenous application of a low amount of cytokinin (0.1 μM). However, the nodulation can be reduced by the high concentration of cytokinin due to its toxic effects. They also observed that *ipt* genes are regulated in response to rhizobial inoculation (Mens et al., 2018).

2.5 Role of cytokinins in the interaction between plants and pathogenic microbes

Nowadays, scientists across the globe are focusing on the mechanism of interaction between the plant and phytohormones and among the hormones themselves (Kieber and Schaller, 2018). The interactions of phytohormones with each other are responsible for the regulation of the signaling pathways involved in metabolism which in turn affects the final outcome of the plant's response to different environmental conditions and plant development (Lubyanova et al., 2021). This cross-talk between hormones involved in signaling is important for a plant to respond to the stress conditions (Yang et al., 2019). There are various synergistic and antagonistic interactions among the hormones which determine the overall behavior of a plant under different conditions. The interaction between phytohormones, JA (jasmonic acid), SA (salicylic acid), and ET (ethylene), is the key to the defense response of plants. The SA defense of plants is enhanced by cytokinin and thus cytokinin and salicylic acid exhibit a positive and beneficial interaction. A high level of cytokinin can modulate the SA signaling by triggering the SA-related gene expression involved in plant defense, thus, providing an enhanced protection to the plant (Lubyanova., 2021; Asif et al., 2022).

2.6 Role of cytokinins in chloroplast development

The controlled and coordinated regulation of the transcription of genes encoded by the chloroplast and nuclear genomes is the foremost requirement for the development and functionality of chloroplast. Cytokinins are known to be the major factor responsible for the expressions of genes encoding plastids and other related proteins. It is well known that CKs regulate the development of chloroplasts under various environmental conditions during different stages of plant development (Andreeva et al., 2020). These molecules regulate the expression of gene-encoding protochlorophyllide oxidoreductase enzyme having a critical role in chlorophyll synthesis. These hormones are also responsible for the synthesis of electron transport chain proteins in the thylakoid membrane (Toribio et al., 2020; Santini et al., 2021).

3 Exogenous sources of cytokinin

Cytokinins may be synthesized in a laboratory which has certain beneficial impacts. The exogenous application of synthetic cytokinins can also enhance plant growth parameters. It can improve the antioxidant enzyme activity and thus reduce the oxidative stress. Synthetic cytokinins such as Thidiazuron (TDZ) and CPPU N-(2-chloro-4-pyridyl)-N'phenylurea (CPPU), that can activate cytokinin receptors are known to improve chlorophyll *b* content and can be used as a cotton defoliant (van Voorthuizen et al., 2021).

The use of synthetic cytokinin, TDZ (0.25 mg/L) has been reported to adversely affect the shoot development in *Hibiscus acetosella* including poor shoot elongation and distortion. In case of the *Acer saccharinum* plant, a high concentration (1.0 mg/L) of TDZ caused fascination, i.e., formation of flat shoots resembling multiple shoots fused together and stunting and drop of leaves were observed (Trigiano and Gray., 2016). In another study, the suppression of shoot elongation was observed in the *Liquidambar* plant in response to TDZ. Another study observed the hyperhydricity of shoot tissues in the *Juglans nigra* plant when exposed to high concentrations of TDZ. In addition, the plants exhibited thicker stems, shorter internodes, as well as elongated, deformed, and brittle leaves, with an

appearance of being soaked and translucent (Trigiano and Gray, 2016). More so, TDZ stimulates the establishment of the abscission zones and leaf abscission, increase in ROS levels, destruction of net photosynthesis, and a substantial decrease in transpiration and stomatal conductance by increasing the activity of cell wall-degrading enzymes and the ethylene content (Jin et al., 2020). On the other hand, the application of CPPU may cause a slight delay in the maturity of plants and ripening of fruits (Fujisawa et al., 2018).

In addition to synthetic sources, there are several natural exogenous sources of cytokinins such as insects, plant pathogenic bacteria, fungi, amoeba, and cyanobacteria (Frébortová and Frébort, 2021). Phytophagous insects are reported to produce cytokinin as evinced by the higher cytokinin content in the body of different insects as compared to plants. Cytokinin production by an insect aims to manipulate the plant defense response against herbivory and also to modify the distribution of nutrients at the site of feeding in a leaf (Andreas et al., 2020). Plant pathogenic microbes produce cytokinins as a virulence factor. For example, *Agrobacterium tumefaciens*, transfers T-DNA (Transfer- DNA, which is a section of the Ti plasmid, present in the bacteria and is responsible for inducing diseases in plants) containing the *ipt* gene into the host cell during infection. As this T-DNA gets integrated into the host genome, it leads to an overproduction of cytokinins and along with increased auxin content results in tumorous cell proliferation. Thus, the bacterium is responsible for crown gall disease. Similarly, *Pseudomonas syringae* and *R. fascians* also cause diseases in plants (Schmülling, 2013). Fungus *Claviceps purpurea*, amoeba *Dictyostellium discoideum,* have also shown the presence of adenylate IPTs (Frébortová et al., 2017).

In addition to plant pathogens, few human pathogens are also known to produce cytokinins. Earlier, the significance of cytokinin production by human pathogens was unknown and a challenge for researchers. Samanovic et al. (2018) demonstrated that the production of cytokinin by a human pathogen, *Mycobacterium tuberculosis*, is necessary to instigate changes in its transcription and physiology. Thus, cytokinin is used as a communication signal by pathogenic microorganisms. Though, these organisms are able to produce cytokinins, their application in agriculture is restricted due to their adverse effects on plants as well as in human health.

Although there are a number of external sources of cytokinin, cyanobacteria may be the most preferred source as these organisms have the ability to impart several other benefits to plants as well. The details of these benefits are given in a later section. Thus, cyanobacteria may be deployed in agricultural fields as biofertilizers leading to the effective enhancement in growth and yield of crops in a sustainable way (Žižková et al., 2017).

4 Cytokinin production by cyanobacteria

Cytokinins are crucial phytohormones responsible for stimulating the interaction between plants and cyanobacteria. There are many experimental evidences that support the cytokinin production by cyanobacteria and its further role in crop improvement. The cyanobacteria such as *Nostoc sp.* PCC7120, *Anabaena variabilis* ATCC 29413, *Microcystis aeruginosa* NIES-843, *Oscillatoria*, *Phormidium,* and *Chroococcidiopsis sp.* are known to produce cytokinins. Table 1 shows the different methods of cytokinin detection in cyanobacterial extract/cell suspension/dried biomass. These hormones are responsible for the induction of shoots as well as adventitious roots at petiolar and internodal segments (Zhao et al., 2021). Thus, cytokinins produced from cyanobacteria can be used for the *in vitro* regeneration of plants (Toribio et al., 2021). Toribio et al. (2020) observed an enhanced radicle and aerial growth of cucumber seedlings after cyanobacterial inoculation with *Trichormus* SAB- M304 and *Nostoc* SAB-M251, due to the cytokinin secretion by these organisms. Frébortová et al. (2017) have studied the effect of light on cytokinin production by *Nostoc* sp. and observed the enhanced production of cytokinins under dark conditions. A microscopic analysis evinced that the cyanobacterium, *Calothrix ghosei*, is capable of establishing a relationship with wheat plant roots by penetrating them and stimulating their growth (Karthikeyan et al., 2009).

Cytokinins are the primary hormones that initiate the cyanobacterial colonization in plant roots as evidenced by a gene knockout study. In this study, the *ipt* gene knockout of cyanobacterium, *Nostoc* sp., was created. The knocked-out organism was unable to colonize the plant root. The hypothesis behind this knockout study was that cytokinin production by the cyanobacterium would be ceased in the absence of the *ipt* gene as it codes for isopentenyl transferase, a biocatalyst critical for cytokinin biosynthesis. Furthermore, the absence of cytokinin would also reduce the root colonization potential of the organism (Lee and Ryu., 2021).

It has been experimentally proved that cyanobacteria can modify the endogenous phytohormone levels in an inoculated plant as observed in wheat. An *in vitro* study conducted by Hussain and Hasnain 2011, concluded that cyanobacterial inoculation led to the endogenous cytokinin and IAA accumulation (Santini et al., 2021). Table 2 shows examples of crops which exhibited resistance to abiotic stresses and enhanced yields when treated with cyanobacterial extracts/cell suspension. However, more research is needed to study the effects of cyanobacterial inoculation on various other crops.

TABLE 1 The techniques used for the detection of cytokinins in different cyanobacterial species.

Cyanobacteria	Process used for sample preparation	Technique used for quantification of cytokinin	References
Arthronema africanum	Cation exchange resin and paper chromatography	GC-MS	Stirk et al. (1999)
Calothrix sp	Ultrasonication	Soybean Callus Bioassay	Stirk et al. (2002)
Chroococcidiopsis	Homogenization, sonication	UPLC–ESI–MS/MS	Hussain et al. (2010)
Chroococcus, Nostoc	Homogenization	HPLC-MS/MS, UHPLC-MS/MS	Žižková et al. (2017)
Nostoc SAB-M251, Trichormus SAB-M304, Nostoc SAB-M612	Sonication	Immunodiagnostic test	Toribio et al. (2020)
Nostoc sp. HK-01	Sonication	HPLC-ESI-MS/MS	Kimura et al. (2020)
Anabaena oryzae, *Nostoc entophytum*	Homogenization	Gas liquid chromatography	EL-Zawawy et al. (2021)

5 Cytokinins in plant tissue culture/ explant regeneration

Plant tissue culture is currently recognized as an important tool of plant biotechnology, since it provides a fresh way to plant production, propagation, conservation and manipulation. Plant nutrition and morphogenesis are increasingly being studied using *in vitro* cell and tissue culture techniques. These methods are based on three primary principles: 1) isolation of the explant from its natural habitat, 2) use of aseptic cultivation conditions to keep these isolates alive in controlled conditions, and 3) *in vitro* maintenance of the physical and chemical environments. To manage the tissue culture environment, a variety of synthetic compounds are used. The phytohormones, such as cytokinins and auxins produced by cyanobacteria, may be used as a culture media supplement to enhance culture growth and shoot regeneration. This is evident from the study of a researcher, who recorded the advantageous effects of adding cyanobacterial biomass to culture media on the development of beet root and pea explants. Similarly, the extract of the cyanobacterium, *Plectonema* sp., when added in the culture medium, stimulated somatic embryogenesis and organogenesis in sandalwood and paddy (Singh, 2014). Moreover, cyanobacterial spent media have also been found effective in generating *in vitro* callus from explants of *Stevia rebaudiana* Bertoni (Blinstrubienė et al., 2020).

6 Metabolism and signal transduction of cytokinins

The biosynthesis and degradation of cytokinin regulate its endogenous levels within the cells. The biosynthesis of isoprenoid cytokinin involves the handover of the isopentenyl chain from DMAPP (Dimethylallyl pyrophosphate) or HMBPP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate) to AMP (bacteria), ADP, or ATP (plants) in the very first step to form isopentenyl -AMP, -ADP, -ATP, respectively. This reaction is catalyzed by the enzyme isopentenyl transferases (IPTs) of two different categories. The first one is adenylate IPT that catalyzes the transfer of the isopentenyl chain to the N^6 amino group of free AMP, ADP, or ATP. The second one, tRNA- IPT, catalyzes its transfer to tRNA-bound AMP (Frébortová et al., 2017). The genes for both, adenylate IPT (*NoIPT1*) and tRNA-IPT (*NoIPT2*) have been identified in *Nostoc* sp. PCC7120 (Frébortová et al., 2017). Several researchers have concluded that there is much similarity between isopentenyl transferases (IPTs) in cyanobacteria and the same in plants (Žižková et al., 2017). However, the preferred substrate for plant IPT is ATP or ADP while AMP is the preferred substrate for cyanobacterial IPT. Furthermore, a majority of the cytokinin synthesis in plants is catalyzed by adenylate IPT, whereas tRNA IPT is the enzyme catalyzing most of the cytokinin synthesis in cyanobacteria. Figures 1A, B show the process of cytokinin synthesis in plants and cyanobacteria. Furthermore, the isopentenyl AMP, -ADP, and -ATP get converted to ribosides cytokinins, like zeatin, by hydroxylation of the isopentenyl side chain using the enzyme cytochrome P450 monooxygenase. The CKs, ribosides 5′monophosphates, are converted to free base CKs by the enzyme phosphoribohydrolase (Lonely guy; LOG). The interconversion of one form of cytokinin to another form continues so as to maintain and regulate the level of active compounds. Conjugates are formed by O and N glycosylations in the cytokinin (Frébortová and Frébort, 2021; Wu et al., 2021).

Although bacterial IPT uses both DMAPP and HMBPP as isopentenyl chain donors, in case of cyanobacteria, *Nostoc sp.* PCC 7120, the IPT enzyme (NoIPT1) was found to be more active when DMAPP was used as a donor. Since the reaction rate was very slow while using HMBPP as a side chain donor, further biochemical characterization in this case could not be done. Thus, it can be concluded that the cyanobacterial IPT

TABLE 2 Physiological effects of cyanobacteria on different crops exposed to abiotic stress.

Cyanobacteria	Forms used	Crop	Relevant response in higher plants	Physiological impacts in higher plants	Responsible metabolites	References
Scytonema hofmanii	Extracellular products	Oryza sativa	Tolerance to salt stress	Hormone homeostasis	Cytokinin	Rodriguez et al. (2006)
Spirulina maxima	Water extract	Triticum	Salinity tolerance	Antioxidant activity, stimulate protein content	Carotenoid tocopherol, phenolic, and protein	Abd El-Baky et al. (2010)
Oscillatoria acuta, *Plectonema boryanum*	Cell suspension	Oryza sativa	Stress tolerance against abiotic stress	Increases enzyme activity of peroxidase, antioxidant activity	Phenylpropanoids and flavonoids	Singh et al. (2011)
Phormidium tenue	Crude extract	Caragana korshinskii	Increases shrub performance in crusted desert parts	Acts as a biofertilizer, seed germination, increase carbohydrate contents and photosynthetic activity	Polysaccharides	Xu et al. (2013)
Calothrix elenkinii, *Anabaena laxa*	Thermotolerant bacteria	*Foeniculum vulgare*, *Coriandrum sativum*, *Cuminum cyminum*	_	Increases the enzyme activity of peroxidase, antioxidant activity, plant growth promotion	-	Kumar et al. (2013)
Microcystis aeruginosa MKR 0105 and *Anabaena* sp. PCC 7120	Biojodis and Cyanobacteria	*Zea mays*	Tolerance to thermal stress	Antioxidant activity tolerance to thermal stress	-	Piotrowski et al. (2016)
Nostoc muscorum, *Anabaena oryzae*	Algal extracts	*Phaseolus vulgaris*	Tolerance to cold and drought stress and nitrogen deficiency	Enhanced growth and photosynthesis	-	Setta et al. (2018)
Spirulina platensis	Exogenously applied cynobacteria	*Vicia faba*	Salt tolerance	Increase photosynthetic activity and protein content	Carotenoids	Selem, (2019)
pirulina platensis	Cell suspension	*Zea mays*	Tolerance to salt stress	Tolerance to cadmium toxicity	-	Seifikalhor et al. (2020)
Arthrospira platensis	Cell hydrolyzate	*Petunia xhybrida*	Tolerance to salt stress	Increased the K+/Na + relationship and Stimulates shoot and bud formation	-	Bayona-Marcillo et al. (2020)
Nostoc piscinale	Cyanobacterium-based biostimulant	*Zea mays* (SY Zephir hybrid)	Stress tolerance	Faster vegetative growth and higher chlorophyll content, higher grain yield	-	Ördög et al. (2021)
Roholtiella sp	Foliar extract	*Capsicum annuum*	Tolerating salt stress	Antioxidants activities and accumulation of proline, shoot length increased, fresh and dry weights chlorophyll a and b increase	Proline	Bello et al. (2021)
Leptolyngbya-1267	Sonicated extracts	*Solanum lycopersicum*	Plant protects against biotic stresses	Biopesticidal effect, mitigation of bacterial canker, promote plant growth, root development	Exo-metabolites	Toribio et al. (2021)

resembles more to a plant's IPT. The difference of IPT (plant and bacterial IPTs) in regard of the substrate may be due to different protein structures (Frébortová and Frébort, 2021; Wu et al., 2021).

Cytokinins can be inactivated or degraded through an enzyme known as cytokinin dehydrogenase (CKX) (Zhang et al., 2021; Mandal et al., 2022) which catalyzes an irreversible, oxidative cleavage of the cytokinin side-chain, which regulates the concentration of active cytokinins in plants. Multiple CKX proteins are found in terrestrial plants, each with its own tissue and subcellular localization as well as substrate specificity. Frébortová et al. (2015) carried out an investigation to explain the functionality of enzyme NoIPT1 along with NoCKX1 in *Nostoc* sp. The organism,

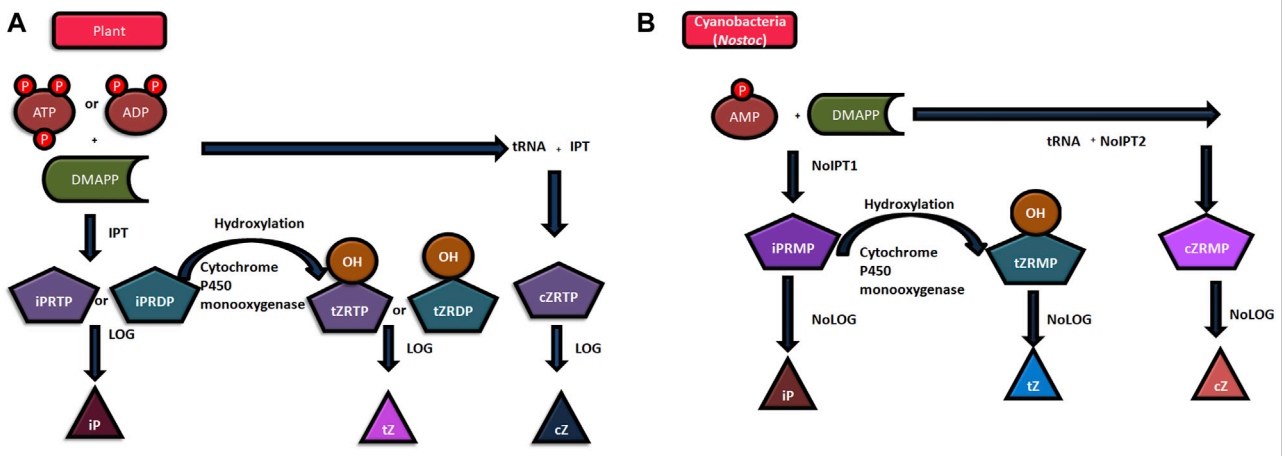

FIGURE 1
Mechanism of cytokinin synthesis **(A)** in plants, and **(B)** in Nostoc sp. PCC 7120. (Abbreviations: ATP- Adenosine triphosphate, ADP- Adenosine diphosphate, AMP- Adenosine monophosphate, DMAPP- dimethylallyl pyrophosphate; iPRMP- isopentenyladenosine-5- monophosphate; iPRDP- isopentenyladenosine-5- diphosphate, iPRTP- isopentenyladenosine-5- triphosphate tZRMP- trans-zeatin riboside 5′-monophosphate; tZRDP- trans-zeatin riboside 5′-diphosphate; tZRTP- trans-zeatin riboside 5′-triphosphate; cZRMP- cis-zeatin riboside 5′-monophosphate; cZRTP- cis-zeatin riboside 5′-triphosphate; iP- N6-(Δ2-isopentenyl) adenine; tZ-trans-zeatin; cZ-cis-zeatin, LOG- LONELY GUY; HMBPP- 4-hydroxy-3-methyl-but-2-enyl pyrophosphate).

Nostoc sp. was selected for the study so as to have a better understanding regarding cytokinin metabolism in cyanobacteria since it is not indulged in any kind of pathogenic activity in plants (Frébortová et al., 2015). The genes, *NoIPT1* and *NoCKX1* were isolated from *Nostoc sp.* and transformed in *E. coli* for the expression of proteins. The study observed that out of the two proteins i.e., NoIPT1 and NoCKX, only NoIPT was functional and had the characteristics of cytokinin dehydrogenase as found in plants. However, the *NoCKX* gene was not functional suggesting that the cyanobacterium was unable to perform cytokinin degradation through the CKX pathway (Frébortová et al., 2015).

Various efforts have been made to determine the signal transduction mechanism of cytokinins. As cytokinins are critical phytohormones, the model plant *Arabidopsis* was selected for studying the cytokinin signaling pathway. Cytokinins are upregulated by the plant cell through the interaction with the CHASE (cyclase/histidine kinase-associated sensing extracellular) domain of CRE1 (cytokinin receptor). The interaction of cytokinin to CRE1 leads to its autophosphorylation as it has a histidine kinase activity. After phosphorylation, it transfers its phosphate group to AHP (Arabidopsis Histidine Phosphotransfer) belonging to a histidine-containing phosphotransfer family. Once phosphorylated, AHP enters the nucleus and in turn activates ARR-B (Arabidopsis Response Regulator) through phosphorylation. The phosphorylated ARR-B further regulates the transcription of cytokinin-response genes (Bidon et al., 2020; Rashotte, 2021). As far as cyanobacteria are concerned, the presence of a cytokinin receptor homolog, all2875, is evident in *Nostoc sp.* PCC 7120. This receptor, all2875, is a histidine kinase receptor, containing the CHASE domain, and is known to bind with iP (isopentenyl adenine). Moreover, this receptor binds with trans-zeatin also but with lower affinity (Bidon et al., 2020). However, the majority of the proteins required for cytokinin signaling are not organized in cyanobacteria in a way that allows them to contribute to phytohormone signaling.

Another technique for limiting CK degradation is to suppress the CK oxidase/dehydrogenase gene (CKX), which induces the removal of the prenyl side chain from the adenine moiety of cytokinin (Khandal et al., 2020). Apart from that, various inhibitors of CKX, such as Thidiazuron, CPPU (*N*-(2-chloro-4-pyridyl)-*N*′-phenylurea), TD-K (*N*-furfuryl-*N*′-1,2,3-thiadiazol-5-yl-urea), and INCYDE (2-chloro-6-(3-methoxyphenyl) aminopurine), are used to prevent the irreversible degradation of cytokinin. These inhibitors can activate the cytokinin receptor as anti-senescence properties (van Voorthuizen et al., 2020). INCYDE is a particularly potent CKX inhibitor among them (Prerostova et al., 2020). INCYDE has been used to improve plant tolerance to abiotic and biotic stresses such as the cadmium challenge in *Bulbine natalensis* and *Rumex crispus* as well as the salt challenge in tomato (van Voorthuizen et al., 2021) and *Verticillium longisporum* (Prerostova et al., 2020). In a recent study, an analog of INCYDE, INCYDE-F was used in the barley field for altering the endogenous cytokinin content (Koprna et al., 2020).

7 Genetic manipulation of the cytokinin biosynthetic pathway

As the climate is changing, the demand for stress-tolerant crops that can adapt to harsh environmental circumstances like drought and heat stress has to be enhanced (Mubarik et al., 2021). The existing crop enhancement approaches, such as grafting (hybridization), polyploidy, backcross method, clonal selection, and mutation breeding are likely to have reached their limits, genetic engineering is projected to be able to increase the crop output even further (Salonia et al., 2020). As a result, the genetic manipulation of plants, widely used in the United States, is offering solutions to farming challenges in dry and tropical economies that rely largely on agriculture. The resource-poor farmers from countries like China, Brazil, and India account for almost 90% of the genetically modified (GM) crop cultivation. Extensive research studies are being carried out in order to generate transgenic plants with enhanced stress resistance. Hereafter, the present article explains the overview of the genetic manipulation strategies for refinement of the plant's responses to various abiotic stressors, such as DNA methylation, post-transcriptional modification of mRNA, post-translational modifications, tagging RNA molecules, micro-RNAs, epigenetic modification, and CRISPR-Cas9 editing (Mandal et al., 2022). Furthermore, the synthetic biology technologies and approaches will expand the capabilities and perspectives of genetic engineering initiatives aimed at improving the abiotic stress tolerance in plants (Sargent et al., 2022).

The traditional approach to engineer plants for improved abiotic stress tolerance entails interruption at various stages of the response, from sensors and signaling/regulatory elements (such as kinases, transcription factors) to effectors like osmo-protectants, phytohormones, antioxidant enzymes, and heat-shock proteins (Tanpure et al., 2021). Ramireddy et al. (2018) created transgenic barley plants with an expanded root system by accelerating cytokinin breakdown in the roots by the production of a cytokinin oxidase/dehydrogenase under the control of root-specific rice promoters. They also showed that, using cytokinins to influence root growth and branching resulted in an increase in macro and microelement levels in transgenic barley plants' leaves and seeds. This was complemented by an increased drought tolerance, demonstrating that root engineering of cereals is an appealing method for avoiding nutrient deficiency in agronomically important plants.

Another tactic for increasing production is to extend the lifespan of plants to delay senescence. For the first time in tobacco transformation, researchers used an *Arabidopsis* senescence-specific promoter (PSAG12) fused with the IPT gene, resulting in better flowering, seed yield, biomass, and reduced leaf and floral senescence (Salvi et al., 2021). SAG promoters such as SAG12 and others have been widely used in plant transformation vectors since then. The Senescence Associated Receptor Protein Kinase (PSARK) promoter, which is induced by stress during maturation, has also been effectively used in the generation of transgenic plants with greater CK content (Salvi et al., 2021). The use of inducible promoters for the conditional expression of CK-biosynthetic genes allows for hormone modulation without the negative consequences of large amounts of this phytohormone on plant growth and development (Salvi et al., 2021).

Recently, it has been observed that the CK biosynthesis pathway in *Nostoc* is activated during the dark period, which results in the gradual increase of CK content once the light phase begins (Frébortová et al., 2017). It was revealed in a transcriptome-based study that the application of cytokinin in the presence of light (CK alone was unsuccessful) changed the transcript levels of numerous genes, such as signal transduction, including two-component sensor histidine kinases and two-component hybrid sensors and regulators in cyanobacteria (*Nostoc* sp. PCC 7120) (Frébortová et al., 2017).

Genetic engineering of cytokinins (CKs), appears to be a viable method for increasing plant productivity, both the synthetic and catabolic routes of phytohormones can be engineered to regulate their levels (Mubarik et al., 2021). Plant yield and productivity have long been thought to be affected by cytokinins, which regulate many aspects of plant growth and development. Genes involved in CK synthesis (*ipt* encoding isopentenyl transferase) and metabolism (CKX encoding cytokinin dehydrogenase and the genes of glucosyltransferases) are the main targets of CK engineering (Mandal et al., 2022).

The applications of nanotechnology for plant transformation have opened new avenues for understanding cytokinin biology. Researchers have demonstrated that tools such as carbon nanotubes and the widely used CRISPR-Cas9 genetic editing might be used to efficiently change the plant's genetic material (Demirer et al., 2021). A wide variety of crops have been successfully modified using the CRISPR/Cas technique due to its precision, simplicity, and low cost (Mandal et al., 2022; Yan et al., 2022). Nanomaterial-mediated gene editing now provides the benefits of species independence, ease of use, acceptable preservation of external nucleic acids, strong biocompatibility, high transformation efficiency, and the potential for plant regeneration (He et al., 2019). Exogenous genes are delivered into the cytoplasm or organelle by nanomaterials via an endocytic or non-endocytic route. Raspor et al. observed that an overexpression of the AtCKX2 gene from *Arabidopsis thaliana* in *Solanum tuberosum* L. cv. Désirée resulted in considerably fewer bioactive cytokinins than the control (Mandal et al., 2022). More so, researchers discovered that an overexpression of the CKX gene causes CK deficit in *Hordeum vulgare*, which in turn hinders blooming and causes morphological alterations such as larger root systems and delayed aerial development (Mandal et al., 2022). Silencing the TaCKX2.4 gene in *Triticum aestivum* lowered

the activity of the cytokinin oxidase in transgenic plants, leading to cytokinin accumulation (Li et al., 2018). Similarly, Tao et al. (2020) reported that CRISPR-edited RGG1 (G proteins) in *O. sativa* lowered endogenous cytokinin levels, grain size, and plant development. Combining rapidly evolving advancements in the CRISPR/Cas tool with knowledge of the IPT or CKX gene function could speed up the development of new, higher-yielding cultivars that could shape future agricultural practices (Jiang et al., 2021).

8 Conclusion

In view of a paradigm shift in climate, and extensive use of chemicals, it is indispensable to cultivate stress-tolerant crops, that too in a sustainable way. Cyanobacteria can be used as a preferred source of exogenous cytokinins to enhance the stress-tolerance capacity, growth, and productivity of plants. These organisms when inoculated in the field may form a symbiotic association with plants and increase the cytokinin level in plants. On the other hand, the cyanobacterial filtrate may also be used directly in the field. Moreover, the biomass or the spent media of cyanobacteria may be used as a source of cytokinin in the tissue culture of various explants. As the metabolism of cytokinin in cyanobacteria is well understood, its production may be enhanced using genetic engineering and synthetic biology approaches. Thus, cyanobacteria, as a source of exogenous cytokinins, have the capability to boost the agro-economies in a sustainable way to fulfill the continuously increasing demand of organic agricultural products. However, more research is needed to ensure the cost-effective availability of cyanobacterial biomass at a large scale as well as the easier techniques for detection and quantification of cytokinins in the biomass. More so, the techniques for co-culturing the cyanobacteria with different plant species should be developed to promote the hassle-free application of cyanobacteria in agriculture.

Author contributions

RKS and SPT planned the manuscript. SU, MB, and PS wrote the first draft of manuscript. All the authors have finalized the manuscript.

Acknowledgments

Authors are thankful to Head, Botany and Microbiology, H.N.B. Garhwal University, Srinagar (Garhwal), Uttarakhand, India, for providing the necessary facilities.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Genetics

TYPE Original Research
PUBLISHED 27 September 2022
DOI 10.3389/fgene.2022.963789

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

RECEIVED 07 June 2022
ACCEPTED 05 August 2022
PUBLISHED 27 September 2022

CITATION
Blume R, Yemets A, Korkhovyi V, Radchuk V, Rakhmetov D and Blume Y (2022), Genome-wide identification and analysis of the cytokinin oxidase/dehydrogenase (ckx) gene family in finger millet (Eleusine coracana). *Front. Genet.* 13:963789. doi: 10.3389/fgene.2022.963789

Genome-wide identification and analysis of the cytokinin oxidase/dehydrogenase (*ckx*) gene family in finger millet (*Eleusine coracana*)

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Cytokinin dehydrogenase/oxidase (CKX) enzymes play a key role in regulating cytokinin (CK) levels in plants by degrading the excess of this phytohormone. *CKX* genes have proven an attractive target for genetic engineering, as their silencing boosts cytokinin accumulation in various tissues, thereby contributing to a rapid increase in biomass and overall plant productivity. We previously reported a similar effect in finger millet (*Eleusine coracana*) somaclonal lines, caused by downregulation of *EcCKX1* and *EcCKX2*. However, the *CKX* gene family has numerous representatives, especially in allopolyploid crop species, such as *E. coracana*. To date, the entire *CKX* gene family of *E. coracana* and its related species has not been characterized. We offer here, for the first time, a comprehensive genome-wide identification and analysis of a panel of *CKX* genes in finger millet. The functional genes identified in the *E. coracana* genome are compared with the previously-identified genes, *EcCKX1* and *EcCKX2*. Exon-intron structural analysis and motif analysis of FAD- and CK-binding domains are performed. The phylogeny of the *EcCKX* genes suggests that *CKX* genes are divided into several distinct groups, corresponding to certain isotypes. Finally, the phenotypic effect of *EcCKX1* and *EcCKX2* in partially silencing the SE7 somaclonal line is investigated, showing that lines deficient in *CKX*-expression demonstrate increased grain yield and greater bushiness, enhanced biomass accumulation, and a shorter vegetation cycle.

KEYWORDS

cytokinin dehydrogenase/oxidase enzymes, *EcCKX* genes, phylogeny, gene expression, *Eleusine coracana*, finger millet, food security

Introduction

Plant growth regulation depends on the balance of phytohormones, among which cytokinins play an important role. Cytokinins are plant hormones that not only take part in cell proliferation and differentiation, but also in the establishment of plant architecture. They regulate many developmental processes, strongly influence grain yield (Zhang et al., 2012; Jameson and Song, 2016; Chen et al., 2020b; Dash and Rai, 2022), and impact the plant signaling pathways of biotic and abiotic stresses (Werner et al., 2006; Blume et al., 2017). Cytokinin homeostasis is regulated by members of several multigene families through a balance between biosynthesis [isopentenyl transferase (IPT)], activation [Lonely Guy (LOG)], inactivation (O-glucosyl transferase), re-activation (β-glucosidase), and degradation [cytokinin oxidase/dehydrogenase (CKX)] (Werner et al., 2006; Jameson and Song, 2016). In particular, *CKX* genes encode the cytokinin oxidase/dehydrogenase enzymes, which catalyze the irreversible degradation of cytokinin. Such enzymes are responsible for the balance of cytokinins in the plants, and for the control of cytokinin-dependent processes (Werner et al., 2006; Dash and Rai, 2022).

It should be noted that CKXs are encoded by a relatively small gene family in different plant species, such as rice (Ashikari et al., 2005; Mameaux et al., 2012), *Arabidopsis* (Galuszka et al., 2007), maize (Gu et al., 2010; Mameaux et al., 2012; Zalabák et al., 2014), foxtail millet (Mameaux et al., 2012; Wang et al., 2014), barley, wheat, sorghum, and brachypodium (Mameaux et al., 2012), Chinese cabbage (Liu et al., 2013), potato (Suttle et al., 2014), oilseed rape (Liu et al., 2018), forage legume barrel medic (*Medicago truncatula*) (Wang et al., 2021), soybean (Liu et al., 2021; Nguyen et al., 2021), cowpea (Liu et al., 2021) and apple (Liu et al., 2022). Following phylogenetic, molecular, and comparative analyses of *CKX* families in sequenced grass species, such as rice, *Brachypodium distachyon*, sorghum, maize and foxtail millet, as well as members identified from wheat and barley transcriptomes/genomes, the phylogenetic analyses identified four Poaceae *CKX* clades (Mameaux et al., 2012). Comparative analysis showed that such phylogenetic groupings of *CKX* can largely be explained by a combination of local gene duplication and a whole-genome duplication event that preceded their speciation. Recently, the evolutionary origin of CKXs has been analyzed and attempts have been made to understand their function in relation to their structure in different organisms (Dabravolski and Isayenkov, 2021).

Even monocots and dicots show significant differences in cytokinin metabolite composition (Jiskrová et al., 2016). Inhibition of *CKXs* can lead to increased plant productivity, and thereby increased crop yields (Zhang et al., 2012; Jameson and Song, 2016; Szala et al., 2020). These changed features were detected by us earlier in finger millet (*Eleusine coracana*) (Radchuk et al., 2012). We found that downregulation of two *CKX* genes (i.e., *EcCKX1*, *EcCKX2*) in the obtained somaclonal variants of *E. coracana* leads to the formation of a dwarf and highly productive phenotype of finger millet (Radchuk et al., 2012). As a consequence of this gene downregulation, attenuated degradation of cytokinins in such *E. coracana* lines leads to higher cytokinin levels, increased accumulation, and stimulation of meristem activity, resulting in the production of more inflorescences and seed setting (Radchuk et al., 2012; Yemets et al., 2020). Similar data for rice were previously reported by Ashikari et al. (2005), where the authors found that a decrease in *OsCKX2* expression leads to the accumulation of cytokinin in the meristems of inflorescences, an increase in the number of reproductive organs, and, as a result, an increase in grain yield. Other studies confirm such an effect: the silencing *ckx2* in barley, for example (Zalewski et al., 2014), with overexpression of this gene leading to a non-flowering phenotype (Mrízová et al., 2013). This gene is described as having the same function in *A. thaliana* (Li et al., 2013) and also in the development of seed pods in *B. napus* (Liu et al., 2018).

Given the role of cytokinins in plant development and productivity, the identification and regulation of *CKX* gene expression in main crops is extremely important from the perspective of increasing yield and food security. Due to the ability of *E. coracana* to grow in arid and semi-arid regions of Central Africa and India, as well as in tropical regions (more than 25 countries in Africa and Asia), its ability to withstand abiotic and biotic (resistance to pathogens) stresses (Gupta et al., 2017); its nutritional value (the grains are rich in amino acids, such as methionine and lysine; minerals such as calcium, iron, zinc, phosphorus, and potassium; vitamins; and fibers), and its good grain storage properties, finger millet is one of the most important crops for sustainable agriculture in developing countries, and a very valuable food resource. Finger millet is gluten free and therefore can be included in the diet of patients suffering from celiac disease (Pagano, 2006). In addition, finger millet can still potentially be used as animal fodder (Gupta et al., 2017), and for bioethanol production (Yemets et al., 2020). To date, there is no accurate information on the global cultivation of this crop, although India is known to be the largest producer, with finger millet grown on 1.19 million hectares and yielding over 1,660 kg/ha (Sood et al., 2019).

The availability of finger millet genomic resources (Hittalmani et al., 2017; Hatakeyama et al., 2018; Zhang et al., 2019b; Bančič et al., 2021; Pendergast et al., 2022) makes it possible to analyze different groups of genes and estimate practical methods for molecular breeding and biotechnological improvement of this crop (Antony Ceasar et al., 2018; Pandian and Ramesh, 2019; Sood et al., 2019). Certainly, the identification and characterization of all CKX family members in *E. coracana* is a very important tool for achieving these practical goals. In this paper, we present for the first time the comprehensive genome-wide identification and analysis of a panel of *CKX* genes in finger millet. These functional genes, identified in the *E. coracana*

genome, are compared with the two previously-identified genes, *EcCKX1* and *EcCKX2*. Exon-intron structural analysis, and a motif analysis of the FAD- and CK-binding domains are carried out. Study of the phylogeny of *EcCKX* genes establishes that *CKX* genes are divided into several distinct groups, according to their specific isotypes. Finally, the phenotypic effect of partial silencing of *EcCKX1* and *EcCKX2* in the somaclonal line of SE7 is investigated, suggesting that lines deficient in *CKX*-expression manifest increased grain yield, higher bushiness, enhanced biomass accumulation, and a shorter vegetation cycle. The bioinformatic data obtained will prove useful for further manipulation of finger millet *CKX* genes so as to improve crop quality and yield.

Materials and methods

Initial identification and analysis of cytokinin dehydrogenase/oxidase genes in the *E. coracana* genome

The initial search for *CKX* genes in the *E. coracana* genome was conducted using a series of BLAST searches of the *Eleusine coracana* annotation release v1.1 (genome ID: 560), which is deposited in the Phytozome v13 database, and which contains the most recent and complete *E. coracana* genome assembly and annotation. Personal permission to use the genome data for the current study was obtained from Prof. Katrien M. Devos, as the current release of the *E. coracana* annotation has a "Reserved Analysis" status, limiting its use for publication.

The BLAST algorithm was used to search the translated nucleotide databases, and the coding regions of the *OsCKX* and *AtCKX* genes (Swarbreck et al., 2008) were used as queries. In addition, sequences of two previously identified genes, *EcCKX1* (HE800184.1) and *EcCKX2* (HE800185.1) of *E. coracana*, were used for the genomic search (Radchuk et al., 2012). Search parameters were: E-value threshold—$1e^{-5}$, comparison matrix—BLOSUM62, and word length—3 (Henikoff and Henikoff, 1992; Altschul, 1993). We analyzed the results and discarded short and insignificant hits.

Information on *E. coracana CKX* genes, including location, genomic coordinates, sequence ID, genomic sequence, protein sequence, and coding sequence (CDS), was acquired from the Phytozome v13 database. Orthologous information for the identified genes was retrieved from the KEGG database (https://www.genome.jp).

The genomic organization and synteny of cytokinin dehydrogenase/oxidase genes

A multiple alignment CDS sequence of the *CKX* genes was performed using the MUSCLE algorithm (Edgar, 2004). Representations of the exon-intron structure of the genes were obtained using the Gene Structure Display Server (http://gsds.gao-lab.org/) (Hu, 2015).

The domain organization of CKX peptides was analyzed using the Pfam tool (https://pfam.xfam.org/) (Mistry et al., 2021), which allowed us to confirm the presence or absence of key FAD- and CK- binding domains. Calculation of the rate of non-synonymous substitution (K_A), the rate of synonymous substitutions (K_S), and their ratio (K_A/K_S), were performed in the TBtools v1.0971 software (Chen et al., 2020a), using CDS sequences of the identified *EcCKX*.

The organization of CK-responsive *cis*-elements was analyzed in the 2 kbp upstream regions of the identified *EcCKX* genes (taking into account the initiation codon), using the PLACE v30.0 tool (https://www.dna.affrc.go.jp/PLACE) (Higo et al., 1999), and the results were compared with the search results in a similar database—PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002). Further data on the presence of particular *cis*-elements motifs were filtered to identify only the CK-responsive elements. The structure of the protein domain organization, and the allocation of CK-responsive *cis*-elements were visualized using TBtools v1.0971 software (Chen et al., 2020a).

Syntenic relationships between homeologous *CKX* genes from different subgenomes of *E. coracana* were analyzed in TBtools v1.0971 software (Chen et al., 2020a), using the MCScanX algorithm (Wang et al., 2012). The results were further visualized as a circos plot.

To explore the syntenic relationships of *E. coracana* orthologous *CKX* genes with *O. sativa* species, the genome data and the gene annotation files of rice (GCA_001433935.1 assembly) were also downloaded from the NCBI database. The synteny analyzing dual plot graphs were constructed using the dual synteny plotter function in TBtools, while inter-genome synteny was inferred using the MCScanX algorithm.

Phylogenetic analysis

Phylogenetic analysis was performed using MEGAX (Kumar et al., 2018). Respective amino acid sequences of CKX proteins from different species (Supplementary Table S1), including *E. coracana*, were aligned using the MUSCLE algorithm (Edgar, 2004). The CKX protein sequences of *Arabidopsis thaliana* and *Oryza sativa* were retrieved from the KEGG genome database, while those of *Setaria italica*, *Hordeum vulgare*, *Populus trichocarpa*, and *Prunus persica* were obtained from the Phytozome v13 database.

Initial isotype determination of *EcCKX* genes was performed using cds sequences as the initial data set, which was then compared to the previously described *EcCKX1* (HE800184.1) and *EcCKX2* (HE800185.1) (Radchuk et al., 2012). This "guide"

tree was constructed using the neighbor joining (NJ) method with the default software settings. The NJ tree was generated with a bootstrap support of 1,000 replicates (Supplementary Figure S1).

Before the maximum likelihood (ML) phylogenetic tree was constructed, the best substitution model analysis was performed. The Jones-Taylor-Thornton model with gamma distribution rate and invariant sites (JTT + G + I) was chosen as optimal (Jones et al., 1992). The selected model was used to infer a phylogenetic tree using the ML estimation method. The initial tree was derived using neighbor-joining analysis, following the nearest-neighbor-interchange heuristic method. The number of discrete gamma categories was four; the treatment for gaps and missing sites was "use all sites." The statistical confidence of tree topology was assessed using a bootstrap test with 1,000 replicates.

Plant material, growth conditions, and agro-morphological evaluation

Field evaluation of the characteristics of finger millet somaclonal mutants was carried out using approx. 100 plants per wild-type (variety Tropikanka) and SE7 (registered now in Ukraine as Yaroslav-8 variety) finger millet lines. Approximately 15–25 seeds were planted in 20-cm diam. pots in a greenhouse at the Leibniz Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany) in the late April. Plants were grown for 2 weeks in a greenhouse and then some of them were transplanted to a field in Gatersleben in May. Spacing between individual plants was 20 cm × 20 cm. Plant height and yields from a random 25 plants were measured in late September 2011. Additional field trials were conducted in Kyiv, Ukraine, at the National Botanical Garden experimental field, under the conditions described earlier (Rakhmetova et al., 2020). Plants were harvested manually and the following parameters were recorded at the plant maturity stage: plant height, 1000-grain weight, number of primary and secondary stems per plant, dry matter content (w/w), dry biomass yield, and seed yield. The obtained data were also extrapolated to rate per hectare.

Tissue sampling for expression analyses

For quantitative reverse transcription-PCR (qRT-PCR) and northern blot analyses of different tissues, total RNA was isolated 4 and 5 days after imbibition (4 and 5 DAI), from the young leaves of seedlings at the tillering stage, and from the old leaves of maturating plants. In addition, tissue samples of the developing inflorescence were taken at three stages: Stage A at a length of 2–3 cm; Stage B at 3–4 cm; and Stage C at > 4 cm. All samples were collected at least in triplicate from biologically-independent plant material.

RNA extraction

Total RNA was extracted from different tissues of the wild type and SE7 mutant of finger millet using Trizol reagent (Invitrogen). To do this, 100 mg of tissue was ground in liquid nitrogen, mixed with 1 ml of preheated Trizol (60°C) for 5 min, and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred into a new tube, mixed with 0.2 ml of chloroform for 2 min, and centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous phase was transferred into a new tube and mixed with 0.6 vol. of isopropanol, incubated at room temperature for 10 min, and then centrifuged at 13,000 rpm for 10 min at 4°C. The pellet was rinsed once with cold 70% ethanol and dissolved in 100 µl of distilled water. The isolated RNA was treated with RNase-free DNase (Qiagen), purified using a RNeasy plant mini kit (Qiagen), and used for the synthesis of cDNA, quantitative RT-PCR, and cDNA array.

Expression analyses

The procedure for cDNA synthesis was identical to that described previously (Radchuk et al., 2012). cDNA fragments of the *EcCKX1.A/B* and *EcCKX2.A/B* genes were further amplified from different tissues of wild-type and mutant lines of finger millet by RT-PCR, using gene-specific primers selected from conserved regions of the corresponding sequences. For distinguishing of *EcCKX1.A/B* genes, the following primers were used: EcCKX1.A/B-For—5′-TGCGCCTCGACGGCCATTTCAG-3′ and EcCKX1.A/B-Rev—5′-GGATCGTACGATTCGCCCTTCC-3′, while for *EcCKX2.A/B* genes — EcCKX2.A/B-For—5′-CACCACCATCGCTGCGTCCAGT-3′ and EcCKX2.A/B-polyT—5′-GTTGGGTNTTTTTTTTTTTTTTTTTT-3′. The actin gene was an internal control, for which primers targeting barley actin were used: qactin_u—5′-ATGGTGGGGATGGGGCAGAAG-3′ and qvactin_r—5′-CTCCTCCGGGGCAACACGAA-3′. Northern blot analysis of the *EcCKX* genes transcript was performed according to the previously-described procedure (Conrad et al., 2007). For qRT-PCR, as described above, 5 µg of the total RNA isolated were used for reverse transcription by SuperScript III reverse transcriptase (Invitrogen), with an oligo (dT) primer. The resulting cDNAs were used as templates for qRT-PCR assays, which were performed as previously described (Radchuk et al., 2011). The PCR efficiency was assessed using LinRegPCR software (Ramakers et al., 2003). Analysis of RT-PCR data was performed according to the procedure described in our previous publication (Radchuk et al., 2012).

Statistical processing of the data

The obtained data were statistically processed using OriginPro 2019b software. Deviations of all means were

TABLE 1 List of the identified *EcCKX* genes in the genome of finger millet.

Gene name	Loci (Phytozome ID)	Gene length (bp)	Putative peptide length (aa)	Chr	Type
EcCKX1.A	ELECO.r07.1AG0046160	1925	523	1A	IIb
EcCKX1.B	ELECO.r07.1BG0096130	1913	523	1B	IIb
EcCKX2.A	ELECO.r07.2AG0128410	3,629	523	2A	IIIb
EcCKX2.B	ELECO.r07.2BG0183810	3,421	523	2B	IIIb
EcCKX3.A	ELECO.r07.5AG0375500	1978	523	5A	IIb
EcCKX3p.B	ELECO.r07.5BG0422940	937	243	5B	IIb
EcCKX4.A	ELECO.r07.4AG0310130	4,675	522	4A	IIIb
EcCKX4.B	ELECO.r07.4BG0341290	3,198	390	4B	IIIb
EcCKX5.A	ELECO.r07.1AG0035370	3,871	535	1A	IIa
EcCKX5.B	ELECO.r07.1BG0085420	3,839	533	1B	IIa
EcCKX6.A	ELECO.r07.8AG0633390	1705	414	8A	IIIa
EcCKX6.B	ELECO.r07.8BG0662450	3,044	609	8B	IIIa
EcCKX7.A	ELECO.r07.1AG0012730	2,118	529	1A	Ib
EcCKX7.B	ELECO.r07.1BG0061400	2038	531	1B	Ib
EcCKX8.A	ELECO.r07.1AG0013320	3,487	551	1A	Ib
EcCKX8.B	ELECO.r07.1BG0062080	3,732	554	1B	Ib
EcCKX9.A	ELECO.r07.2AG0106510	1862	543	2A	Ia
EcCKX9p.B	ELECO.r07.2BG0160030	1,106	332	2B	Ia
EcCKX10.A	ELECO.r07.6AG0543440	1782	541	6A	Ia
EcCKX10.B	ELECO.r07.6BG0496700	1,694	518	6B	Ia

calculated as a standard deviation (SD). To reveal the significance of differences in various parameters between the studied genotypes, one-way ANOVA was used, which included the calculation of Fisher's least significant differences (LSDs). The LSDs were used to determine homogeneous groups for values of specific morphological or productive parameters at different level of significance, $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results

The cytokinin dehydrogenase/oxidase gene family in finger millet

As the result of a thorough search of the finger millet genome, deposited in the Phytozome v13.0 database, 20 representatives of the *CKX* gene family were identified (Table 1). The length of the genes varied from 937 to 3,871 bp, and the length of the encoding peptides ranged from 243 to 609 aa. MUSCLE alignment of the previously-described *EcCKX1* and *EcCKX2* (Radchuk et al., 2012) against cds of the identified *EcCKX* panel revealed the existing orthologs of *EcCKX1* and *EcCKX2* in the finger millet genome, which were then named *EcCKX1.A*/*B* and *EcCKX2.A*/*B*, respectively (Supplementary Figure S1). Types of the encoded EcCKX proteins were determined on the basis of the phylogenetic analysis that will be described separately.

All identified *EcCKX* were present in doublets and were allocated on homologous chromosomes from subgenomes A and B of *E. coracana*. As this species has an allotetraploid nature, all gene pairs were defined as homologous. In the vast majority of cases, the homologous genes possessed similar gene length and encoded highly identical peptides (92.55%–98.85%). Two genes, *EcCKX3p.B* and *EcCKX9p.B*, were of significantly reduced length, 937 bp and 1,106 bp, respectively, compared to other *EcCKX*, and thus encoded shortened peptides, 243 aa and 332 aa, respectively. It is also worth noting that four *EcCKX*, encoding different isotypes of this enzyme, were located on the same 1A chromosome (and their four homeologs on 1B). *EcCKX1.A* and *EcCKX5.A* were located relatively close at 7.11 Mbp, while *EcCKX7.A* and *EcCKX8.A*—were only 710 kbp apart. Also, *EcCKX1.A*/*B* and *EcCKX5.A*/*B* both belong to type II of CKXs, while both *EcCKX7.A*/*B* and *EcCKX8.A*/*B* are type I members. Co-location of pairs of these genes on the same chromosome could possibly correspond to their potentially paralogous nature.

Exon-intron structure of *EcCKX*, and domain distribution in their peptides

Next, the exon-intron structure of the identified *EcCKX* (Figure 1), as well as the domain organization of the encoded

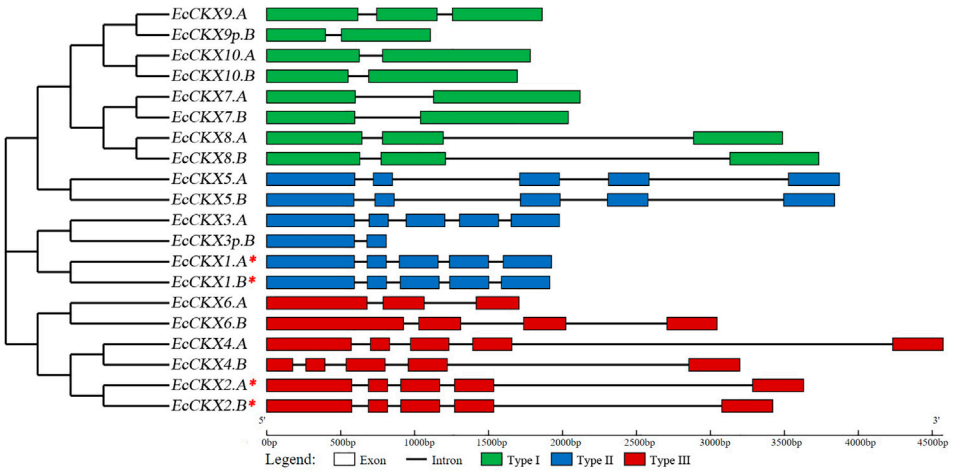

FIGURE 1
Exon-intron structure of the identified *CKX* genes within the *E. coracana* genome. The NJ phylogenetic tree was constructed with bootstrap support of 1,000 iterations (all branches had support of 65% and higher), based on cds sequences of the identified genes. A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

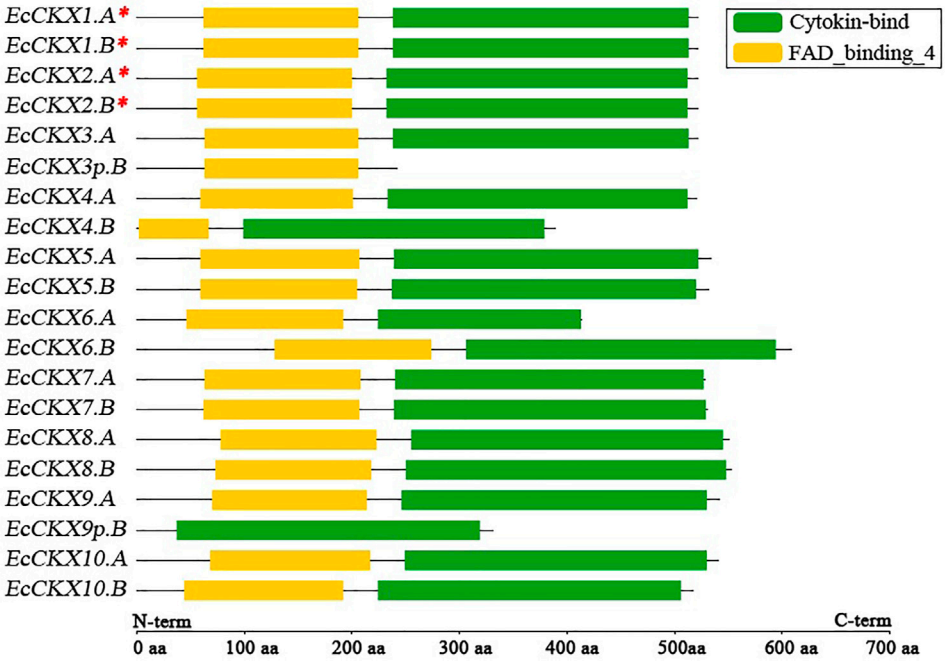

FIGURE 2
Putative motif/domain distribution in 20 translated peptide sequences, derived from identified *EcCKX* genes. A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

EcCKX peptides were investigated (Figure 2). The number of exons was not conserved among members of the CKX gene family. Type I members, such as *EcCKX9.A* and *EcCKX8.A/B*, had three exons, while others, *EcCKX7.A/B* and *EcCKX10.A/B*, had only two exon structures, most likely due to the reduction of the intron2. On the other hand, most representatives of type II and III had five exons, with a few exceptions, such as *EcCKX6.A* and *EcCKX6.B*, which had 3- and 4-exon structures respectively.

TABLE 2 Rates of (non-)synonymous substitutions within homologous pairs of *EcCKX*.

Pair of homeologs	KA	KS	KA/KS
EcCKX1.A/B	0.010	0.066	0.155
EcCKX2.A/B	0.005	0.019	0.265
EcCKX3.A/p.B[a]	0.022	0.056	0.399
EcCKX4.A/B	0.027	0.142	0.193
EcCKX5.A/B	0.009	0.047	0.180
EcCKX6.A/B	0.020	0.069	0.291
EcCKX7.A/B	0.036	0.081	0.447
EcCKX8.A/B	0.018	0.062	0.287
EcCKX9.A/p.B[a]	0.025	0.104	0.235
EcCKX10.A/B	0.023	0.107	0.217

[a]One of the genes is a pseudogene.

EcCKX3p.B and *EcCKX9p.B* genes, which showed a significant reduction in their length, lacked several exons in their structure. For example, *EcCKX3p.B* had only exon1 and exon2, while exons3-5 were eliminated. At the same time, exon1 was lost in *EcCKX9p.B* if compared to its homeolog, *EcCKX9.A*.

Typically, CKX proteins should contain two major domains (motifs) in their structure, which are responsible for the FAD- and CK-binding activity of the enzymes. Analysis of the translated peptides of the identified *EcCKX* revealed the presence and location of these crucial structural elements (Figure 2). Most of the identified genes contained both FAD- and CK-binding domains, despite the significant difference in the exon-intron structure of the genes. Usually, the FAD-binding domain is located above the CK-binding ones. Notably, *EcCKX6.B*, which had an enlarged exon1, also had a longer variable sequence, upstream to the FAD-binding domain, while the *EcCKX6.B* peptide had the longest sequence of 609 amino acids—almost 55 amino acids longer than the typical *EcCKX* protein, and 195 amino acids longer than the peptide of its homeolog *EcCKX6.A*.

It is also worth noting that *EcCKX1.A/B* and *EcCKX2.A/B*, orthologs of the previously-described *EcCKX1* and *EcCKX2*, had a conserved protein length of 523 amino acids and domain organization, despite the significant sequence differences that led to their classification into distinct CKX types. Most of the homeologous pairs of genes have preserved the identical domain organization of their peptides. However, such representatives as *EcCKX3p.B* lacked the CK-binding domain, which correlates well with the loss of exons3-5 of this gene. Similarly, the peptide of *EcCKX9p.B* lacked the FAD-binding domain, while the gene did not contain exon1, which should be present in a normal gene, e.g., in its homeolog. Based on these findings, it is hypothesized that *EcCKX3p.B* and *EcCKX9p.B* may be pseudogenes that potentially faced selective pressure after the allopolyploidization of the *Eleusine* species, leading to the origin of *E. coracana*.

Further, analyses were performed of the rate of non-synonymous substitution (K_A), the rate of synonymous substitutions (K_S), and their ratio (K_A/K_S) (Table 2). All of the *EcCKX* homeologous pairs showed extremely low K_A values—in a range of 0.005–0.036—while the rate of synonymous substitutions was higher: K_S = 0.019–0.142. Interestingly, *EcCKX2.A/B* possessed the lowest rates of substitutions (K_A = 0.005; K_S = 0.019) among all identified *EcCKX*. *EcCKX7.A/B* showed the highest rate of non-synonymous substitutions (K_A = 0.036), which led to the highest K_A/K_S ratio—0.447. All homeologous pairs of *EcCKX* showed K_A/K_S values lower than 1 (in a range of 0.155–0.447), meaning that all of these duplicated genes were under purifying or stabilizing selection. *EcCKX1. A/B* possessed the lowest value of K_A/K_S at 0.155. Such low values of K_A/K_S ratios among *EcCKX* homeologs may suggest that the duplicated genes will be further preserved in their current state, and not face loss of function or neofunctionalization.

Organization of *cis*-elements in upstream regions of *EcCKX*

To identify the putative *cis*-acting regulatory elements, the 2 kbp upstream sequences (from the start codon) were analyzed for all sets of the identified *EcCKX* (Figure 3). The upstream regions of various *EcCKX*s appear to be rich in various *cis*-regulatory elements, responsible for inducing expression in response to a wide range of factors. However, from the perspective of this study, the most interesting elements are those involved in cytokinin-mediated induction of expression. The PLACE database provides for analysis of only four known and confirmed *cis*-elements associated with CK-response: ARR1 (PLACE ID: S000454), AS1LIKECSHPRA (S000260), CPBCSPOR (S000491), and CYTOSITECSHPRA (S000260). Although the sequences of these *cis*-elements may vary depending on their type, all of them have a common tetranucleotide motif -NGAT- (more often -AGAT-). Only two types of *cis*-acting regulatory elements were identified in *EcCKX*: CPBCSPOR (-TATTAGN-, containing an inverted NGAT motif at 3′) and ARR1 (NGATT), with the latter being the most common. No AS1LIKECSHPRA or CYTOSITECSHPRA were detected.

On average, all identified *EcCKXs* contained 9–21 ARR1 and 1–3 CPBCSPOR elements in the 2 kbp upstream regions. It is interesting to note that type III members contained almost no CPBCSPOR elements in their upstream regions, except for *EcCKX4. A*, which had CPBCSPOR at position 1754–1759 bp, and overlapped another ARR1 element located at 1758–1762 bp. Often ARR1 elements were closely located, formed clusters (containing 2–3 ARR1 elements), or overlapped each other. However, the presence of two overlapping ARR1 (one straight and one inverted element),

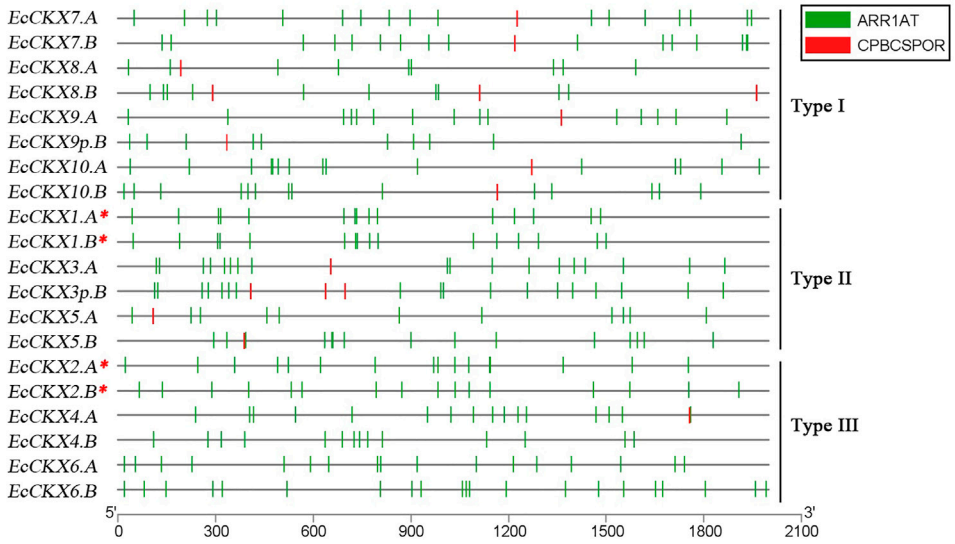

FIGURE 3
Presence of *cis*-elements, responsible for CK-mediated expression induction, in the 2 kb upstream region of the identified *EcCKX* genes of finger millet. A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

was identified in the upstream region of *EcCKX10.A* [463–473 (+) and 473–477 (-)], thus forming a -NGATTTAGN- palindromic sequence motif. For most of the CKX genes, the presence of a double placement of ARR1 elements in the upstream region (2–4 bp away from each other) was found, but their sequence had same direction, thus not forming palindromic motifs.

Different types of *CKX* genes had different combinations of regulatory *cis*-elements. For example, type I *EcCKX* had the lowest number of ARR1 elements, ranging from 9 to 17 ARR1 motifs per promoter region of each gene, whereas all type I genes, without exception, contained one to three CPBCSPOR motifs. For example, type I *EcCKX* had the lowest number of ARR1 elements, ranging from 9 to 17 ARR1 motifs per promoter region of each gene, whereas all type I genes contained from one to three CPBCSPOR motifs, without exception. In contrast, type III genes had the highest number of ARR1 regulatory elements in the upstream region (14–21 per gene), whereas CPBCSPOR motifs were absent. Only gene *EcCKX4.* had one CPBCSPOR element, which, however, overlapped the ARR1 motif sequence. Some of the genes had clusters of ARR1 motifs, where on a 100 bp sequence three or more ARR1 elements were present (e.g., *EcCKX6.B*).

Not all homeologous pairs shared the same patterns of ARR1 motif distribution. The structure of the upstream regions of *EcCKX1.A*/*B* was almost identical, while in the case of *EcCKX2.A*/*B*, it was similar only in a certain range—approx. 900–1,200 bp, etc. *EcCKX3p.B* displayed a very similar pattern of CK-responsive *cis*-elements to that of *EcCKX3.A*, whereas another possible pseudogene, *EcCKX9p.B*, had a distinct pattern of ARR1 allocation from its functional homeolog. Despite its pseudogenic nature, *EcCKX9p.B* had 10 ARR1, which is comparable to the upstream regions of other functional genes. It is unknown whether they were influenced by the process of gene disruption (possible loss of exon1 and its associated regions), or arose due to stochastic causes.

Phylogenetic analysis of the cytokinin dehydrogenase/oxidase gene family

The next step was to analyze the phylogenic relationships between the identified *EcCKX* and members of this gene family in other species (Figure 4). In order to perform this analysis, 83 CKX amino acid sequences were aligned, and further phylogenic analysis was performed using the maximum likelihood (ML) method, which allowed us to obtain a tree with high confidence at all crucial branching nodes (bootstrap values for main nodes were 85 and higher). The results allowed us to conclude that CKX proteins can be divided into three main types, which include sequences of both monocotyledonous and dicotyledonous species. Seven functional EcCKX proteins and one reduced (EcCKX9p.B) were associated with type I CKX. Among the type I proteins, the vast majority came from monocot species, in which type I panel proteins expanded rapidly compared to dicot species. Dicot proteins (e.g., AtCKX2, AtCKX3, and AtCKX4) formed a separate branch of CKX, specific only to this taxonomic group (named Dicot type I). At the same time, monocot CKX proteins formed four minor clades, which could be grouped into subtype Ia of monocots

belonging to EcCKX9.A, EcCKX10.A/B, and subtype Ib of monocots containing EcCKX7.A/B and EcCKX8.A/B.

Proteins of each minor clade Ia-1, Ia-2, Ib-1, and Ib-2, represent groups of sub-orthologs, as each clade contains the respective proteins from the analyzed monocot species: *O. sativa*, *Z. mays*, *S. italica*, *H. vulgare,* and *E. coracana*. This allows us to assume that diversification of these minor subtypes of CKX in monocots occurred at least before the emergence of the Poaceae species. Such diversity could potentially result from local (in the case of *EcCKX7.A/B* and *EcCKX8.A/B*) or genome-wide duplication.

Type II of CKX is comprised of two major subtypes: IIa and IIb, which can be further divided into three minor clades of Dicot-specific IIb, and Monocot IIb-1 and IIb-2. Similar to Type I, the diversity of the monocot CKX was higher than in the dicot. However, in the case of Type 2 CKX, dicot proteins did not group distinctly, but were present in subtype IIa (AtCKX5), IIb (AtCKX1, and AtCKX6) subtype clades. This indicates that *EcCKX5.A/B* appears to be an ancient paralog in relation to representatives of the IIb subtype (*EcCKX1.A/B* and *EcCKX3.A*), if the close allocation of *EcCKX5.A/B* and *EcCKX1.A/B* on the same chromosome is taken into account.

Proteins of Type III were separated into two branches. The primary branch (IIIa) included several monocot proteins and their orthologs from dicots (e.g., AtCKX7). *EcCKX6.A/B* were also attributed to this subtype. The second branch, subtype IIIb, contained only monocot CKX, and formed two minor clades representing distinct lineages of CKX peptide paralogs: IIIb-1 and IIIb-2, which included *EcCKX4.A/B* and *EcCKX2.A/B*, respectively. The previously-identified *EcCKX1* and *EcCKX2* were attributed to different types of CKX, despite their similarity in protein length, genomic structure, and potentially similar function. It is also worth noting that the branches within the IIb and IIIb clades were the shortest observed, compared to other subtypes of CKX, suggesting that *EcCKX1* and *EcCKX2* orthologs are conserved in monocots (at least among the Poaceae species). However, the fact that these genes were not eliminated, despite their paralogous nature, may indicate that multiple copies of particular *EcCKX* have undergone sub-functionalization, or that an increased number of their copies confer a selective advantage.

Synteny of *EcCKX*

Furthermore, interchromosomal synteny of finger millet *CKX* was inferred (Figures 5, 6), which is of additional interest, since *E. coracana* is considered an allotetraploid species and has an expanded panel of *EcCKX*. Figure 6 shows the syntenic relations between the identified *EcCKX* genes. Thus, all the genes formed syntenic pairs with their homeologs from A and B subgenomes, which further confirms the hypothesis that the rapid expansion of the *CKX* gene family in *E. coracana* is the result of an allopolyploidization event. Only genes from Type I formed pairs, not only between homeologs, but also with paralogs of the same type. For example. *EcCKX10.A/B* formed syntenic pairs with *EcCKX9.A*. This suggests that the sequences of these genes remained sufficiently conserved, and that they were recognized as possible duplicates during the synteny analysis. No syntenic pairs between genes of different CKX isotypes of Type II or III were detected.

Intergenomic synteny between *E. coracana* and the model crop species *O. sativa* made it possible to identify orthologs of *EcCKX* (Figure 5). The potential duplicates *EcCKX7.A/B* and *EcCKX8A/B* share the same loci on chromosomes 1A and 1B of finger millet, similar to their orthologs *OsCKX1* and *OsCKX2* in the rice genome. At the same time, the orthologs of *EcCKX1.A/B* and *EcCKX5.A/B—OsCKX5* and *OsCKX4*—were located distantly on Os1 in the rice genome and formed syntenic pairs with only one of their direct orthologs in *E. coracana*. Taking into account the significant phylogenetic distance between the *EcCKX1.A/B-OsCKX5* and *EcCKX5.A/B-OsCKX4* clades, it may be concluded that the lineages of these genes did not arise as a result of a recent duplication event.

At the same time, members of Type III, *EcCKX2.A/B* and *EcCKX4.A/B*, each formed syntenic pairs with both of their orthologs in rice: *OsCKX3* and *OsCKX8*. Unlike the previous case, this finding confirms the paralogous nature of the relatively recent duplicates *EcCKX2* and *EcCKX4*. Similarly, members of Type III, *EcCKX9.A/B* and *EcCKX10.A/B*, formed double pairs with *OsCKX6* and *OsCKX7*. It is worth noting that *OsCKX6* and *OsCKX7* are located relatively close on the Os2 chromosome, which may indicate the origin of these paralogs from a tandem duplication event.

Apart from the other genes, *EcCKX6.A/B* formed a syntenic pair only with *OsCKX11*. Both of these genes belong to the sub-Type IIIa (Figure 5), the clade which also contains dicot orthologs. This confirms that the present gene of this CKX isotype was evolutionarily preserved and diversified from other Type III genes, at least in the last common ancestor of dicots and monocots.

Expression profiling of *EcCKX*

Using the previously confirmed *CKX*-deficient lines of *E. coracana* somaclonal mutants (Radchuk et al., 2012; Bayer et al., 2014), we performed an expression profiling of *EcCKX* and *EcCKX1.A/B* genes, in particular of *EcCKX2.A/B* (Figure 7). As mentioned above, homeologous pairs of *EcCKX1.A/B* and *EcCKX2.A/B* encode highly conserved peptide products with sequence similarities up to 97.7% and 98.85%, respectively. It is the highest degree of conservation among all identified *EcCKX* homeolog pairs. Since the transcripts of these genes are hard to distinguish and are highly conserved, the expression of

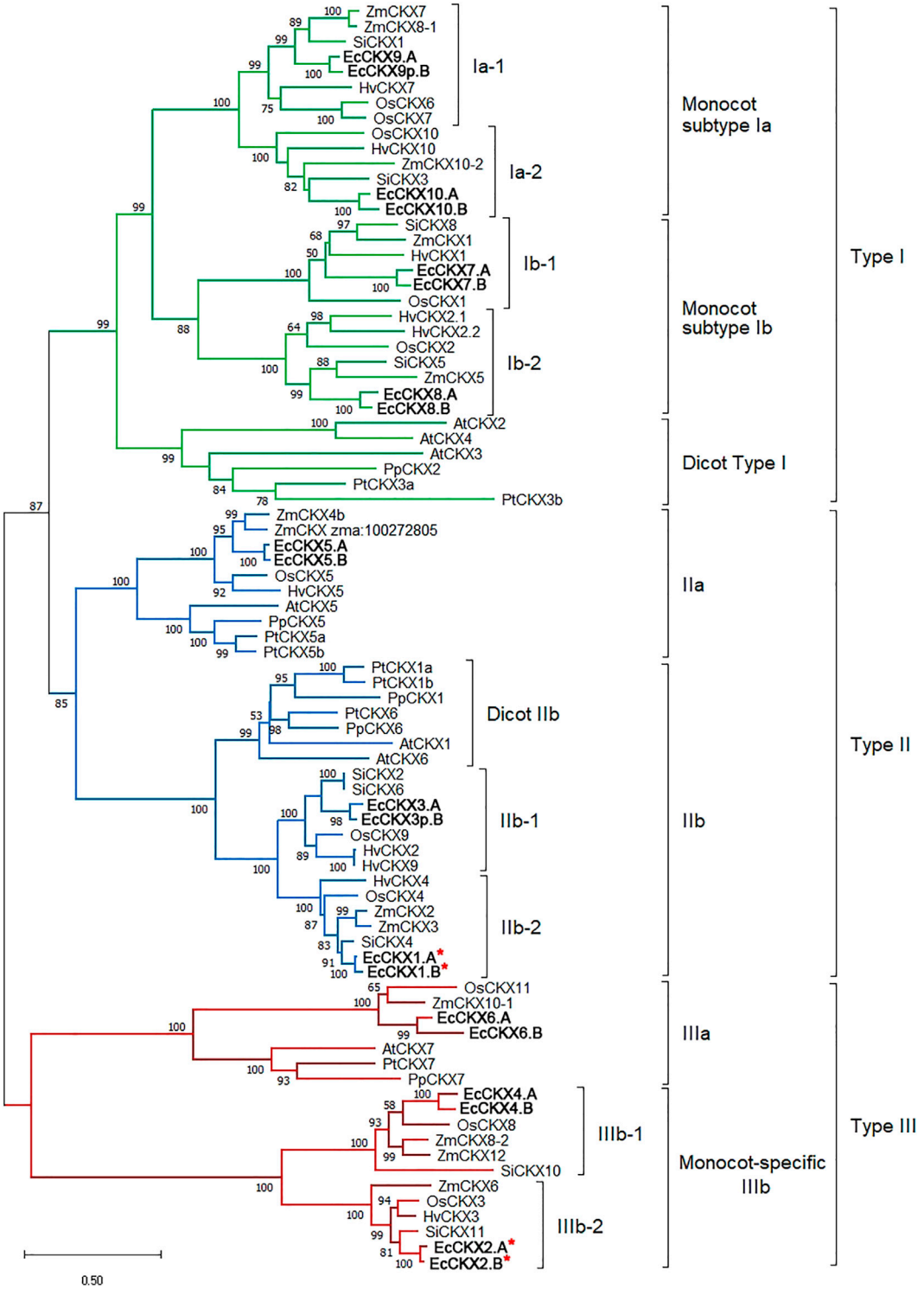

FIGURE 4
Unrooted ML phylogenetic tree for dicot (*A. thaliana*, *P. persica*, and *P. trichocarpa*) and monocot (*O. sativa*, *Z. mays*, *H. vulgare*, and *S. italica*) CKX proteins, as well as translated peptides of identified genes in *E. coracana* genome. The tree was constructed with bootstrap support of 1,000 iterations (50% and higher values are displayed). A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

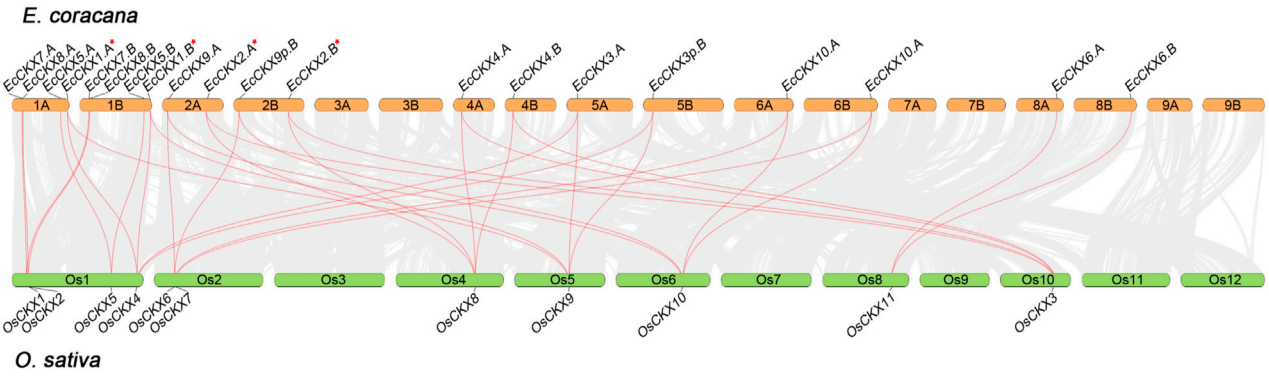

FIGURE 5
Synteny analysis of *CKX* genes between *E. coracana* and *O. sativa* species. The collinear blocks between *O. sativa* and *E. coracana* species are shown with gray lines, while syntenic pairs of *CKX* genes are highlighted in red. The chromosome number is indicated above each respective chromosome. A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

EcCKX1.A/B and *EcCKX2.A/B* was analyzed jointly for both homologous copies (Figure 7B).

The northern blot array allowed us to determine the total level of *EcCKX* transcripts in different *E. coracana* tissues (Figure 7A). For example, the upper leaves of the SE7 line were found to have higher levels of *EcCKX* transcripts than the WT line. Similarly, young roots of SE7 plants had higher levels of *EcCKX* transcripts compared to the WT, while no significant differences in *EcCKX* levels were found at 4 and 5 DAI in either line. However, at early stages of panicle development, SE7 plants had a lower level of *EcCKX* expression than the WT, whereas at later stages, the total levels of expression were comparable, or the *EcCKX* expression in SE7 could even be higher. The total *EcCKX* expression in the nodes and internodes of SE7 was lower than in the WT.

Previously, total transcriptomic analysis revealed the genes responsible for the semi-dwarf and bushy phenotype of SE7 (Radchuk et al., 2012). In this study, a detailed analysis of the expression of *EcCKX1.A/B* and *EcCKX2.A/B* reveals patterns of changes in the expression of these genes in the SE7 mutant, especially given that the SE7 has normal rates of CK synthesis (Radchuk et al., 2012). The most dramatic decrease in *EcCKX2.A/B* expression is observed in young leaves of SE7 (more than 8-fold), compared to the wild type, whereas the levels of *EcCKX1.A/B* remain unchanged (Figure 7B). At the same time, these genes exhibit no significant changes in expression in old leaves. However, in seedlings (5 DAI), the expression of both *EcCKX1.A/B* and *EcCKX2.A/B* decreases 3.1 to 3.2-fold, whereas analysis of total *EcCKX* expression shows no significant difference. It is possible that during germination the total level of these CK-degrading enzymes remains high, thus partially compensating for the deficiency of the *EcCKX1.A/B* and *EcCKX2.A/B* isotypes, possibly through the increased expression of their paralogs.

Most interestingly, *EcCKX1.A/B* expression levels change during early panicle development, while no differences in *EcCKX2.A/B* expression are found in the SE7 and WT lines. The transcript levels of *EcCKX1.A/B* are found to be nearly 3.9-fold lower in SE7 inflorescences during the early inflorescence development (stage A). It is noteworthy that at later stages the expression of *EcCKX1.A/B,* as well as that of *EcCKX2.A/B,* remains unchanged. However, in the later stage of inflorescence development (stage C), *EcCKX2.A/B* shows a 2.13-fold increase in expression. This correlates well with the observed increase in the total *EcCKX* level of transcripts at late stages of inflorescence development. Perhaps this observation can serve as a partial confirmation of the stage-dependent specificity of the expression of these two genes. Most likely, the early stages of panicle growth, such as stage A, are the key phases, in which high levels of CK determine subsequent panicle/inflorescence size, thus shaping crop grain productivity.

Phenotypic changes in cytokinin dehydrogenase/oxidase-deficient somaclonal mutants of *E. coracana*

For field evaluation of the phenotype, around 100 wild type and SE7 finger millet plants were grown for 2 weeks in a greenhouse and then transplanted in May to a field in Gatersleben, Germany, using 20 cm plant spacing. Plant height and the yields from 25 plants were measured in late September. For greenhouse phenotype evaluation, 25 SE7 mutant and wild-type plants were grown in a greenhouse during the same period (Figure 8).

SE7 somatic mutant plants grown in both the field and the greenhouse were characterized by significantly lower plant

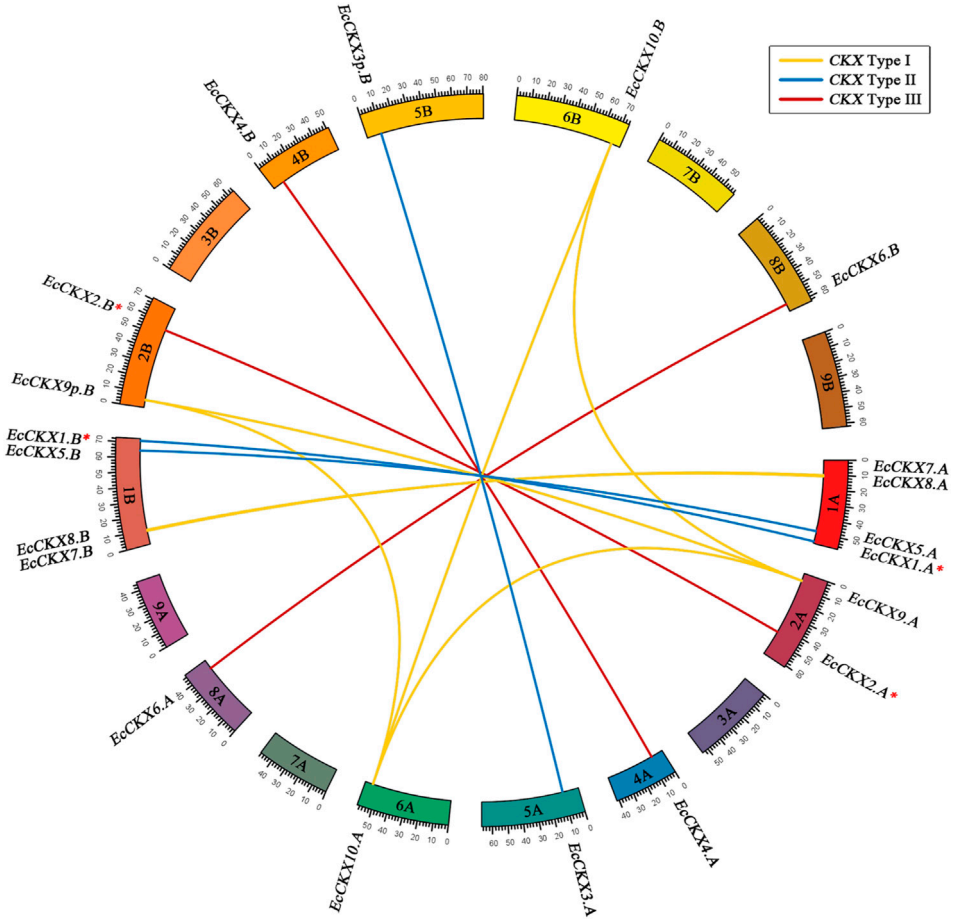

FIGURE 6
Synteny analysis of interchromosomal relationships of *EcCKX* of various types from different subgenomes of *E. coracana*. Syntenic gene pairs are colored according to the type of CKX, to which they are attributed. A red asterix denotes genes identical to previously sequenced and characterized *EcCKX1* and *EcCKX2*.

height (Figure 8) than the wild type, whereas greenhouse-grown plants differed more. The total number of inflorescences per plant also increased, indicating that more vegetative meristems were formed in SE7 plants (Figure 8). Surprisingly, greenhouse-grown plants did not show any significant difference in this parameter, probably due to altered growing conditions. Also, the *CKX*-deficient mutant line had a shortened vegetation cycle.

The mutant plants also produced more seeds per plant compared to the wild type, indicating extensive production of generative meristems in the SE7 line in response to higher levels of CK, which were not degraded (Figure 8). Increased numbers of inflorescences and flowers led to a significantly higher grain yield per plant in field conditions. However, this effect was less significant among the greenhouse-grown plants, since under such conditions, both lines produced more inflorescences, and thus showed higher grain productivity (Figure 8). The less significant difference in total plant productivity in the greenhouse could be explained by the fact that greenhouse-grown SE7 plants had a significantly lower grain mass. In contrast, 100-grain weight was not significantly different between field-grown SE7 and WT plants. It can therefore be concluded that the increase in the number of inflorescences is a key factor in determining the increased grain productivity (per plant) of the SE7 line (Figure 8).

Following the successful comparison of SE7 plant performance, and confirmation that the increased plant productivity is also found in field-grown plants, a full-scale field trial was conducted using the SE7 line and its WT (Figure 9). The most prominent features of the obtained somaclonal line SE7 were significantly reduced height, compared to the parental genotype cv. Tropikanka, and a much greater bush density, due to an increased number of primary and lateral stems. However, the somaclones did not show the reduced grain size. It was established that the SE7 line had a reduced height of almost 40%, compared to the WT (60.4±8.8 v. 100.1±5.5 cm, Figure 9A). Similar to the

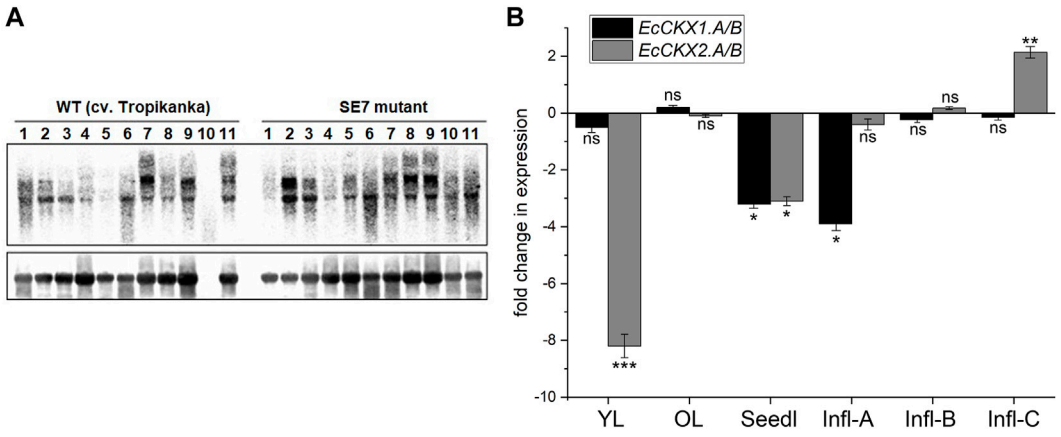

FIGURE 7
Expression profiling of *EcCKX* genes in the *E. coracana* mutant SE7 line. **(A)**—Northern blot of different tissues and developing stages of wild type and mutant SE7 finger millet plants hybridized with the *EcCKX* sequence as a probe (above): 1—node; 2—upper leaf; 3—lower leaf; 4—internode; 5—young root; 6—seedling 4 DAI; 7—developing panicle, < 1 cm in length; 8—developing panicle, 2–3 cm; 9—developing panicle, 3–4 cm; 10—developing panicle, > 4 cm; 11—seedling, 5 DAI. The signals were quantified by sequential hybridization with a ribosomal fragment as probe (below). **(B)**—Transcript profiling of the *EcCKX1. A/B* and *EcCKX2. A/B* genes in the developing inflorescences and young leaves of finger millet determined by quantitative RT-PCR analysis: YL—young leaf; OL—old leaf; SeedI—seedling 5 DAI; Infl-A/B/C—inflorescence tissue at stage A, B, and C. Data shown as fold change of upregulation of downregulation of respective gene in SE7 mutant line, compared to WT: ns—difference not statistically significant, *—significant at $p < 0.05$; **—$p < 0.01$; ***—$p < 0.001$.

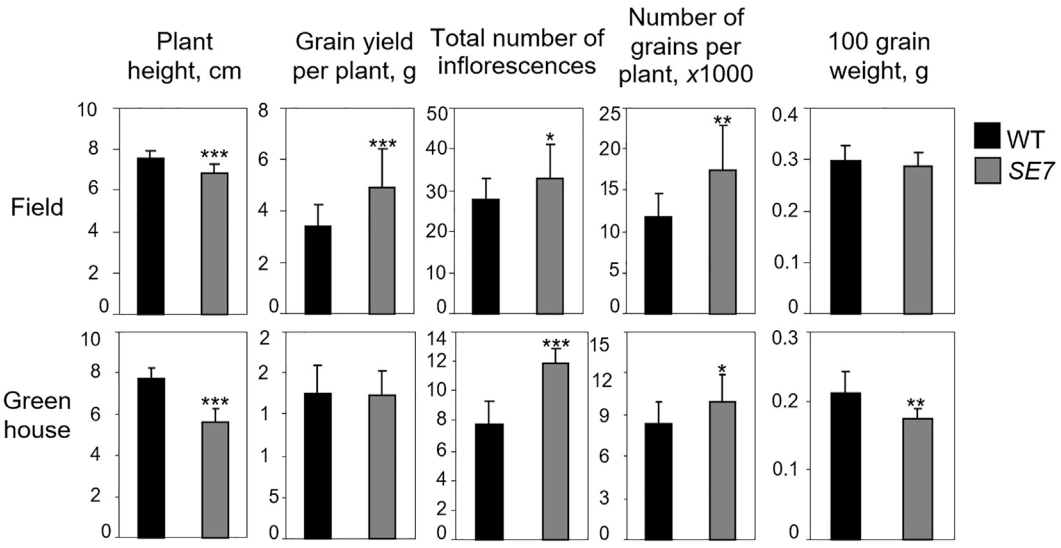

FIGURE 8
Productivity trait difference of field-grown (upper panel) and greenhouse-grown (lower panel) SE7 somaclonal mutant plants compared to the wild type of finger millet. Statistically significant differences are marked with *—significant at $p < 0.05$; **—$p < 0.01$; ***—$p < 0.001$.

comparison shown in Figure 8, the weight of 1,000 grains manifest no significant change during field trials (Figure 9B).

At the same time, the SE7 had a significantly greater number of primary stems (48±2.9 v. 29±1.9 in WT), while the number of secondary stems was similar for both the SE7 and WT—nine on average. The number of grains per spike increased by 34.3% in the SE7 line (236±16 vs. 175±13), compared to the WT (Figures 9C,D). From the concluding observations of two independent studies, it can be summarized that the key factors shaping the increased productivity of the *CKX*-deficient line are an increase

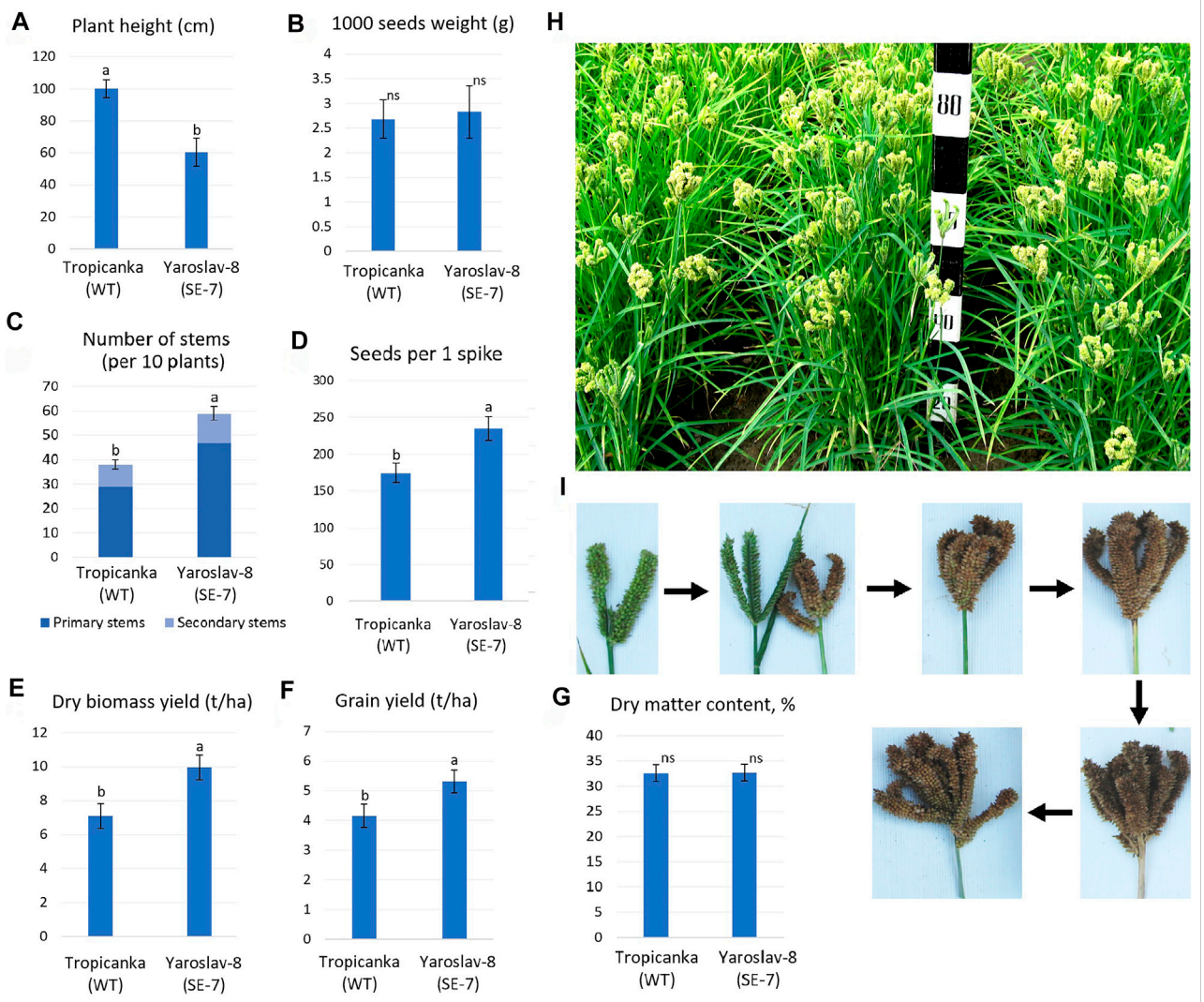

FIGURE 9
Performance of the SE7 mutant line in comparison with the WT during field trials. **(A–D)**, **(G)**—measurements of different productivity traits; **(E,F)**—grain and biomass yield; **(H)**— plants of the SE7 line during maturation; **(I)**—different stages of finger millet panicle maturation. Different letters within each parameter **(A–G)**: a, b—significantly different values; ns—difference not significant (at $p < 0.05$, Fischer LSD).

in the number of inflorescences (due to the larger number of primary stems), and an increase in the number of seeds per panicle, with grain weight not significantly affecting the resulting plant productivity.

The resulting grain productivity of the SE7 line reached 5,320 kg/ha on average, 27.9% higher than the WT, which produced 4,159 kg/ha of grain (Figure 9F). In addition, the SE7 line showed increased levels of biomass accumulation, which is associated with the previously-identified downregulation of *CKX*. The SE7 line showed almost 10 t/ha of straw (absolutely dry mass), which is higher than that of the WT at 7.1 t/ha. Such a substantial increase in biomass productivity is most likely associated with the appearance of additional stems.

Discussion

A total of 20 *EcCKX*s were identified in the latest *E. coracana* assembly. Two of all the identified genes are considered pseudogenes, as they lack important structural domains: FAD- and CK-binding domains. The rapid expansion of the CKX gene family in finger millet can be associated with the evolutionary history of this species, which includes at least one

allopolyploidization event giving rise to modern *E. coracana* (Zhang et al., 2019a; 2019b). *EcCKXs* are represented by complete homeologous pairs, indicating that the majority of these duplicated genes were not eliminated, but most likely passed through subfunctionalization. Using the example of *Brassica rapa,* it is shown that *CKX*, resulting from segmental duplications, tend to change their expression patterns and thus potentially gain new functions (Liu et al., 2013). It is obvious that in such a case, the duplicated genes are not eliminated or pseudogenized over time.

Other species closely related to *E. coracana* contain a lower number of *CKX* in their genomes. For example, *S. italica* has only 11 *CKX* genes (Wang et al., 2014); *H. vulgare* has 13–14 genes (Zalewski et al., 2014); *O. sativa* has 11; *Brachypodium distachyon* has 13; *Sorghum bicolor* has 12; and *Z. mays* has 16 (Mameaux et al., 2012). Genomes of dicot species can contain 7 *CKX* genes, as is observed in *A. thaliana*, while some species can contain a lower number of *CKX* genes, such as *P. persica* which has only 6; or more, e.g., 8 genes in *P. trichocarpa* (Immanen et al., 2013). Polyploid dicots possess much larger panels of *CKX* genes, for example *Glycine max* has 16 genes (Liu et al., 2021), while polyploid *Brassica* species have a much larger gene family compared to diploids. While *B. rapa* has only 12 *CKX* genes (Liu et al., 2013), amphidiploid *B. napus* contains 23 (Liu et al., 2018), while *B. oleracea* accounts for up to 36 *CKX* genes (Zhu et al., 2022).

Analysis of *cis*-regulatory elements make it possible to reveal the patterns of distribution of CK-responsive elements within the identified genes. Understanding of the diversity of CK-responsive *cis*-elements is currently limited to only a few validated motifs, such as ARR1 (AGAT [T,C] motif) (Brenner and Schmülling, 2015). However, it is evident that the expression of *CKX* is specifically induced by CK (Curaba et al., 2014), hence, new types of CK-responsive *cis*-elements have yet to be described. In this study we find unconventional motif organization, such as the presence of palindromic ARR1s or overlapping inverted ARR1-CPBCSPOR motifs. However, it is important to note that *in silico* identification of *cis*-regulatory elements, especially short-sequence motifs (such as ARR1, discussed above), is not enough for prediction of the binding site of functional proteins involved in transcriptional regulation (Hernandez-Garcia and Finer, 2014). Despite the presence of numerous copies of short-sequence motifs in the genome, only a small part of them are accessible for protein binding and further regulation of gene expression (Hardison and Taylor, 2012). Similarly, as analyzed in our study, ARR1 elements consist of 3–4 nucleotides. It may be expected that only a few of the identified *cis*-elements might be involved in CK-mediated expression of *EcCKX*. This could be a question for further separate research.

It is not clear what the role of such a broad diversity of these genes is in different taxa. However, the so-called "ancient" *CKXs* (which have direct orthologs in non-angiosperm species) have recently been found to preferentially degrade *cis*-zeatin (*c*Z)-type cytokinins, while non-ancient (relatively recent duplicates) mostly target N^6-(Δ^2-isopentenyl) adenines (iPs) and *trans*-zeatins (tZs) (Wang et al., 2021). In addition, it has been hypothesized that non-ancient CKX and their substrates (iP) may regulate the development of plant organs, especially flowers, and may regulate stress responses (Wang et al., 2021). Rapidly expanded type I *CKXs* belong to such non-ancient type genes, since their entire group is sub-orthologous to *AtCKX4*, which is considered one of the remarkable representatives of recent *CKXs*. Similarly, groups IIa, IIb, and IIIb are also orthologous to such non-ancient *CKX*, including *EcCKX1.A*/*B* and *EcCKX2.A*/*B* (widely discussed in this study), the deficiency of which results in increased inflorescence/panicle. By contrast, the phylogenetic group IIIa is orthologous with *AtCKX7*—the gene of ancient-type *CKX*. Genes *EcCKX6.A*/*B* belong to this group. Notably, this *CKX* homeolog pair has very strict orthology with *OsCKX11* of rice, unlike other *EcCKX*. In a detailed study on Poaceae *CKX*, a clade containing *OsCKX11* orthologs, clustered as basal to all monocot *CKXs* (Mameaux et al., 2012), further supports the assignment to "ancient" type genes.

Measurement of CK species in finger millet identifies iP as the major form of CKs in developing inflorescences of this species (Radchuk et al., 2012). When we analyzed the somaclonal variant *SE7*, produced previously (Baer et al., 2007), the levels of iP and the iP precursor riboside phosphate were strongly increased early in inflorescences of this genotype, but did not change compared to the wild type later on (Radchuk et al., 2012). We hypothesized that increased iP levels are probably due to impaired degradation, as evidenced by lower *EcCKX* expression in young inflorescences. Such disturbed CK homeostasis, brought about by either reduced CK degradation or biosynthesis, can stimulate plant productivity. As observed in the present study, effects of *EcCKX* deficiency in the SE7 mutant may contribute to an understanding of the phenotypic effects caused by *CKX* downregulation. Analyzed *EcCKX1.A*/*B* and *EcCKX2.A*/*B* were preferentially down-regulated in actively growing and generative tissues, leading to rapid increase of grain and biomass productivity of the mutant. Increased plant productivity of the SE7 line may be beneficial not only for food production, but may also open ways to use finger millet for biofuel production, in particular, the production of second-generation bioethanol (Yemets et al., 2020; Blume et al., 2021).

Initially, down-regulated *CKX* expression was shown to decrease CK-degradation activity and increase CK levels in rice, leading to an increase of seed numbers per plant and 1000-grain weight (Ashikari et al., 2005). The same evidence was then obtained in experiments with RNAi-based silencing of the *HvCKX1* in barley, resulting in reduced cytokinin oxidase/dehydrogenase levels and higher plant productivity (Zalewski

et al., 2010). This is confirmed in other publications by Zalewski et al. (2012; 2014).

Later, *HvCKX1* and *HvCKX9*, predominantly expressed in the aleurone layer of maturing grains and leaf vasculature, were used for stable *Agrobacterium tumefaciens*-mediated transformation of the barley cultivar Golden Promise (Mrízová et al., 2013). As a result, constitutive overexpression of these two genes independently induced morphological changes in barley plants and prevented their transition to flowering. In all obtained transgenic lines, roots proliferated more rapidly and root-to-shoot ratios were higher than in wild-type plants. Depleted CK levels during early phases of development are restored by down-regulation of endogenous *CKX* and reinforced *de novo* biosynthesis of CKs. When barley plants were transformed with *Arabidopsis AtCKX1* under a mild root-preferred promoter, the obtained transgenic lines were found to have higher drought tolerance then the control (Pospíšilová et al., 2016). These transgenic plants can maintain higher yield parameters following revitalization compared to wild type plants under stress conditions. Knockdown of the inflorescence meristem-specific cytokinin oxidase in rice (*OsCKX2*) regulates primary flowering activity, and modulates rice grain yield under normal conditions as well as under abiotic stress conditions by controlling cytokinin levels (Joshi et al., 2018).

Several recent studies have demonstrated that RNAi-based silencing of *CKX* leads to increased grain yields in other cereals. In particular, a significantly increased grain number per spike was found as the effect of *TaCKX2.4* silencing by RNAi in wheat (Li et al., 2018). Coordinated effects of *TaCKX1* silencing on the expression of other *TaCKX*, wheat spike phytohormone levels, and yield-related traits were demonstrated in silenced T2 lines (Jabłoński et al., 2020). In the moss *Physcomitrella patens*, an evolutionarily early divergent plant, it was shown that under normal growth conditions overexpression of *PpCKX1* caused many phenotypic changes at different developmental stages, and that increased growth of the rhizoid could affect these changes. In addition, evidence is provided that the *PpCKX1*-overexpressing plants show enhanced dehydration and salt stress tolerance. Taken together, the authors suggested that *PpCKX1* plays a regulatory role in the development and adaptation to abiotic stresses of this evolutionarily early land plant species (Hyoung et al., 2020).

Finally, knockout mutation of *HvCKX1* by CRISPR/Cas9 editing in barley had a limited effect on yield productivity. However, significant reductions in CKX enzyme activity in young spikelets and 10-day-old roots corresponded to greater root length, number of root hairs, and increased surface area (Gasparis et al., 2019). In contrast, the roots of *ckx3* knockout mutants were smaller. The observed changes suggest that the knockout of a single *CKX* in barley may not be sufficient to disrupt cytokinin homeostasis or increase grain yields. These authors then demonstrated that underlying mechanisms of co-regulation of the expression of *TaCKX* family members were similar in different spring wheat cultivars, but, depending on the content and composition of phytohormones, regulation of yield-related traits was differentially affected (Jablonski et al., 2021). On the other hand, identification and characterization of all members of the *CKX* family, not only in *E. coracana*, but in closely-related species with already characterized genomes and transcriptomes (Zhang et al., 2019a; Zhang et al., 2019b; Hall et al., 2021), may provide more information about functional features of different *CKX* types.

Successful application of genome editing technics, targeting *CKX* genes in different taxa, will require a comprehensive understanding of genomic organization, phylogenetic classification, and evolution of *CKX* genes, in order to efficiently affect all paralogous copies with similar function and achieve desirable effects of genetic engineering. The greater diversity of *CKX* in monocots compared to dicots, and the relationship between different metabolic pathways of different forms of cytokinins, remains unclear. Understanding of these matters may become an important tool for targeted crop design to ensure global food security in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

RB, AY, and YB wrote the original draft of the manuscript and contributed to the conception and design of the study. VK, VR, and DR contributed to particular sections of the manuscript. RB, AY, VK, VR, and DR performed the collection of the experimental data, while RB and YB analyzed and interpreted the obtained results. RB, VK, and VR performed the statistical analysis. All authors listed made a substantial and direct intellectual contribution to the work, and approved it for publication.

Acknowledgments

We are grateful to Prof. Katrien M. Devos, who generously provided permission to use the *E. coracana* sequence data produced under NSF-BREAD award IOS-1543901. We acknowledge the suggestions by Dr. V. Kyrylenko (Institute of Food Biotechnology and Genomics, National Academy of Sciences of Ukraine, Kyiv), aimed at improving the language of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.963789/full#supplementary-material

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frontiers | Frontiers in Genetics

TYPE Systematic Review
PUBLISHED 12 October 2022
DOI 10.3389/fgene.2022.940660

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

RECEIVED 10 May 2022
ACCEPTED 11 July 2022
PUBLISHED 12 October 2022

CITATION
Sharma S, Kaur P and Gaikwad K (2022), Role of cytokinins in seed development in pulses and oilseed crops: Current status and future perspective. *Front. Genet.* 13:940660. doi: 10.3389/fgene.2022.940660

Role of cytokinins in seed development in pulses and oilseed crops: Current status and future perspective

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Cytokinins constitutes a vital group of plant hormones regulating several developmental processes, including growth and cell division, and have a strong influence on grain yield. Chemically, they are the derivatives of adenine and are the most complex and diverse group of hormones affecting plant physiology. In this review, we have provided a molecular understanding of the role of cytokinins in developing seeds, with special emphasis on pulses and oilseed crops. The importance of cytokinin-responsive genes including cytokinin oxidases and dehydrogenases (*CKX*), isopentenyl transferase (*IPT*), and cytokinin-mediated genetic regulation of seed size are described in detail. In addition, cytokinin expression in germinating seeds, its biosynthesis, source-sink dynamics, cytokinin signaling, and spatial expression of cytokinin family genes in oilseeds and pulses have been discussed in context to its impact on increasing economy yields. Recently, it has been shown that manipulation of the cytokinin-responsive genes by mutation, RNA interference, or genome editing has a significant effect on seed number and/or weight in several crops. Nevertheless, the usage of cytokinins in improving crop quality and yield remains significantly underutilized. This is primarily due to the multigene control of cytokinin expression. The information summarized in this review will help the researchers in innovating newer and more efficient ways of manipulating cytokinin expression including *CKX* genes with the aim to improve crop production, specifically of pulses and oilseed crops.

KEYWORDS

cytokinin, yield contributing traits, pulses and oilseeds, seed development, CKX, cytokinin oxidase/dehydrogenase

Introduction

The importance of oilseeds and pulses in the human diet cannot be overstated. Wherein oilseeds comprise high-energy food with double the amount of energy as carbohydrate and protein (AGRIS FAO, 2022), pulses fulfill protein demands of the majority of people, and both the crop varieties are important for agriculture and livestock. Hormones constitute a pivotal component of regulatory mechanisms directing plant development and have been extensively studied with respect to various seed attributes.

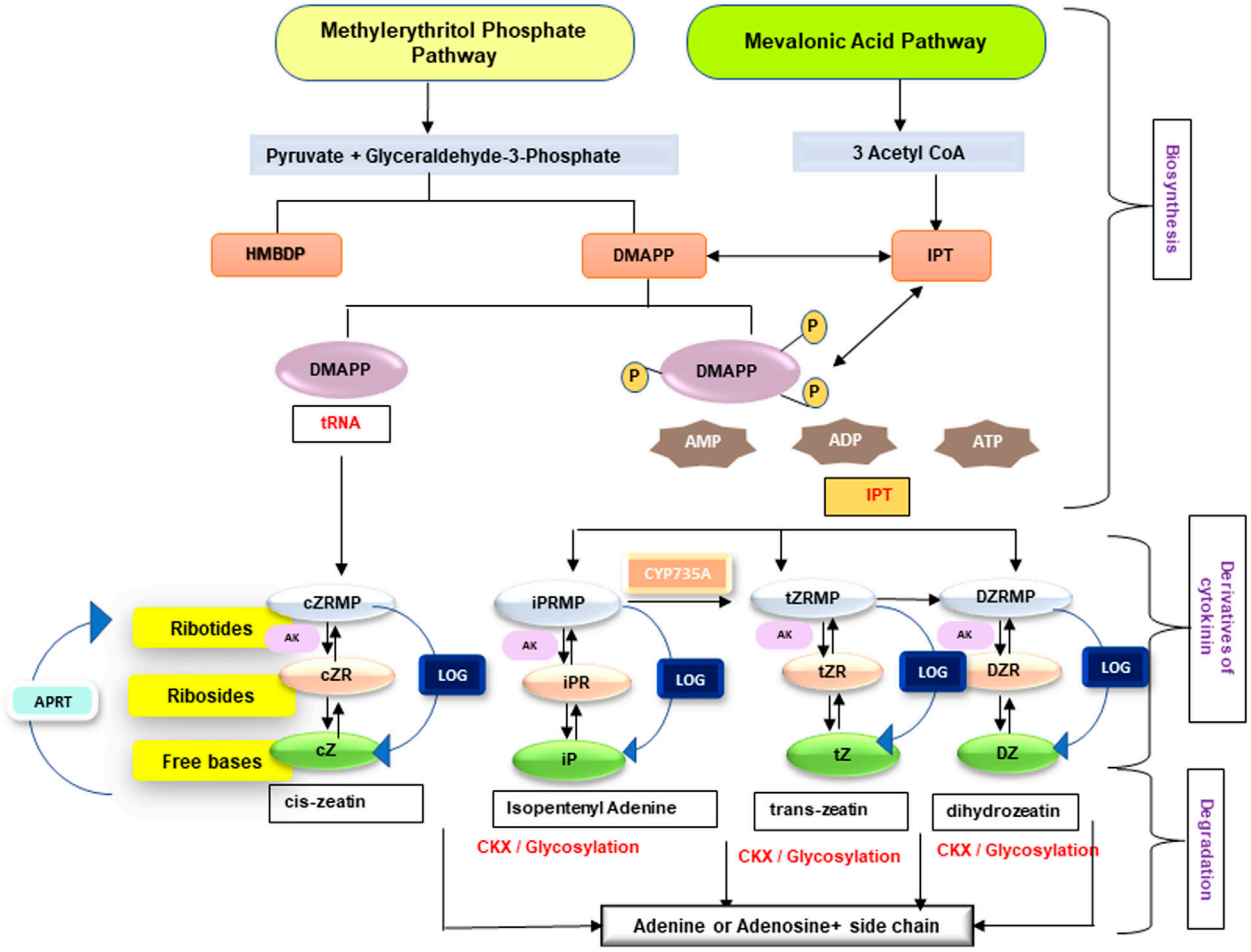

FIGURE 1
An overview on biosynthesis and enzymes of cytokinin homeostasis (iP, isopentenyladenine; DHZ, dihydrozeatin; cZ, cis-zeatin; tZ, trans-zeatin; DMAPP, dimethylallyl pyrophosphate; AMP, adenosine monophosphate) cytokinin biosynthetic pathways—mevalonic acid pathway (operates in cytosol and mitochondria) and methylerythritol pathway (operates in plastids). Enzymes involved are 1) IPT (isopentenyl transferase), which uses ATP, ADP, or ATP as acceptor and forms iPRTP (riboside 5′-diphosphate), iPRDP (riboside 5′-diphosphate), and iPRMP (riboside 5′-monophosphate); 2) CYP735A converts CK nucleotides in to tZ nucleotides; 3) AK (adenosine kinase) causes the phosphorylation of ribosides iPR, tZR, cZR, and DZR; 4) LOG (LONELY GUY) produces free bases from ribotides; 5) APRT (adenine phosphoribosyltransferase) catalyzes the conversion of CK bases to nucleotides; and 6) CKX (cytokinin oxidase/dehydrogenase) causes catabolism of free bases (iP, tZ, DZ, and cZ) to adenine or adenosine (for references, see Takei et al., 2004; Kudo et al., 2010; Sakakibara 2006; Kurakawa et al., 2007; Schoor et al., 2011).

In India, pulses and oilseeds are vital components of the food and nutritional security. India's dietary habits are still predominantly vegetarian, and the country relies primarily on plant-based sources to achieve its daily protein and other nutritional needs. In addition, according to the FAO, pulses are an important part of a balanced diet. Pulses have been linked to lower risk factors for chronic disease. Apart from being an important aspect of human nutrition, pulses also play a key role in sustainable agriculture and climate change mitigation (Fao 2017; Ha et al., 2014; Jayalath et al., 2014; Kim et al., 2016; Sievenpiper et al., 2009; Viguiliouk et al., 2017; Calles, 2016; Foyer et al., 2016). Over the last 15 years, India has made significant success in increasing pulse production. In 2005–06, India's total pulse production was 13.38 million metric tonnes (MT), which rose to 25.58 million MT in 2020–21. This represents a 91% increase, or a compound annual growth rate (CAGR) of 4.42%. Regarding oilseeds production, India grows roughly 15%–20% of the world's total output, produces 6%–7% of vegetable oils, and consumes 9%–10% of all edible oils. Oilseeds are only second to food grains in terms of acreage, production, and economic worth.

Cytokinins are one of the most well studied plant hormones, exercising huge physiological and molecular impact throughout the life cycle of a plant. Cytokinins regulate several functions, such as root development, formation and maintenance of shoot meristem, organ formation, seed germination, seed and fruit development, senescence delay, and response to abiotic and biotic stress. An overview about cytokinins in terms of their

biosynthesis, types, bioavailability, and storage forms is described in Figure 1. Cytokinin homeostasis is maintained through action of various enzymes involved in their activation, irreversible conjugation, and degradation. Cytokinins are present throughout the parts of higher plants, though abundantly in the tips of roots, apical meristem of the shoots, and the immature seeds. Majority of higher plants have more than a dozen cytokinins forms which are capable of interconversion. A plethora of previously undertaken studies (Jameson, et al., 1987; Werner and Schmülling, 2009; Kudo et al., 2010; Spichal and Spichal 2012; Schaller et al., 2014; Zwack and Rashotte, 2015) have provided a detailed and extensive overview of cytokinin.

Attempts have been made toward understanding the cytokinin-mediated molecular mechanism regulating important agronomic traits including plant height, plant density, date of flowering, number of primary and secondary branches, seed number per pod/silique, number of pod per plants, pod length, thousand seed weight, and seed size including seed length and seed width.

Cytokinin biosynthetic pathways and its regulation

Naturally occurring cytokinins are derivatives of adenine with an aromatic or an isoprenoid side chain of isopentenyladenine [N6-(D2-isopentenyl) adenine] and hydroxylated either at *cis*- or *trans*-terminal position, thereby forming zeatin, named after its discovery in maize (Strnad 1997; Mok and Mok 2001). Among these, cytokinins with the r side chains are widespread in nature. Later in mid-1970s, the presence of benzyladenine was reported in pea, a leguminous plant (Gaudinova' et al., 2005). Numerous researchers in the past have reported the dominance of *trans*-zeatin and isopentenyladenine derivatives in nature, whereas *cis*-isomer is present in very low concentration and has very little or no activity. In contrast to this, recent studies on phytohormones revealed the abundance of *cis*-isomers in several legume species (Emery et al., 1998, 2000; Quesnelle and Emery, 2007) and other plants such as rice (Takagi et al., 1985) and maize (Veach et al., 2003; Vyroubalova' et al., 2009). Naturally occurring derivatives of cytokinin encompass N6-(2-isopentenyl) adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ), which are a part of isoprenoid cytokinins, whereas benzyladenine (BA) along with its hydroxylated derivatives ortho- and metatopolin (oT and mT) and their methoxy-derivatives are included under the aromatic cytokinins group. Theoretically, 26 molecular species of cytokinin have been discovered (Murai, 2014). In total, seven distinct modifications at positions including, N9-position ribosides in adenine mono-, di-, and tri-phosphate ribonucleotides; at N7- and N9-positions glucosides; and O-glucoside in the isopentenyl side chain have been observed so far (Mok and Mok 1994; 2004; Sakakibara 2006; Kojima et al., 2009).

Essentially, two pathways are involved in the biosynthesis of cytokinin precursors, that is, isopentenyladenine-dependent pathway (mevalonic acid pathway) in the cytosol and mitochondria and isopentenyladenine-independent pathway (methylerythritol pathway) in the plastids in order to form isopentenyl transferase (IPT) and dimethylallyl pyrophosphate (DMAPP) as precursors in cytokinin biosynthesis (Figure 1). Subsequently, isopentenyl transferase (IPT) catalyzes transfer of isopentenyl moiety from DMAPP or hydroxymethyl butenyldiphosphate (HMBDP) to either AMP, ADP, or ATP for the formation of biologically active cytokinins and constitutes the rate limiting step. Furthermore, zeatin-type cytokinins are produced by the hydroxylation of the isopentenyl side chain. Alternatively, the hydroxylated side chains can be inserted straight to the N6-position of adenine moiety leading to formation of adenylate IPT. The isopentenyladenine derivative thus produced is then converted to *trans*-zeatin adenine by an action of a root localized cytochrome P450 monooxygenases (Takei et al., 2004; Kiba et al., 2013), thereby limiting *trans*-zeatin synthesis to roots but readily transported to other plant parts *via* xylem. The interconversion between the *cis* and the *trans*-isomer of zeatin is mediated by the enzyme *cis-trans* zeatin isomerase. Macro-concentration of cytokinin in plants is controlled by action of IPT and CKX enzymes; however, conversion of cytokinin nucleotides to cytokinin bases is catalyzed by enzymes, that is, cytokinin phosphoribosyl hydrolase (LOG) (Kurakawa et al., 2007; Kuroha et al., 2009; Tokunaga et al., 2012) and reverse action, that is, conversion from cytokinin bases to nucleotides is catalyzed by adenine phosphoribosyl transferases (APRTs) enzymes (Zhang et al., 2013). All the genes involved in cytokinin homeostasis exists as multi-gene families, with the individual members being differentially expressed in space and time. For instance, 8 and 9 *IPT* cDNAs and genes, respectively, have been reported in *Arabidopsis* genome. Of these, seven *IPT* genes use adenine nucleotides as substrate for transfer reaction, except *IPT2*. In addition, three of the *IPT* genes are expressed in the plastids (*IPT1*, 5, and 8), whereas the rest are localized to the cytoplasm. Furthermore, each gene has different spatial expression profile, such as *IPT6* expresses in siliques, *IPT4* in immature seeds, and *IPT3* in phloem tissues.

Role of kinases in cytokinin signaling

Cytokinins act by regulating the expression of several genes downstream of the signaling cascade. The signaling mechanism of cytokinins in plants is unique and is very similar to the bacterial two component system (Stock et al., 2000; Cheung and Hendrickson, 2010). It involves a transmembrane histidine

kinase receptor which dimerizes on ligand binding followed by autophosphorylation of the receptor. This leads to recruitment of an intermediate histidine phosphotransfer protein (HP) which in turns causes phosphorylation of downstream proteins known as nuclear response regulators in the nucleus which then execute cytokinin action. They either regulate the expression of cytokinin response genes as transcription factors or activate different downstream proteins as protein kinases. A detailed characterization of these response regulators in *Arabidopsis*, which in conjugation, led to their classification in four different classes: type-A, type-B, type-C, and pseudo response regulators (PRRs). The type-B response regulators are DNA-binding transcription factors that promote the expression of cytokinin primary response genes.

Recent evidences suggest the presence of cytokinin response factors in *Arabidopsis* and in conjugation with response regulators mediate the expression of cytokinin-responsive genes. The mode of signaling involved is either paracrine (local signal in meristematic tissues) or distal signaling (for signaling of availability of nutrients). Furthermore, the selective transport of the two most common cytokinins-*trans*-zeatin (tz) and isopentenyladenine (iP) is mediated specifically by xylem and phloem, respectively. The histidine kinases involved in the cytokinin signaling cascade are the transmembrane receptors that comprises of an extracellular CHASE domain (cyclases/histidine kinases–associated sensory extracellular) on which the ligand binds leading to the dimerization and hence activation of the receptor (Inoue et al., 2001; Nishimura et al., 2004, Nishimura et al., 2004 C.; Higuchi et al., 2004). The conserved histidine kinase domain is essential for the activation through autophosphorylation. The receiver domain comprises of a conserved aspartate domain which plays a key role in the transfer of phosphate group from HK domain to the histidine phosphotransfer protein (HP) that further conveys the signal specifically to the type-B response regulator (Appleby et al., 1996; Schaller et al., 2011) in the nucleus. The type-B response regulators possess a DNA-binding domain, thereby acting as transcription factors. In contrast to this, type-A response regulators do not have a DNA-binding domain and their downstream proteins are still undetermined. Once activated, the type-B response regulators are able to activate all the cytokinin-responsive genes including the type-A response regulators, which in turn suppress cytokinin signaling, thereby providing a negative feedback loop during the signaling pathway (Pareek et al., 2006; Du et al., 2007; Pils and Heyl, 2009; Kieber and Schaller, 2014).

Cytokinin-mediated genetic and epigenetic regulation of seed size

One of the key aspects of increasing crop productivity is the seed size. The major emphasis of crop production or improvement since time immemorial has been the selection of crops with bigger seed size (Song et al., 2007; Shomura et al., 2008; Fan et al., 2009). Seed size varies dramatically between species. The endosperm makes up the majority of the mature seed in monocots like rice and wheat. Majority of the dicots, for e.g., *Arabidopsis thaliana* and *Brassica napus* develop their endosperm rapidly at the initial stage; as a consequence, the embryo occupies the larger part of the developed mature seed. In flowering plants, the development of seeds is influenced by complex interactions between maternal tissues, embryo, and endosperm. It has been observed that the endosperm has a major role in regulating seed size. The endosperm exhibits more expeditive growth as compared to embryo during the early phase of seed development, and the seed volume also increases vis a vis the endosperm's growth (Sundaresan, 2005). Several genes and transcription factors regulating growth of endosperm have also been reported to regulate the seed size in *Arabidopsis*. For example, *HAIKU1 (IKU1)*, *HAIKU2* (*IKU2*), and *MINISEED3* (*MINI3*) have been reported to function synergistically in the same genetic pathway to enhance the endosperm size and embryo development (Garcia et al., 2003; Luo et al., 2005; Zhou et al., 2009). Promoters for *MINI3* and *IKU2* also associate with Short Hypocotyl Under Blue 1 (*SHB1*) transcription factor (recruited by *WRKY10*) to promote endosperm development (Zhou et al., 2009). A number of reviews have detailed out the role of these genes in the genetic regulation of seed size (Sundaresan, 2005; Sun et al., 2010; Kesavan et al., 2013). In addition, several maternally derived factors have also been shown to regulate seed size in various plants (Jofuku et al., 2005; Li et al., 2008; Adamski et al., 2009; Ohto et al., 2009; Xia et al., 2013; Singh et al., 2017). Recent studies have highlighted the involvement of various transcription factors, G protein-coupled hormone signaling and ubiquitin-mediated pathway in maternal control of seed size in *Arabidopsis*. The cytokinin oxidase 2 (*CKX2*) gene produces a protein that destroys active cytokinin present in the cell in an irreversible manner. The IKU pathway controls seed size *via* regulating endosperm growth, and *CKX2* has been identified as a direct transcriptional target of the IKU system (Li et al., 2013). Overexpression of *CKX2* resulted in the recovery from the decrease in seed size phenotypes, indicating the involvement of *CKX2* in regulating seed size in a positive way. DNA methyltransferase 1 (*MET1*) (causes methylation of cytosine in CG) regulates *CKX2* as well as epigenetic maternal imprinting (Li et al., 2013). Membrane-bound cytokinin receptors are encoded by the arabidopsis histidine kinases (AHK) family, and *AHK2*, *AHK3*, and *CRE1* (cytokinin response 1)/*AHK4* are the three histidine kinases that bind cytokinin. Although the deletion of one or both of these receptors had no effect on seed size, *ahk1 ahk2*, and *ahk3* triple mutant seeds exhibited a 250% higher volume, with embryo cell number and size increasing by 15% and 30%, respectively (Riefler et al., 2006). Furthermore, it was suggested that the cytokinin-mediated regulation of seed size mostly occurs due to maternal and/or zygotic tissues.

Exogenous application of cytokinin has also been proven beneficial in improving the yield-related traits in pulses and oil seed crops. Application of 100–200 μM BAP (6-benzylaminopurine) increased ovule and seed number in *Brassica napus* and also restored the replum development in wild-type *B. napus* and in the *A. thaliana* rpl ntt double mutant (Zuniga-Mayo et al., 2018). Moreover, when different forms of cytokinin *viz.,* [6-benzyladenine (BA), N-(2-chloro-4-pyridyl)-n-phenylurea (CPPU), 6-furfurylaminopurine (KT), and thidiazuron (TDZ)] were applied on *B. juncea*, highest frequency of shoot regeneration was noticed in combinatorial treatment of thidiazuron (TDZ)] and NAA (Guo et al., 2005). Similarly, combination of BAP and IAA resulted in significantly high biomass and seed yield in *Guizotia abyssinica* (L.f.) Cass. (niger seed plant); a multipurpose oil seed crop (Talukdar et al., 2022). Application of 3.4 × 10–7 mol of 6-benzylaminopurine (BA) resulted in a 79% increase in soybean seed yield compared with controls (Nagel et al., 2001).

Cytokinin-induced/boosting number of seed per pod/pod set/pod development

Among yield-related traits, the number of seeds per/pod and/or number of seed per unit area determines the overall yield (Schou et al., 1978; Kokubun, 1988). The number of seeds per pod or number of seeds produced per unit area is directly proportional to the number of flowers that further develop in to mature pod. Leguminous plants, such as soybean, pigeonpea, and chickpea, produce a higher number of flowers, but most of them abort before reaching maturity (Abernethy et al., 1977). Likely causes include the lack of nutrition, vascular constrictions, and certain hormones (Heindl et al., 1982; Antos and Wiebold, 1984; Brun and Betts, 1984; Kokubun and Honda, 2000). Competition for photosynthesis among seeds and organs is also thought to be a major cause of abortion (Shibles et al., 1975). There are various reports highlighting the exogenous and/or endogenous cytokinin-mediated boost in flower and pod formation (Crosby, 1981; Peterson et al., 1984; Dyer et al., 1987; Mosjidis et al., 1993; Nagel et al., 2001; Yashima et al., 2015).

Figure 2 highlights various aspects of yield and yield-related traits as mediated by cytokinins. Increase in pod number was observed after application of exogenous BA4 to the floral raceme of mung bean (Clifford 1981) and two soybean cultivars (Crosby, 1981; Peterson, et al., 1984) The cultivar Shore, exhibited significant increases in pod number than Essex soybean cultivar. It was speculated that the difference in response was due to Shore ovules having a lower endogenous level of cytokinin-like activity than that in Essex ovules at the time of BA application. Similarly, the administration of three BA on the top of the nodes of field grown soybean resulted in an increase in total number of pod per plant by 27% and seed weight by 18% (Carlson et al., 1986). Furthermore, an increase in the total number of flowers, pods, and number of ovules per gynoecium observed in soybean and oilseed rape after exogenous application of cytokinin (Dyer et al., 1987; Honig et al., 2018) showed that the cytokinin system could be effectively utilized as a target for improving yield and yield-related traits in dicots as well. Bartrina et al., 2011 and Gailbiati et al., 2013 reported that an increased cytokinin levels in the *ckx3 ckx5* double mutant result in a larger gynoecium and production of more ovules. Similarly, sextuple *ckx3 ckx5* mutants were observed to have higher cytokinin concentrations with larger and highly active inflorescence meristems. They also produced up to 72% more flowers on the main stem, with the gynoecia had 32% and 54% more ovules pods, respectively. In addition, the weight of seeds extracted from the main stem of plants was found to be heavier by 20–32% (Schwarz et al., 2020). Furthermore, cytokinins have been shown to increase ovule quantity in other Brassicaceae species, implying that genetic manipulation of cytokinin metabolism could be an effective technique for increasing seed yield (Cucinotta et al., 2016; Zuniga-Mayo et al., 2018) Surprisingly, it was known recently that high amounts of cytokinin hindered fruit elongation in *ckx7* mutants (Di Marzo et al., 2020). Niemann et al., 2015 highlighted the formation of more number of flowers and siliques as a result of mutation in the *ROCK1* gene (*REPRESSOR OF CYTOKININ DEFICIENCY1*), which is essential for full *CKX* function. Furthermore, in the *ugt85a3* (*UDP-GLUCOSYL TRANSFERASE 85A3*) mutant, lower cytokinin inactivation resulted in the development of more ovules per gynoecium (Cucinotta et al., 2018). In soybean, higher levels of cytokinin were found in the reproductive tissues during the pod set and seed filling phases (Jameson and Song, 2016; Liu et al., 2018; Zuniga-Mayo et al., 2018). Metabolite profiling of 27 cultivars of field-grown soybeans (pod and seed tissues) revealed that high producing varieties maintained a constant supply of cytokinins *via de novo* biosynthesis into later stages of development as compared to low yielding soybean genotypes. In addition, zeatin-type cytokinins are required for pod/seed set, whereas isopentenyladenine-type cytokinins have a role in seed filling (Kambhampati et al., 2017). Numerous studies in various crops have reported the significant impact of cytokinins in mediating yield and its related traits through a range of studies, such as exogenous cytokinin application, NGS, transgenic expression studies, advanced chromatographic techniques, mass spectrometry, and many others (Table 1).

Source-sink pathways are always in as dynamic state during the entire life cycle of a plant. Initially starting their life cycle as sinks, leaves mature into source for the seeds, with the latter acted upon as a source to begin with, that is, providing energy and nutrients during germination. Central to these relationships are the availability and partitioning of two major resources: carbon and nitrogen. Sucrose and amino acid mobilization are controlled by *SWEET* (sugars will eventually be exported) and *SUT* (sucrose transporters)/*SUCs* (sucrose carriers) transporters and cell wall invertases for the

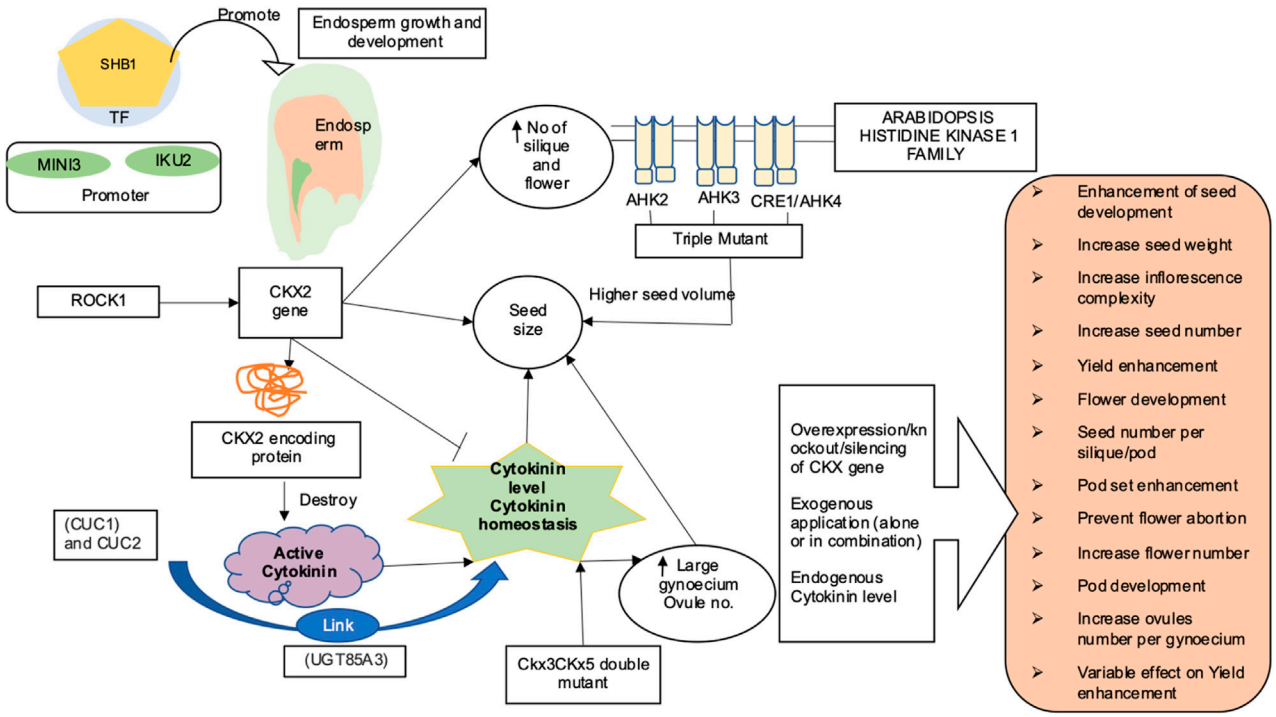

FIGURE 2
Cytokinin-mediated regulation of yield and yield-related traits (SHB1, Short Hypocotyl Under Blue1; ROCK1, Repressor of cytokinin deficiency 1; AHK1, Arabidopsis histidine kinase1 family; *CKX2*, Cytokinin oxidase/dehydrogenase; *CUC*, Cup-shaped cotyledon; *MINI3*, *MINISEED 3*; *IKU2*, *HAIKU2*) (Riefler et al., 2006; Zhou et al., 2009; Li et al., 2013). Various strategies such as overexpression of gene *CKX/IPT* (Werner et al., 2003), knockdown of *CKX* gene family (Ashikari et al., 2005), gene silencing (Heyl et al., 2008; Schoor et al., 2011), and exogenous application of CK (Nagel et al., 2001; Liu et al., 2018; Talukdar et al., 2022) have been attempted to enhance yield-related trait in pulses and oilseed crop. Dynamic role of cytokinin in orange box-in Arabidopsis; CKs are involved in formation of more number of flower, silique, seed (Bartrina et al., 2011; Niemann et al., 2015), ovule number, and seed yield (Werner et al., 2003; Cucinotta et al., 2018; Nguyen et al., 2021b; Zu et al., 2022) In soybean, CKs are involved in preventing flower abortion and in pod setting (Nonokawa et al., 2012), associated with seed development and fatty acid biosynthesis (Nguyen et al., 2016), increase seed yield (Kambhampati et al., 2017), pod set and seed filling (Jameson and Song 2016; Kambhampati et al., 2017; Cai et al., 2018; Liu et al., 2018), and reduction in floral abscission and increase pod number and seed weight (Carlson et al., 1986; Nagel et al., 2001) In *Brassica* increase in seed number per ovule number, pod number, seed weight (Zuniga-Mayo et al., 2018; Schwarz et al., 2020), more pod number, and increase in seed yield was observed (Atkins et al., 2011)

former and primarily *AAPs* (amino acid permease) for the latter. Cytokinins have also been observed to regulate sink number as well as sink size in various legumes, cereals, and Arabidopsis. These signaling molecules are reported to increase the strength of sink tissues and magnetize the assimilates through either influencing sucrose metabolism and transport or promoting cell division (Brenner and Chiekh 1995; Emery and Atkins 2006) through upregulation of cyclins controlling check points of cell cycle (Riou-Khanlichi et al., 1999) and increased phloem unloading in seed coat (Brenner and Chiekh 1995). The insight into cytokinin-mediated influence on source-sink relations and nutrient allocation is depicted in Figure 3. Developing seeds have been documented with the highest cytokinins level among all plant tissues; thus, they are rich source of the former metabolites (Emery et al., 2000). As reported in *Arabidopsis*, *IPT* gene expression in the endosperm continues till early heart stage of its developing seeds, making them sites of cytokinin biosynthesis (Miyawaki et al., 2004), with minor quantities being translocated from xylem or phloem (Emery et al., 2000). Jameson et al., 2016 conducted expression profiling *of SWEET*, *SUT*, *AAP*, *CWINV* (cell wall invertases), *IPT*, *LOG* (Lonely Guy/cytokinin phosphoribosyl hydrolases) and *CKX* genes to ascertain source-sink dynamics in germinating seeds of *P. sativum*. They reported an active expression of cytokinins in imbibing seeds and its biosynthesis in germinating seedlings as well as strong expression of specific genes regulating source-sink dynamics in plants.

Ectopic expression of an IPT gene (isopentenyltransferase) has been observed to increase seed yield. In addition, the inherent expression of cytokinins is highly dynamic and changes rapidly over time, as observed in developing cereal grains (Morris et al., 1993). In wheat, expression profile of specific members of the cytokinin biosynthesis (IPT), degradation (CKX), O-glucosylation, and ß-glucosidase gene families have been implicated in the changing cytokinins levels (Song et al., 2012). In legumes, the level of cytokinins also influence the pod set. Application of cytokinins in lupin has been observed to prevent abortion

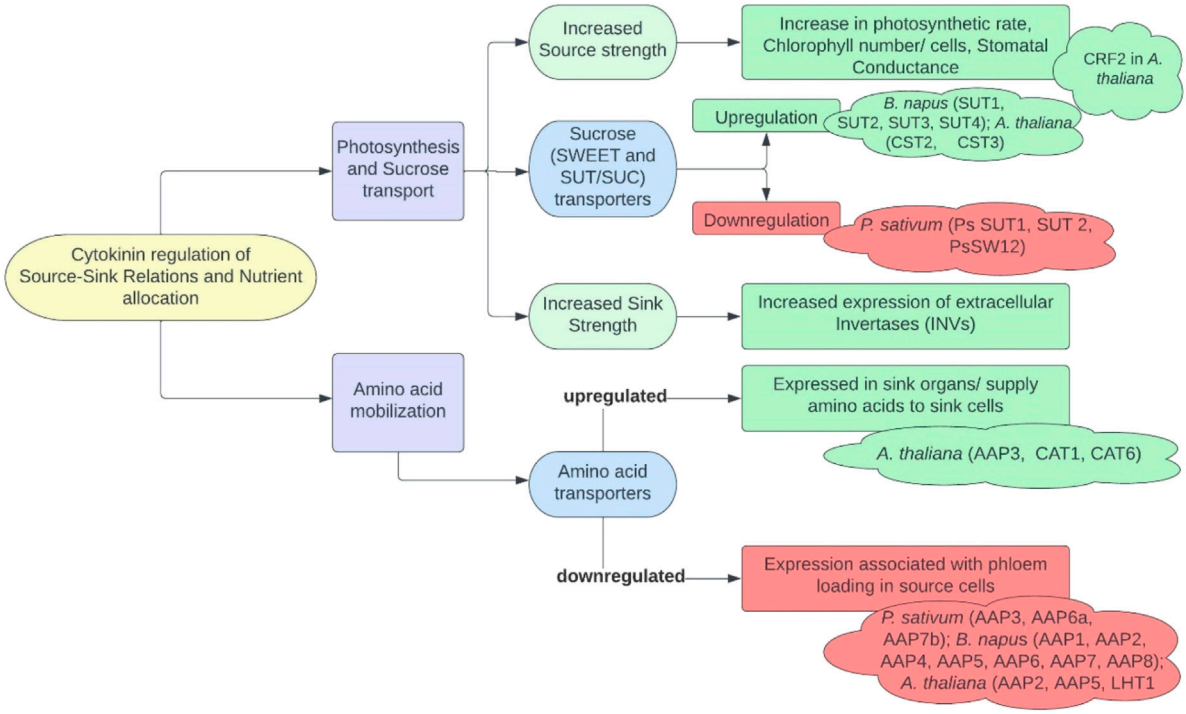

FIGURE 3
Insights into cytokinin-mediated source-sink relationship and nutrient allocation (SWEET, SUGARS WILL EVENTUALLY be EXPORTED TRANSPORTERS; SUT/SUCs, SUCROSE TRANSPORTERS/CARRIERS; *AAP*, amino acid permease; *LHT*, lysine and histidine transporters; *CAT*, cationic amino acid transporters) (for references, see McIntyre et al., 2021; Harms et al., 1994; Ninan et al., 2017; Song et al., 2015; Ehness and Roitsch, 1997; Godt and Roitsch, 1997; Yang et al., 2014; Chernyad'ev, 2000; Ahanger et al., 2020; Boasson and Laetsch, 1969; Okazaki et al., 2009; Lee et al., 2007; Kiba et al., 2011; Kiba et al., 2005; Yokoyama et al., 2007; and Jian et al., 2016)

(Aitkins and Pigeaire, 1993). Emery et al. (2000) investigated correlation of cytokinins with abortion in developing flowers and pod set by increasing an *IPT* gene expression (Atkins et al., 2011). A detailed GC-MS analysis on developing white lupin revealed the expression nature of cytokinins and showed that the peak and transient cytokinin expression occurs in the liquid endosperm of developing seeds (Emery et al., 2000). Several evidences have pointed out the fact that in legumes maternally derived cytokinins are restricted to the pod and seed set (Emery et al., 2000), but in the developing embryo cytokinin biosynthesis is active in the filial tissues (Singh et al., 1988; Emery et al., 2000). In Arabidopsis, Day et al. (2008) observed that the cell cycle genes and cytokinin biosynthesis (IPT8) genes play a crucial role in the syncytial endosperm development.

Spatial expression of cytokinin gene regulating seed/seed number per pod development

Active sites for cytokinin biosynthesis include developing seeds, pod walls and seed coats. Cytokinins have been demonstrated to influence yield by inducing flowering, increasing silique/seed number/pod, and seed size. Various members of the cytokinin gene family viz cytokinin biosynthesis related gene family (*BnIPT1*, 2, 3, 5, 7, 8, and 9), cytokinin degradation gene family (*BnCKX1* to *BnCKX7*), cell wall invertase gene family (*BnCWINV1* to *BnCWINV6*), sugar transporter gene family (*BnSUT1* to *BnSUT6*), and amino acid permease–related gene family (*BnAAP1* to *BnAAP8*) have been identified in *Brassica napus* as a target for breeding (Song et al., 2015; Ninan et al., 2017). It has been reported that developing seeds are the major site of cytokinins and the filial tissues of developing legume seeds have been shown to rely on cytokinin biosynthesis (Singh, 1988; Jameson et al., 2016), whereas pod walls and seed coats have been observed to have significant amounts as well as different forms of cytokinin (Davey and van Staden, 1977, 1978, 1979; Zhang and Letham 1990; Emery et al., 2000; Song et al., 2015). In *Arabidopsis*, many studies have observed dynamic and differential spatiotemporal expression patterns of *IPT* gene family members (Miyawaki et al., 2004; Belmonte et al., 2013) as well as in other members of Brassicaceae family (O'Keefe et al., 2011; Liu et al., 2013). After profiling all seven *AtIPT* gene family members using RT-PCR, Miyawaki et al. (2004) observed the presence of the cytokinin biosynthesis pathway in most plant organs. However, distinct tissue specificity

was observed in reporter gene (GUS) constructs used. But still, each family member was found to be expressed at several regions. Liu Z. et al. (2013) observed *BrIPT1*, *3*, *5*, and *7* genes to be strongly expressed in the roots, whereas *BrIPT8-1* was largely confined to immature siliques and *BrIPT8-2* in stamens of *Brassica rapa*. These evidences match well with the data on the developing seeds of the *Arabidopsis*, where expression of genes like *AtIPT8* and *AtIPT4* is localized to the chalazal region (Miyawaki et al., 2004; Day et al., 2008; Belmonte et al., 2013).

Cytokinin and seed germination.

Crop stand and productivity is a manifestation of seed germination and seedling establishment, with the germination process categorized into three phases (water uptake by seeds; mobilization of food reserves and reactivation of metabolism; and radical protrusion) and involves numerous physiological, morphological, and biochemical changes upon favorable conditions, which are regulated by endogenous and exogenous factors. One of the important internal components affecting germination is hormones. Emergence of seeds and buds from dormancy involves reduction in the levels of inhibitors and gradual buildup of growth promoters; thus, extensive changes in seed metabolome repertoire. Role of hormones in mediating a shift of an inert quiescent embryo to a rapidly metabolizing system, that is, seed germination and post germinative seedling growth is well elucidated, particularly that of abscisic acid (ABA) and gibberellins. However, several studies have implicated the regulatory role of cytokinins in seed germination. Different forms and activity of cytokinin are a function of developmental stages, tissues, and plant species in question (Kieber and Eric Schaller, 2010). Cytokinin application was observed to revert the ABA-induced inhibition of seed germination in *Brassica oleraceae* (Khan, 1971), suggesting permissive role of these hormones in removing the blocks (inhibitors) present in seeds. However, the study also suggested that if these inhibitors are absent, role of cytokinins to mediate germination becomes redundant. Water imbibition by seeds exclusively determines radical emergence; however, seedling growth is a feature of remobilization of stored food reserves to zones of growth and mitosis. Several research studies have implicated both of the latter processes to be possibly regulated by cytokinins (Gepstein and Ilan, 1980; Hepler, 1986; MacGaw and Borch, 1995). For instance, reserve material mobilization in *Cicer arietinum* seeds coincides with the period of supply of cytokinin from the embryonic axis to the cotyledons, that is, first 12 h s after the start of imbibition (Martin et al., 1987; Pino et al., 1991). Exogenous cytokinin application has been known to influence the development of embryonic axis as cited by several researchers (Tzou et al., 1973; Alvarez et al., 1987). Gallego et al., 1991 revealed variation in the endogenous level and compartmentalization of different cytokinin groups in embryonic axis of *C. arientinum* L. in response to exogenous cytokinin application and calcium treatment during germination. Zeatin, 2-isopentyl adenosine, and 2-isopentyl adenine application induces germinative changes peculiar to those under normal conditions, such as delay in epicotyl emergence and short and thick embryonic axis with reduced dry weight, whereas application of zeatin riboside and dihydro derivatives did not induce such changes. Furthermore, controlled conditions revealed high amounts of conjugated cytokinins (storage and inactive forms) in the basal regions of epicotyl and hypocotyl of embryonic axis, which are hydrolyzed to free bases followed by their transportation to apical zones, wherein their transformation to dihydro derivatives (most stable form) takes place, as the later form is resistant to the action of cytokinin oxidase enzyme present in the embryonic axis (MacGaw and Burch, 1995). However, a germination medium supplemented with calcium increased the level of dihydro derivatives, whereas exogenous cytokinin application leads to faster appearance but lower levels of these derivatives in embryonic axis segments displaying maximum growth. The later could be attributed to the fact that exogenous cytokinin could lead to stress or produce compounds resistant to enzymes (Whitty and Hall 1974).

ABI5 (abscisic acid insensitive 5) encodes for a basic leucine zipper transcription factor that is best characterized as a key component involved in ABA signaling and early seedling development. ABI5 transcripts buildup during germination and degrade to basal amount post seed germination (Lopez-Molina et al., 2001; Cho et al., 2002). A total of two mechanisms have been proposed wherein cytokinin targets ABI5 to regulate seed germination, as shown in the Figure 4. One route explains marginal regulation by cytokinin through repression of ABI5 expression in an ABA-independent manner (Wang et al., 2011). Second mechanism pertains to the role of cytokinin in promoting plastid differentiation. In *Arabidopsis*, Guan et al. (2014) demonstrated that cytokinin mediates ABI5 protein degradation, thereby alleviating ABA-induced inhibition of cotyledon greening, a focal point during post germination growth and marking the transition from heterotrophy to autotrophy and establishing photosynthetic capacity. In cytokinin signaling, hybrid histidine protein kinases (AHKs) autophosphorylate upon sensing cytokinins through receptor binding. This phosphorelay system comprises of transfer of the phosphate group from the receptors to the downstream components, initially to AHPs (Arabidopsis histidine phosphotransfer proteins), followed by phosphorylation and activation of Myb transcription factor encoding type B ARRs (response regulators). The latter induces expression of cytokinin response gene, including type A ARRs as characterized in *Arabidopsis* (Nishimura et al., 2004; Riefler et al., 2006). When phosphorylated, type A ARRs, through unknown mechanism, negatively regulates cytokinin signaling, thus forming a feedback regulatory loop (Muller and Sheen 2007; To and Kieber 2008; Hwang et al., 2012). To et al. (2007) and Ren et al. (2009) reported cytokinin to positively regulate steady level

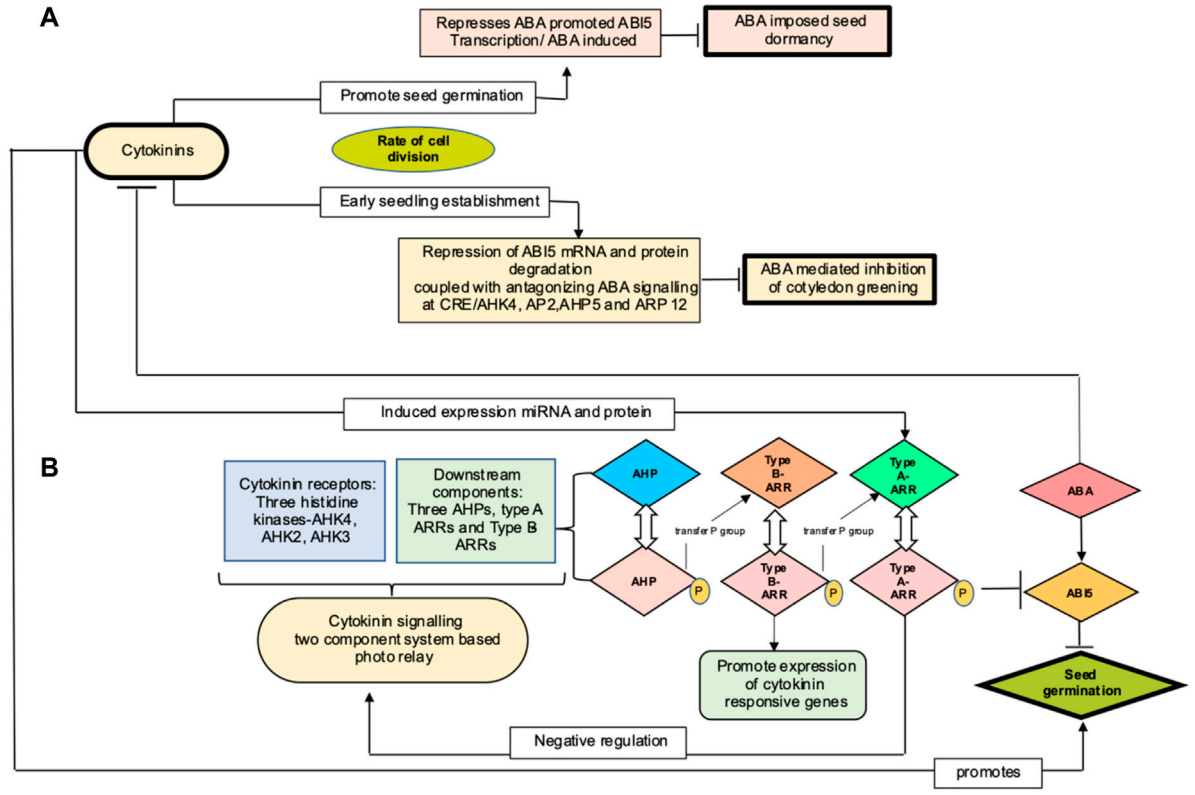

FIGURE 4
(A) Proposed mechanism for cytokinin action in regulating seed germination in *Arabidopsis* by Wang et al. (2011) and Guan et al. (2014), represented at top (light pink (marginal mechanism) and yellow (major mechanism) color boxes, respectively). Cytokinin influences cell division to promote seed germination and seedling establishment and thereby activates ABA-imposed seed dormancy and inhibition of cotyledon greening. **(B)** represents cytokinin signaling components and their interaction with *ABI5* to mediate seed germination (AHK, *Arabidopsis* histidine kinases, ARRs, *Arabidopsis* response regulators; ABA, abscisic acid; *ABI5*, abscisic acid insensitive protein 5)

of type A- ARRs mRNA by increasing the stability of protein in a phosphorylation-dependent manner and further postulated type A ARR–ABI5 complex to inhibit *ABI5* protein interaction with proteasome degradation. Cytokinin promotes seed germination by mimicking the action of auxin, stimulating *ABI5* protein degradation and thus uplifting the abscisic acid inhibitory effect on post germination growth of *Arabidopsis*. Thereby, degradation of *ABI5* protein rather than its mRNA is a major step in cytokinin-mediated ABA signaling.

Convergence of cytokinin with light signaling has been demonstrated through interaction of type-A ARRs with phytochrome b (phyb) (Sweere et al., 2001) and a bZIP transcription factor, HY5, in *Arabidopsis* (Ang et al., 1998). Hutchison et al. (2006) and Riefler et al. (2006) emphasized role of cytokinin receptor genes and *AHPs* in far red light regulated seed germination, implicating cytokinin repressed ABA signaling as an important regulatory mechanism to coordinate early seedling establishment. An enhanced cell division rate as a function of seed priming with cytokinin, particularly kinetin has also been related to improved germination and robustness of seeds (Sawan et al., 2000; Tahaei et al., 2016). Wang et al., 2011 characterized *Arabidopsis gim1* (germination insensitive to ABA mutant 1) mutants' deficit for *AtIPT8* gene encoding for isopentenyl transferases, catalyzing a rate limiting step in the cytokinin biosynthetic pathway (Sun et al., 2003; Miyawaki et al., 2006). These mutants were characterized for reduced expression of *ABI5* gene, and the expression could not be restored with exogenous ABA treatment. However, ectopic expression of *AtIPT8* (ecotypic expression) in OE-2/Com1 transgenic plants was observed to raise the cytokinin level and ABA insensitive seed germination features of *gim1* mutants was observed.

Several advanced studies have also been conducted to elucidate the role and establish mechanism of cytokinin in germination of oil seed crops and legumes. For instance, first study underpinning legume metabolism in relation to seed germination was conducted by Araujo et al. (2019). Priming of *Medicago truncatula* seeds (50 mM) kinetin throughout germination followed by detailed metabolome and physiological characterization was observed to speed up

TABLE 1 Impact of cytokinins on yield and yield-related trait in oilseed and pulses.

Trait under study	Crops	Cytokinin types/Conc	Methodology used/ parameter estimated	Results	References
Other traits	*Arabidopsis thaliana*	NA	Cytokinin response assay and various inhibitors of known signaling pathways were tested	Primary alcohols that specifically inhibit phospholipase D (PLD) partially prevented cytokinin-induced GUS activity and reduced the accumulation of ARR5 gene transcripts	Romanov et al. (2002)
Yield-related traits	*Arabidopsis thaliana*	NA	*AtCKX*-overexpression	Plants with increased number of flowers and siliques, small leaf buds and apical meristems, and expanded root system	Werner et al. (2003)
Other traits	*Arabidopsis thaliana*	NA	Overexpression of an aldose-like enzyme (ALL)	Elevated CK signaling (increased ARR4 and ARR5 expression), dwarfism, reduced apical dominance, and dark green rolled leaves	Jung et al. (2005)
Other traits	*Arabidopsis thaliana*	NA	Gene silencing 35S:ARR1-SDRX	Insensitivity to active CKs arising from loss of the B-type Arabidopsis response regulator 1 *via* gene silencing	Heyl et al. (2008)
	Arabidopsis thaliana	NA	Induced mutations in ↓*Atckx3ckx5*	Formation of more no of flower, Silique number and seed number	Bartrina et al. (2011)
Other traits	*Arabidopsis thaliana*	NA	SiRNA- and artificial miRNA-mediated silencing of ADK (adenosine kinase) Comprehensive HPLC-tandem MS analysis	In ADK-deficient roots and leaves, cell division was irregular. The metabolic studies of ADK-deficient lines revealed an irregular organization of root tip and root cap cells, decreased meristem diameters, and expanded cells in the elongation zone, highlighting the importance of ADK in CK homeostasis *in vivo*	Schoor et al. (2011)
Yield-related traits	*Arabidopsis thaliana*	NA	Mutation in the *ROCK1* gene (*REPRESSOR OF CYTOKININ DEFICIENCY1*)	Enhanced SAM activity and formation of more number of flowers and siliques	Niemann et al. (2015)
Yield-related traits	*Arabidopsis thaliana*	NA	Functional characterization of *UDP-GLUCOSYL TRANSFERASE 85A3 (UGT85A3)* and *UGT73C1*	*CUP-SHAPED COTYLEDON1* (CUC1) and CUC2 regulate cytokinin homeostasis by interacting with UGTs to determine ovule number thus seed yield	Cucinotta et al. (2018)
Yield-related traits	*Arabidopsis thaliana*	6-BA and eBL 10 mmol/L (30 min 1 μmol/L eBL and 30 min 1 μmol/L BRZ)	Crossed BR-and CK-related mutants to test if these two phyto-hormones functions together in ovule initiation	Increasing BR and CK levels at the same time resulted in more ovules and seeds than increasing BR or CK individually. *BZR1*, a BR-response transcription factor, interacted directly with ARR1, to increase ovule initiation. Brassinosteroid-cytokinin interaction improved ovule initiation and increases seed quantity per silique	Zu et al. (2022)
Other traits	*Arabidopsis thaliana*	NA	*CYTOKININ-RESPONSIVE GROWTH REGULATOR (CKG)*, mediates CK-dependent regulation of cell expansion and cell cycle progression in *Arabidopsis thaliana*	From embryonic through reproductive phases, CKG promoted organ development in a pleiotropic manner, especially in cotyledons. Conversely, cotyledons were smaller in ckg loss-of-function mutants. CKG primarily controls the expression	Park et al. (2021)

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TABLE 1 (*Continued*) Impact of cytokinins on yield and yield-related trait in oilseed and pulses.

Trait under study	Crops	Cytokinin types/Conc	Methodology used/ parameter estimated	Results	References
				of cell cycle-related genes such as *WEE1* (a cell cycle promoting factor)	
Yield-related traits	*Arabidopsis thaliana*	NA	Impact of an altered epidermal cytokinin metabolism on Arabidopsis shoot development	This cytokinin action was primarily mediated by the AHK3 receptor and the transcription factor ARR1.Increased cytokinin production in the outer layer of reproductive tissues and the placenta resulted in the placenta producing more ovules and longer siliques. As a result, more seeds in longer pods, leading to higher seed yield per plant	Werner et al. (2021)
Other trait	*Arabidopsis thaliana*	NA	The effect of light intensity on the cold response in *Arabidopsis thaliana*	Transcription of genes related to CK metabolism and signaling showed a tendency to re-establish, CK homeostasis in both transformants. Up-regulation of strigolactone-related genes indicated their role in suppressing shoot growth. The analysis of leaf proteome revealed over 20,000 peptides, representing 3,800 proteins and 2,212 protein families	Prerostova et al. (2021)
Oilseed and pulses					
Yield-related traits	Oilseed Rape	NA	Measurement of various cytokinin during pod development with high performance liquid chromatography and immunoenzymic (enzyme-linked immunosorbent assay, ELISA) techniques	Variable effect on yield enhancement were noticed such as increase flower number, increase ovules number per gynoecium and pod development	de Bouille et al. (1989)
Yield-related traits	Oilseed Rape (*Brassica napus L.*)	NA	Constitutive expression of *IPT* gene under Slightly leaky maize heat-shock (hsp70)	Increase in seed number and seed weight were found	Roeckel et al. (1997)
Shoot regeneration	Oilseed Rape (*Brassica juncea* var.)	[6-Benzyladenine (BA), *N*-(2-chloro-4-pyridyl)-*n*-phenylurea (CPPU), 6-furfurylaminopurine (KT) and thidiazuron (TDZ)]	The shoot regeneration frequency of cotyledon and leaf	The highest frequency of shoot regeneration was 61.3%–67.9% in cotyledon and 40.7%–52.4% in leaf segment respectively when 2.27 or 4.54 μM TDZ was combined with 5.37 μM NAA	Guo et al. (2005)
Leaf senescence and yield	Canola (*Brassica napus L.*)	NA	Regulated Expression of a IPT gene using AtMYB32 promoter Evaluation of seed quality parameters; fatty acids (% of oil content)	The yield was increased from 16 to 23%. Oleicacid content was increased in all transgenic lines, with higher oil content and reduced glucosinolate levels in one particular transgenic line. Increase the number of flowers, siliques, and overall yield	Kant et al. (2015)
Pod development and stress responses	Oilseed Rape (*Brassica napus L.*)	Cytokinin 6-benzylaminopurine (6-BA) and the auxin indole-3-acetic acid (IAA)	Genome-wide identification and expression profiling of *CKX* Genes	A total of 23 BnCKX genes were identified and the expression levels of *BnCKX5*-1, 5–2, 6–*1*, and 7–1 significantly differed between the two lines and changed during pod development. Also exhibited role	Liu et al., (2018)

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TABLE 1 (*Continued*) Impact of cytokinins on yield and yield-related trait in oilseed and pulses.

Trait under study	Crops	Cytokinin types/Conc	Methodology used/ parameter estimated	Results	References
				in increasing silique length and pod development	
Flower and fruit development traits	*Brassica napus and Arabidopsis thaliana*	100–200 μM BAP (6-benzylaminopurine	Hormone treatment, microscopy, parameters related with fruit development	Cytokinin affects stamen filament elongation and anther maturation, and causes a conspicuous overgrowth of tissue in petals and gynoecia. Also increases in ovule and seed number was observed	Zuniga-Mayo et al. (2018)
Yield-related traits	*(Brassica napus)* Mutant *ckx3* and *ckx5*	NA	RNA-seq analysis and *in situ* hybridization	Increased cytokinin concentration and larger inflorescence meristems. Increase in no of flowers, ovules, no of pods and seed weight were noted	Schwarz et al. (2020)
Yield-related traits	Chickpea (*Cicer arietinum*)	NA	Estimation of cytokinin at four developmental stages in chickpea using gas chromatography–mass spectrometry	Enhancement of seed development and Increase seed weight	Emery et al. (1998)
Yield-related traits	*Guizotiaabyssinica* (L.f.) Cass. (Multipurpose oil seed crop)	6-Benzyl aminopurine (BAP) 25, 50, 75, and 100 mg L^{-1}	Physio-chemical properties of soil of experimental site, FA composition and yield-related traits in (niger seed plant)	The combination of IAA (50 mg L^{-1}) and BAP (100 mg L^{-1}; $I_{50}B_{100}$) yielded significantly high biomass (38 and 40 g $plant^{-1}$) and seed yield (13.24 and 12.67 g $plant^{-1}$) in 2014 and 2015, respectively	Talukdar et al. (2022)
Yield-related traits	Lupin	NA	Flower-specific expression of *IPT* gene	More pod number and increase in seed yield was observed	Atkins et al. (2011)
Yield-related trait	Soybean (*Glycine max*)	Applications of BA	Growth characteristics and agronomic traits, including abscission, pod number and seed weight	Reduction in floral abscission and increase in total pod number and seed weight by 27 and 18%, respectively	Carlson et al. (1986)
Yield flower and pod Set	Soybean (*Glycine max*)	6-benzylaminopurine (BA)	Number of pods, seeds per pod, and the total seed weight per plant were measured	In the greenhouse, application of 3.4×10^{-7} mol of BA resulted in a 79% increase in seed yield compared with controls. Pod set enhancement and increase seed weight	Nagel et al. (2001)
Yield-related trait pod and seed development	Soybean (*Glycine max*)	2-(2,4-dichlorophenoxy) propanoic acid (2,4-DP) and 6-benzylaminopurine (BAP),0.12mM, 0.08 mM, 0.04 mM, and 1.5mM, 1 mM, 0.5 mM	Determination of patterns of flower, pod and seed development. Association of reproductive abscission with growth characteristics, including seed yield and weight in two genotypes	BAP (0.5 mM) dramatically decreased flower abortion and delayed pod abscission, leading in higher pod setting rates. 1 mM BAP raised 100-seed weight to 22.3 g at R1 in Manlee (big seeded) and 11.9 g at R3 in Pungsan under field circumstances utilizing intermediate concentrations. BAP (1 mM) at R3 in Pungsan (small seeded) considerably boosted seed yield (40.1 g plant1)	Cho et al. (2002)
Yield-related traits	Soybean (*Glycine max*)	Cytokinin (6-benzylaminopurine, BA)	The endogenous cytokinin (transzeatin riboside) content of individual florets was measured at the 1, 3, 5, 7th position every 3 days after anthesis and the pod-set%age were calculated in racemes of soybean genotype IX93-100	Cytokinin was detected only from the florets at 9 DAA, and the content was higher in the more proximal florets than in the 7th floret. These findings imply that increasing the quantity of cytokinin in individual florets may improve the pod setting of the florets positioned at the	Nonokawa et al. (2012)

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TABLE 1 (*Continued*) Impact of cytokinins on yield and yield-related trait in oilseed and pulses.

Trait under study	Crops	Cytokinin types/Conc	Methodology used/ parameter estimated	Results	References
				middle or distal part within the raceme	
Yield	Soybean (*Glycine max*)	NA	Identification and quantification CK using (HPLC–MS/MS) at three stages of reproductive development in 27 cultivars of Glycine max	Levels of cytokinins strongly correlated with yield and associated traits at stages critical for reproductive development. Isopentenyladenine type cytokinins increase seed filling whereas zeatin type cytokinins exhibited role in pod/seed set.	Kambhampati et al., (2017)
Yield and biological nitrogen fixation	Soybean (*Glycine max*)	Cytokinin was applied (seed or foliar	Nitrogen source, use efficiency and harvest index, tested in two commercial soybean genotypes (DM50I17 and DM40R16)	In the field, DMR50I7 achieved consistent yields across sowing dates because increased Biological Nitrogen Fixation compensated for limited soil N uptake in early sowing dates, also leading to 25% higher nitrogen use efficiency (NUE)	Kempster et al. (2021)
Cytokinin content and seed yield	Soybean (*Glycine max*)	NA	Genome-wide identification and expression profiling of *CKX* Genes and CK metabolite profiling	A total of 7 *GmCKX* GFMs were identified. Natural variations in SNP were found in five of the seventeen identified *GmCKX GFMs*. Soybean lines with this mutation exhibited higher CK content and desired yield characteristics	Nguyen et al., (2021a)
Other traits	*Medicago truncatula*	Cytokinin 6-benzylaminopurine (6-BA) and indole-3-acetic acid (IAA)	Genome-wide identification and expression profiling of *CKX* Genes	A total of 9 putative *CKX* homologues were discovered. Disruption of Medtr4g126160, which is mostly expressed in roots, resulted in reduction in primary root length and increase in lateral root number, showing the specific roles of cytokinin in regulating root architecture	Wang et al. (2021)
Yield-related trait	Soybean (*Glycine max*) and Cowpea (*Vigna unguiculata*)	NA	Integrated Bioinformatics Analyses of *PIN1*, *CKX*, and yield-related genes for the trait seed number per pod	Although the two genes involved in embryo development interact with the *CKX* gene family, VuACX4 demonstrated a substantially higher relative expression level than *GmACX4*. Following then, a tandem duplication in legumes resulted in the separation of *CKX3* into *CKX*3a and *CKX3b*, with *CKX3a* being a critical gene controlling ovule number	Liu et al. (2021)

radical protrusion caused an impairment of seedling growth at the root level. Kinetin affected content of 27 metabolites at radicle emergence stage, chiefly associated with rapid decline of metabolites linked to germination and stress indicating the role of kinetin as both stress agent and inducer of seed germination. Such targeted studies hold potential in identifying the point, wherein priming of cytokinin needs to be stopped for preventing genotoxicity. However, Riefler et al. (2006) demonstrated role of cytokinin in seed germination, shoot and root development, seed size, and senescence through loss of function mutants for cytokinin receptors (*AHK2*, *AHK3*, and *CRE1/AHK4*) in *Arabidopsis*. Rapid germination, decreased sensitivity to far-red light and increased dark germination were observed in the mutants, revealing functions of these cytokinin receptors in regulating these processes. Cytokinin, thus, was observed to negatively regulate light-dependent seed germination in *Arabidopsis*. Similarly, Oh et al. (2009) conducted genome wide chromatin immunoprecipitation (ChIP)—chip analysis in *Arabidopsis* targeting role of phytochrome interacting factor 3–*LIKE5* (*PIL5*), a basic helix-loop-helix transcription factor (TF) in seed germination. It was observed that phytochromes when activated mediated

TABLE 2 Tissue-specific expression of genes involve in seed/silique per pod/seed development.

Gene name	Crop	Tissue (site of expression)	References
BrIPT1, 3, 5, and 7	Field mustard (*Brassica rapa*)	Expressed in root	Liu et al. (2013b)
BrIPT8_1		Expressed in immature siliques	
BrIPT8-2		Expressed in stamens	
BnIPT1-2/1–3 and BnIPT8-1/8–3		Expressed in siliques and developing seeds	
tRNA IPT genes, BrIPT2, BrIPT9-1, and BrIPT9-2		Ubiquitously expressed	
BrIPT1-1 and BrIPT1-2		High expression in small and medium-sized buds while low expression in big bud	
BrIPT7-1 and BrIPT7-2		Expressed in stamen and root	
BrIPT5-1 and BrIPT5-2		Expressed in root	
BrIPT8-1		Highest expression in siliques	
BrIPT8-2		Mainly expressed in stamens	
BrCKX7-1 and *BrCKX7-2*		Uniformly expressed at high levels in sepals and petals	
BrCKX1-1		Expressed in root	
BrCKX1-2		Expressed in stamen, flower, and petal	
BrCKX3-		Highly expressed in petals, stamens, and flowers	
BrCKX3-2		Mainly expressed in the floral buds	
BrCKX4		Highly expressed in root	
BrCKX5		Highly expressed in stamen	
BrCKX6		Expressed in root, leave, and sepals	
BnIPT1		Not detected in immature siliques	
BnIPT8		Expressed in developing siliques and low expression in seed maturation	
BrCKX2-2		Expressed in reproductive tissues	
BnCKX2-1 and 2–2		Expression was restricted to siliques and seeds	
BnCKX5-1 and *5–2*	Oilseed brassica (*Brassica napus L.*)	Expressed in seeds and silique pericarps	Liu et al. (2018)
BnCKX6-1 and *6–2*		Expressed in leaves, stems, and silique pericarps but were not expressed in seeds or buds	
BnCKX7-1		Highly expressed in stems and leaves	
BnCKX3-1 and 3–4		Expressed specifically in buds and seeds	
AtIPT8 and AtIPT4	*Arabidopsis thaliana*	Expressed in chalazal region of developing seeds	Miyawaki et al. (2004), Day et al., (2008), Belmonte et al. (2013), Kakimoto, (2001), Liu et al. (2013b), Werner et al. (2003), Werner et al. (2006); Mason et al. (2005); Yokoyama et al. (2007); Tajima et al. (2004), Miyawaki et al. (2004), Ye et al. (2002); Takei et al. (2004), D'Agostino et al. (2000), Kiba et al. (2002), Ferreira and Kieber. (2005), Yokoyama et al., (2007)
AtIPT1		Expressed in siliques, integument and seed coat of immature seeds	
AtIPT6		Regarded as pseudogenes	
AtCKX1		Expressed at lateral root junction	
AtCKX1 and 5		Expressed in young floral tissue	
AtCKX1, 2, and 4		Expressed in trichome	
AtCKX6		Expressed in leaf vasculature and root vasculature	
AtCKX4		Expressed in root cap	
AtCKX4 and 6		Expressed in stomata	
AtCKX5		Expressed in root procambium and axillary bud	
AtCKX5 and 6		Expressed in root primordium and mature floral tissue	
ARR5, 8, and 9		Expressed in root meristem	
ARR1, 2, 10, 11, 12, 18, and 20; ARR5 and 6		Expressed in shoot meristem	
ARR1, 2, 10, 11, 12, 13, 14, and 18		Expressed in young leaf	

(Continued on following page)

TABLE 2 (*Continued*) Tissue-specific expression of genes involve in seed/silique per pod/seed development.

Gene name	Crop	Tissue (site of expression)	References
ARR 2, 10, and 12		Expressed in lateral root junction	
ARR3, 4, 6, 8, and 9; ARR1, 2, 10, 12, 13, and 20		Expressed in leaf vasculature	
ARR1, 13, 18, and 20		Expressed in mature floral tissue	
ARR20 and ARR21		Expressed in reproductive organs	
ARR1, 13, 18		Expressed in young floral tissue	
ARR5, 8, and 9		Expressed in root cap	
AtIPT4 and *AtIPT8*		Expressed in developing seeds, highest expression in the CZE	
AtIPT1		Expressed in distal part of cotyledons and cell files in the procambium linking to the xylem, root procambium	
AtIPT1 and *7*		Expressed in mature floral tissue	
AtIPT5		Expressed in root cap in primary or lateral roots at their early developmental stages and in root primordia	
AtIPT1, *AtIPT5*, and *AtIPT1*		Expressed in lateral buds (areal portion) and axillary bud	
AtIPT3		Expressed in phloem	
AtIPT7		Endodermis, Trichome	
AtIPT2 and *AtIPT9*		Ubiquitously with higher expression levels in proliferating tissues	
VuCKX5	Cowpea (*Vigna unguiculata*)	Expressed in flowers, roots, and pods	Liu., et al. (2021), Nguyen et al. (2020), Galbiati. et al., (2013), Mens et al. (2018), Nguyen et al. (2021a)
VuCKX6 and *VuCKX7*		Expressed in root	
VuCKX3a		Expressed in flowers	
PvCKX7-1 and *PvCKX6-*	Kidney bean (*Phaseolus vulgaris*)	Expressed in roots	
PvCKX3a		Expressed in flowers	
GmCKX14	Soybean (*Glycine max*)	Highest expression in all seed developmental stages	
GmCKX3a, *GmCKX7-2*, and *GmCKX6-1*		Expressed mainly in flowers	
GmCKX3b-2 and *GmCKX3b-3*		Expressed in roots	
GmCKX08		Expressed in pod	
GmCKX13		Expressed in vegetative tissue	
GmIPT1 and GmIPT2		Root	
GmCKX7-1 and *GmCKX1-2*		Expression level in all three seed stages. transcripts of GmCKX7-1 and	
Ps CKX2	Pea (*Pisum sativum*)	Highest expression in the pod wall and whole seed (Early stages)	Grant et al. (2021)
PsCKX 1		Expressed in 5-day pod wall and 10-day pod wall	
PsCKX 7		Highest expression in whole pod including seed 1DAP and also in 7-day and 10-day pod wall	
Ps IPT1, *Ps IPT2*, and *Ps IPT4*		Expressed in whole pod including seed 1DAP	
PsIPT 4		Expressed in seed coat during developmental stages 12–18 days	
PsCKX1, *PsCKX2*, *PsCKX5*, and *PsCKX7*		Throughout seed coat development 12–30 days	
PsCKX1 and *PsCKX7*		Highly expressed in 30-d cotyledon	

degradation of *PIL5* and enabled seed germination. *PIL5* was identified to directly or indirectly regulate gene expression of several hormonal signaling networks, including GA, ABA, JA, ethylene, and cytokinins, and also impacting the expressions of various genes encoding cell enzymes involved in cell wall modification. Of interest, PIL 5 was observed to directly

downregulate expression of cytokinin response factors, that is, *CRF1*, *CRF2*, and *CRF3* genes as well as to upregulate the expression of *AHP5* gene. *CRFs* (AP2 domain TFs) and *AHP5* are known to positively upregulate subset of cytokinin responses (Rashotte et al., 2006) and cytokinin signaling, respectively; therefore, *PIL5*-mediated reverse regulation of these cytokinin positive signaling genes.

Future perspectives

It has been observed that when cytokinin levels rise, their degradation also kicks in. This is the one of the most important considerations for researchers aiming to target increased cytokinin content as plants will respond to it by accelerating the cytokinin degradation as a result of active homeostasis. Key participants to cytokinin degradation are the *CKX* catalyzing degradation of several cytokinin forms (Werner et al., 2006; Gajdošová et al., 2011). Table 2 shows the expression of the members of *IPT* and *CKX* gene family in different tissues of oilseed and pulses. These gene families have been the preferred targets for yield improvement in several crops. It has been shown that natural or artificial induction of cytokinins result in increased activity of *CKX* (Brugière et al., 2008; Motyka et al., 2003; Liu et al., 2013a, Liu et al., 2013b). Pertinently, a detailed characterization of the spatiotemporal expression of cytokinin gene family need to be performed in oilseeds and pulse crops for shortlisting significant genes that can be further targeted for genome engineering with the aim to boost the yield. However, appropriate genome editing strategies need to be developed as genes involved in cytokinin biosynthesis and its catabolism belong to multigene family and are pleiotropic in nature.

In the past decade, several reviews have focussed on summarizing the role of cytokinins in biotic and abiotic stresses (Cortleven et al., 2019), nitrogen nutrition (Gu et al., 2018), senescence (Zwack and Rashotte, 2013; Honig et al., 2018), and seed yield (Jameson et al., 2016). In addition, the significance of cytokinin dehydrogenase: a genetic target for yield improvement in wheat crop (Chen et al., 2020) has also been extensively discussed. However, the role of cytokinins in oilseeds and pulses has not been reviewed so far. In this review, we have tried to gather all the relevant information concerning cytokinin-regulated yield traits in these crops. Nevertheless, information on cytokinin metabolomics also needs to be reviewed which might open new avenues for cytokinin-targeted research for improving crop yield and quality.

Quantification and qualitative analysis of cytokinins is pivotal to determine their association with agronomically important traits. Several studies have substantiated the relation between yield, associated parameters, and cytokinins and described them as evolutionary conserved and of utmost functional significance. However, advanced multidisciplinary approaches targeting cytokinin purification, profiling, quantification, and underlying molecular mechanisms are required to be undertaken in pulses and legumes, specifically. Omics tools and techniques provides a peek through in cytokinin gene families, spaciotemporal gene expression patterns, and metabolite characterization, to further dissect cytokinin-mediated source-sink relations and associated metabolic pathways in details. Identifying and targeting candidate genes will further pave the road to enhance the endogenous cytokinins level using transgenic and breeding approaches. Cytokinin homeostasis and its regulatory networking could also be fine-tuned in pulses and oilseeds through detailed characterization of its gene families-, tissue-, and stage-specific expression data, detecting novel mutations and their applicability to harness the potential of techniques such as MAS, TILING, and CRISPR and many more for developing new germplasm, breeding lines, and varieties with maintained quality traits. Comprehensive depiction of the inhibitors and activators of key genes of cytokinins homeostasis, such as *CKX* and *IPT*, and standardization of studies conducting their exogenous applications could also be utilized for enhancing abiotic stress tolerance as well as *in vitro* organogenesis and holds immense potential toward micropropagation in numerous agricultural, horticultural crops, and in forestry.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

Authors are grateful to the Director ICAR- NIPB, New Delhi for providing funding and laboratory facilities.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Genetics

TYPE Original Research
PUBLISHED 10 November 2022
DOI 10.3389/fgene.2022.972696

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Genomics, a section of the journal Frontiers in Genetics

RECEIVED 18 June 2022
ACCEPTED 19 October 2022
PUBLISHED 10 November 2022

CITATION
Ullah S, Khan MN, Lodhi SS, Ahmed I, Tayyab M, Mehmood T, Din IU, Khan M, Sohail Q and Akram M (2022), Targeted metabolomics reveals fatty acid abundance adjustments as playing a crucial role in drought-stress response and post-drought recovery in wheat. *Front. Genet.* 13:972696. doi: 10.3389/fgene.2022.972696

Targeted metabolomics reveals fatty acid abundance adjustments as playing a crucial role in drought-stress response and post-drought recovery in wheat

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Drought stress is one of the abiotic stresses restricting plant development, reproductive growth, and survival. In the present study, the effect of drought stress and post-drought recovery for the selected local wheat cultivar, Atta Habib, was studied. Wheat was grown for 16 days followed by drought stress for 7 days and allowed to recover for 7 days after the removal of the drought stress. Same-aged untreated plants were also grown as a control. The effect of drought stress and post-drought recovery on morphology (root length, shoot length, root weight, and shoot weight), enzymatic activity, and fatty acid profile were analyzed. The results showed that shoot weight (93.1 mg), root weight (85.2 mg), and shoot length (11.1 cm) decreased in the stressed plants but increased steadily in the recovered plants compared to the same-aged control plants, while root length showed a higher increase (14.0 cm) during drought stress and tended to normalize during the recovery phase (13.4 cm). The ascorbate peroxidase activity increased in the stressed plants (5.44 unit/mg protein) compared to the control, while gradually normalizing in the recovery phase (5.41 unit/mg protein). Gas chromatography coupled mass spectrometric analysis revealed abundance changes in important fatty acids, such as palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. Palmitic acid (39.1%) and oleic acid (2.11%) increased in the drought-stressed plants, while a reduction in linoleic acid (6.85%) and linolenic acid (51.18%) was observed compared to the same-aged control plants, i.e., palmitic (33.71%), oleic (0.95%), linoleic (7.52%), and linolenic acid (55.23%). The results suggest that wheat tries to recover in the post-drought stage by repairing oxidative damage through ascorbate peroxidase, and by adjusting fatty acid

abundances under drought stress and during the post-drought phase in an effort to maintain membranes' integrity and a suitable fat metabolism route, thus helping recovery. Targeted metabolomics may be further used to explore the role of other metabolites in the drought-stress response mechanism in wheat. Furthermore, this relatively little explored avenue of post-drought recovery needs more detailed studies involving multiple stress durations.

KEYWORDS

wheat, drought, Atta Habib, metabolomics, fatty acids, recovery

Introduction

Wheat (*Triticum aestivum* L.) is attracting worldwide attention due to the rising global population and comprehensive environmental changes (Lucas et al., 2011). Wheat, a greatest energy source, is composed of 58.2% starch, and also it has an adequate amount of body fats and sugar. The worldwide wheat production in 2020–21 was 778.60 million metric tons, with major producers being China, India, Russia, the United States, Australia, France, Canada, and Ukraine (USDA, 2021). Leading wheat consumers include China, the European Union, India, Russia, the United States, and Pakistan (USDA, 2021). In Pakistan, wheat is used as a staple food and accounts for 8.7% of value addition in agriculture and 1.7% of GDP. In 2020–21, wheat production in Pakistan was recorded as 27.293 million metric tons according to the Pakistan Economic Survey 2021 released by the Ministry of Finance (PES, 2021).

Climatic changes have led to abiotic stresses such as drought being more frequent and severe for a number of important crops, such as rice, maize, cotton, tea, sorghum, soybean, and wheat (Farooq et al., 2009). Global climatic changes have led to an increase in the global temperature over the years, which has led to the aggravation of drought events (Lal et al., 2021). Drought restricts plants' reproductive growth, development, and survival. Drought is associated with a water-supply restriction throughout the reproductive, growth, and developmental stages (Flexas and Medrano, 2002). More than 70% of fertile land around the globe is affected by drought, and the yield loss related to drought stress has gained much attention in recent years. Drought decreases the leaf size and the number of leaves per plant, as well as reducing the leaf longevity (Shao et al., 2008). Drought exerts many physiological effects on plants, including decreased photosynthetic activity (Qureshi et al., 2007), increased oxidative stress, altered cell-wall elasticity (Caruso et al., 2009), abscisic acid accumulation, and toxic metabolite generation (Ahuja et al., 2010). Plants accumulate biomolecules such as proline and melatonin (Tiwari et al., 2021). The most essential organ of a plant is its roots, which have the ability to search for, and supply water to, the plant (Hawes et al., 2000). It is the first organ to be affected by water-limiting stress (Shimazaki et al., 2005). Roots continue to develop to find water in the drought-stress state, but the aerial organs of the plant are restricted in their growth.

The stress responses of plants signify a highly dynamic and complex method seeking to establish a unique homeostasis under adverse growth conditions. The mechanisms that are drought-sensitive include fatty acid metabolism, amino acid metabolism, modulation of cell structure, regulation of gene expression, scavenging of oxygen-reactive species, synthesis of osmolyte, nitrogen assimilation, metabolism of energy and carbohydrate, induction of hormones, kinase cascade signaling, and ion channels activation (Wang et al., 2016). The improvement of the water-deficit tolerance in crops has emerged as a key challenge for today's plant scientists (Budak et al., 2013). Plants recognize and respond to stress conditions with a variety of biological signals at appropriate times and speeds for their survival (Takahashi and Shinozaki, 2019). Higher plants achieve sophisticated responses and adaptations to abiotic stresses, including drought, to maintain optimal growth under stress conditions. For these complex physiological responses in plants, a variety of cellular and molecular regulatory mechanisms are required for short-term responses to prevent water loss *via* transpiration from guard cells and for long-term adaptations to acquire stress resistance at the whole-plant level (Nakashima et al., 2014; Takahashi et al., 2018).

Targeted metabolomics, through utilizing powerful techniques such gas chromatography-mass spectrometry (GC-MS), is an outstanding analytical method for providing valuable separation and resolution. The combination of excellent recognition and separation of the GC-MS technique facilitates a comparatively balanced analysis of a number of known and unknown metabolites (Desbrosses et al., 2005).

Fatty acids play vital roles in the membrane structure of lipids in all living cells. These hydrophobic compounds can also play particular roles in signaling events and metabolic processes. Triacylglycerol is a lipid that is a common type of high-energy compound for storage in many organisms, including plants, where it is present in the seeds of many species (Graham and Eastmond, 2002). In all plant cells, the

glycerol-lipid structural membranes include almost entirely 16-carbon and 18-carbon fatty acids, which usually have three methylene-interrupted double bonds (16:0, 16:1*, 18:0, 18:1, 18:2, 18:3, and in some species 16:3) (Ruiz-Lopez et al., 2015). It is equally important to analyze the post-drought recovery stage to reveal valuable information regarding the mechanisms that occur in plants to recover from drought stress. Several oxidative stress-related proteins, including superoxide dismutase, oxidoreductase, and aldehyde reductase, have been found to increase in the roots of *Vigna radiata* in response to drought stress and recovery (Sengupta and Reddy, 2011). In the present study, in order to investigate the response of wheat to drought stress and post-drought recovery, morphological, enzymatic, and GC-MS-based metabolite analyses are performed.

Materials and methods

Plant growth and treatment

Seeds of a wheat cultivar, Atta Habib, were sown in small pots in a greenhouse at the Institute of Biotechnology & Genetic Engineering, University of Agriculture, Peshawar. About 7–8 seeds per pot, with 16 pots per replication, were sown. Sampling was performed for three replications for morphological, enzymatic, and metabolomic analyses. After 16 days of sowing, drought stress was applied for 7 days while keeping some as a control. Stress was removed after 7 days, and some of the stressed pots were kept for the recovery phase for 7 days. Two kinds of samples were collected at the end of stress period, i.e., one control (control-1) and one stressed (after 23 days), and two kinds of samples were collected after 31 days, i.e., control-2 and the post-drought recovery sample (Figure 1).

Morphological analysis

The weights and lengths of the shoots and roots were measured. For this purpose, the roots were taken with extreme care in order to avoid root damage. Soil was removed from roots by washing with tap water. At each time point collection, this same process was repeated.

Ascorbate peroxidase assay

A weight of 200 mg of the sample was homogenized in 2.5 ml of 25 mM potassium phosphate buffer (pH 7.8) containing 2% polyvinyl-pyrrolidone, 0.4 mM EDTA, and 1 mM ascorbic acid. The solution was centrifuged at 15,000 g for 20 min at 4°C; the clear supernatant was collected to measure APX activity. Protein content was measured by Bradford assay (Bradford, 1976), using bovine serum albumin as the standard. For the measurement of APX activity, the reaction mixture comprised 25 mM potassium phosphate buffer (pH 7.0), 0.25 mM ascorbic acid, 0.4 mM EDTA, and 0.1 mM H_2O_2. APX activity was determined following the depletion in absorbance at 290 nm using a UV/Vis spectrophotometer (Nakano and Asada, 1987).

Fatty acid analysis using gas chromatography-mass spectrometry (GC-MS)

Fatty acid composition was analyzed using GC-MS (PERKIN ELMER SQ8S). For the extraction and preparation of the standard and samples, the protocol described by Katoch (2011) was used. For fatty acids extraction, weights of 120 mg of the plant sample and the powdered standard form were put in glass vials. 1 ml of petroleum spirit was added in each and Polytron was used for crushing. Ethanol was used to rinse the Polytron between the samples. The samples were centrifuged at ambient temperature for 5 min at 5,000 rpm. A clear supernatant of 1 ml after centrifugation was transferred for transmetylation in another tube. In the next step, 0.5 ml of sodium methylated solution (10 g of CH_3ONa in 500 ml methanol) was added, and this was incubated for 30 min at room temperature to complete the transmetylation reaction. In the last step of sample preparation, 0.5 ml 1 M NaCl solution was added, and after 5 min the mixture was divided into two separate layers. The upper layer was used for further fatty acid analysis. The following conditions of GC-MS were maintained: column temperature, 120°C–200°C; injector temperature, 250°C; and ion source temperature, 200°C. GC-MS analysis was programmed for 45.67 min. Peaks of the fatty acid methyl esters were identified through the NIST mass spectral library and comparing their retention time with that of the known standards run under similar separation conditions.

Statistical analysis

One-way ANOVA was applied for analyzing data variations among groups at different analyzed time points, followed by Tukey's HSD post-hoc test, utilizing the SPSS software package.

Results

Seeds of a wheat cultivar; Atta Habib, were sown in small pots in a greenhouse. After 16 days of sowing, stress was applied for 7 days while keeping some as a control. Stress was removed after

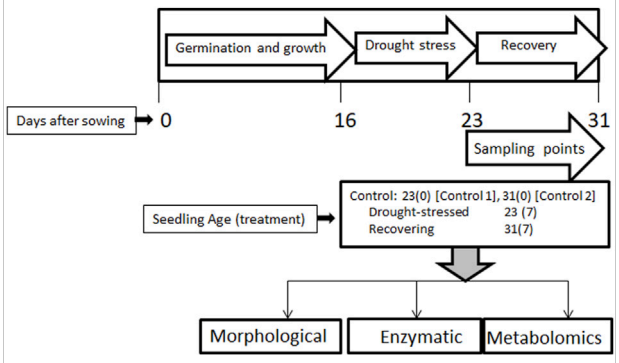

FIGURE 1
The experimental design of the study showing wheat germination, drought-stress application, post-drought recovery stages, and types of analyses performed. Wheat seeds were sown and grown till 16 days. Drought stress was applied on these seedlings for 7 days, followed by 7 days of recovery. Age-matched control plants were also sampled, along with drought-stressed and post-drought recovery plants. 23(0) and 31(0) represent control-1 and control-2 plants, whereas 23(7) and 31(7) represent the drought-stressed and post-drought recovery plants after 7 days of drought stress, respectively.

7 days and some of the stressed pots were kept for the recovery phase for 7 days. Three types of analyses, i.e., morphological, enzymatic, and GC-MS based metabolomics, were performed for fatty acid profiling.

Effect of drought stress on the Atta Habib phenotype

The leaves of the wheat in the control plants were green in color. The leaves turned to pale yellow when they were exposed to drought stress. After recovery from drought stress, the leaves' color changed to light green (Figure 2).

Effect of drought stress on wheat shoot length and weight and its post-drought recovery

The shoot length was shorter after the 7-day drought stress (11.1 cm) compared to the control plants (12.3 cm), although this decrease of 10.81% was not statistically significant (Figure 3A). After the 7-day recovery, the shoot length increased to 12.5 cm, an increase of 12.61%, which represents a significant recovery compared to drought-stressed stage. Similarly, shoot weight was significantly lower under drought stress (93.1 mg) compared to the same-aged control plants' shoot weight of 113 mg, i.e., a decrease of 21.38% (Figure 3B). After the 7-day recovery, the shoot weight increased considerably to 102.2 mg (an increase of 9.77%).

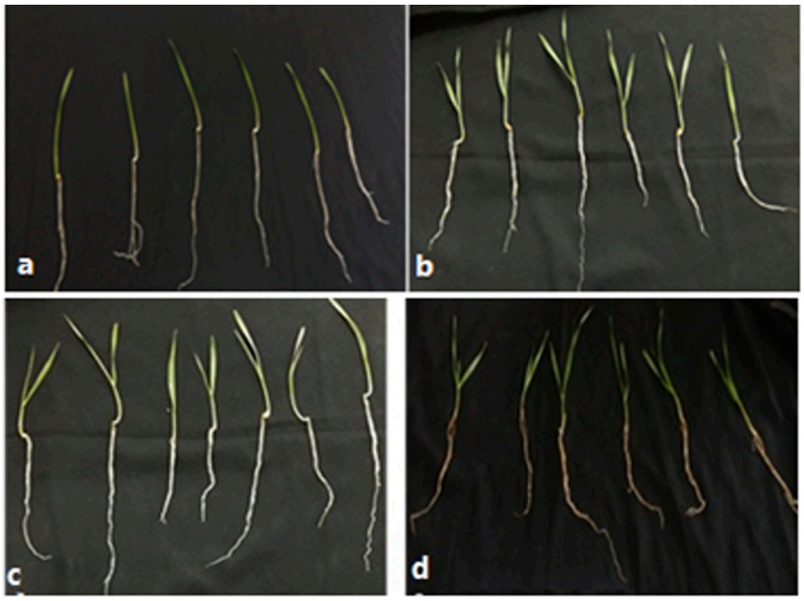

FIGURE 2
Effect of drought stress on leaf pigmentation: **(A)** control-1 plants [23(0)]; **(B)** drought-stressed plants [23(7)]; **(C)** control-2 plants [31(0)]; **(D)** post-drought recovery plants [31(7)].

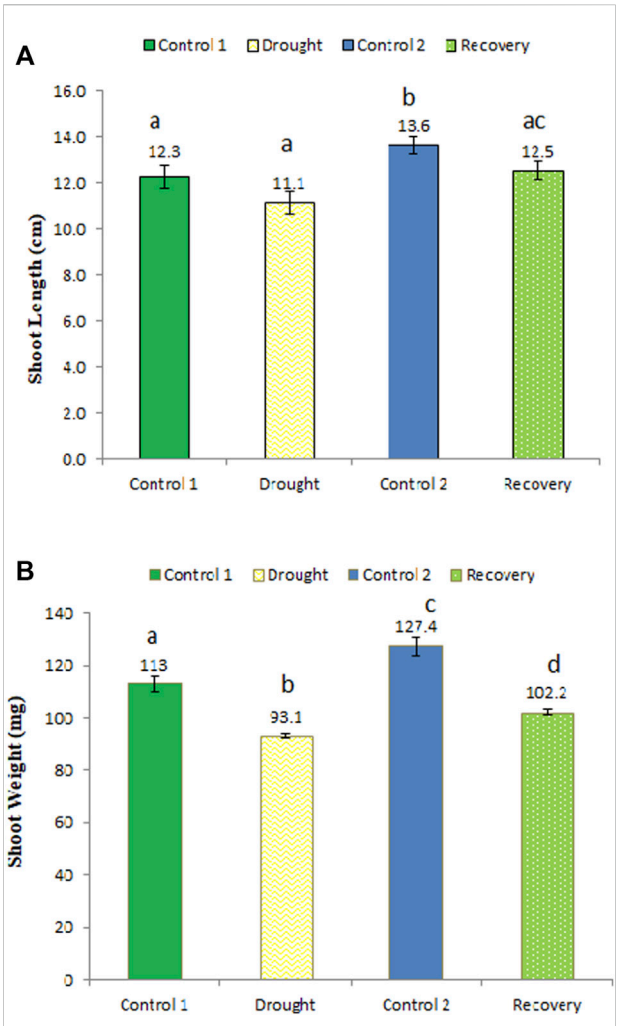

FIGURE 3
Effect of drought stress on the shoot length **(A)** and weight **(B)** of wheat and its recovery after drought stress. Length is measured in centimeters and weight in milligrams. The data represent the mean of three independent replications. Different letters above the column indicate significant changes when determined through Tukey's HSD test.

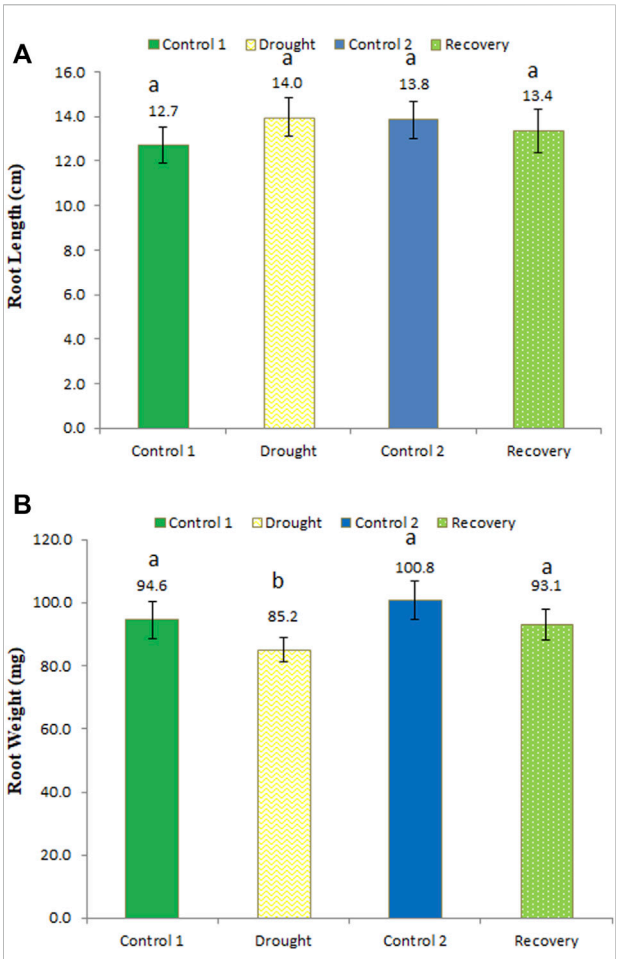

FIGURE 4
Effect of drought stress on the root length **(A)** and weight **(B)** of wheat and its recovery after drought stress. Length is measured in centimeters and weight in milligrams. The data represent the mean of three independent replications. Different letters above the column indicate significant changes when determined through Tukey's HSD test.

Effect of drought stress on wheat root length and weight and its post-drought recovery

An longer root length (14 cm) was recorded under drought stress compared to the same-aged control plants (12.7 cm) (Figure 4A) (an increase of 10.24%). After the 7-day recovery, the root length observed was 13.4 cm, which was restored and normalized to the control plants. These changes in root length were not statistically significant. Drought stress significantly reduced root weight to 85.2 mg compared to the control plants of the same age (94.6 mg) (a 9.94% decrease in weight), while in the recovery stage, the root weight significantly increased to 93.1 mg (an increase of 9.27%) (Figure 4B).

Effect of drought stress on ascorbate peroxidase activity in wheat

Ascorbate peroxidase activity was determined at different time points, i.e., under control, drought, and recovery conditions, in the wheat roots. After the 7-day drought, APX activity was slightly increased. APX activity under drought stress was 5.44 unit/mg protein, while in the recovery phase the enzyme activity was a little lower (5.41 unit/mg protein) (Figure 5A). APX activity at the recovery stage was significantly higher

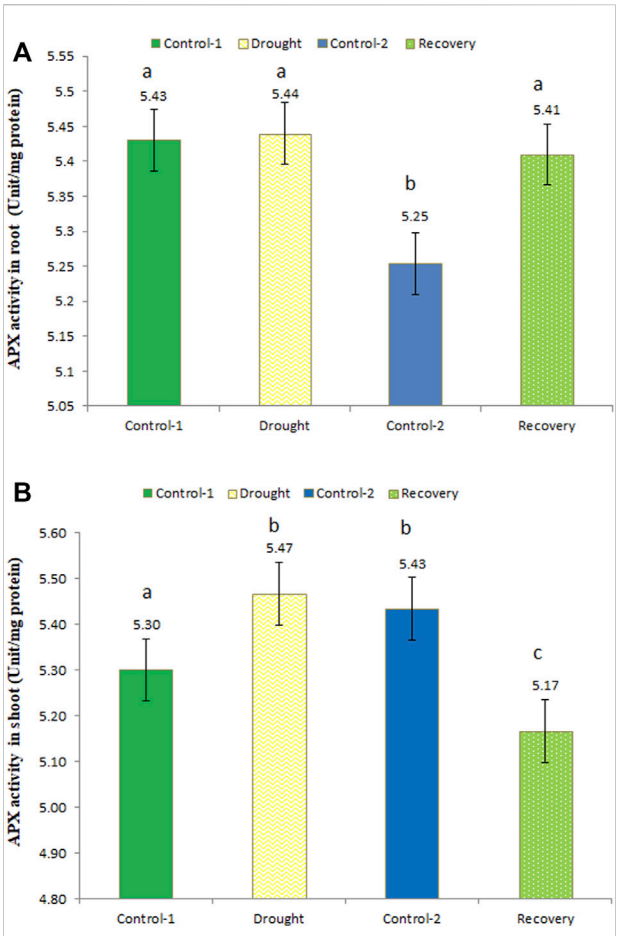

FIGURE 5
Enzyme activity of ascorbate peroxidase (APX) in the roots **(A)** and shoots **(B)** of wheat under control, drought, and recovery stages. The data represent the mean of three independent replications. Different letters above the column indicate significant changes when determined through Tukey's HSD test.

compared to age-matched control-2 plants, whose activity was recorded as 5.25 unit/mg protein.

In drought-stressed wheat shoots, APX activity was higher compared to the control (5.47 unit/mg protein; an increase of 3.21%), while in recovery stage, the activity was lower (5.17 unit/mg protein) (Figure 5B). APX activity changes b/w for age-matched control-1 and drought-stressed as well as control-2 and post-drought recovery plants were statistically significant in wheat shoots.

Effect of drought stress on fatty acids profile

Fatty acid composition was detected in the shoots of the wheat cultivar at different time points, i.e., control, drought, and recovery conditions.

Fatty acids percent composition changes in control, drought-stressed, and post-drought recovery wheat

Fatty acids were detected under control conditions. Control-1 was 16-day old plants, while control-2 was 31-day old plants. The main fatty acids detected in the control plants were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and behenic acid (C22:0) (Table 1). The fatty acid which was detected in the highest amount in control-1 was linolenic acid (55.23%), while oleic acid was found in the lowest amount (0.95%). The same trend was followed in control-2 plants collected after 31 days of sowing, where the highest amount was linolenic acid (49.06%), while the fatty acid detected with the lowest amount was oleic acid (2.60%). Stearic and behenic acids were not detected (ND) in the control-2 plants.

The fatty acid composition changed when analyzed at the end of the 7-day drought stress compared to the age-matched control plants. The main fatty acids detected under drought conditions were palmitic acid, oleic acid, linoleic acid, and linolenic acid. During stress conditions, the linolenic acid was observed with the highest quantity (51.18%), while oleic acid was detected with the lowest amount (2.11%). The results revealed 39.91% palmitic acid and 6.85% linoleic acid in the drought-stressed plants (Table 1).

The fatty acid percent composition was analyzed at the end of the 7-day recovery period. The main fatty acids detected under the recovery stage were lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. In the post-drought recovery plants, lauric acid was quantified as the highest (55.42%), while the acid with the lowest quantity was myristic acid (0.13%) (Table 1). The amounts of palmitic acid (23.25%) and linolenic acid (40.08%) were lower compared to the control plants of the same age.

Percent compositions of fatty acids commonly detected in control, drought-stressed, and recovery wheat

Palmitic acid, linolenic acid, linoleic acid, and oleic acid were commonly identified and quantified at all analyzed time points (Figure 6). The quantities of palmitic acid (from 33.71 to 39.91%) and oleic acid (from 0.95 to 2.11%) increased under drought stress but decreased when analyzed at the end of post-drought recovery stage compared to age-matched control plants. On the other hand, slight changes in linoleic acid and linolenic acid concentrations were observed among different analyzed stages.

TABLE 1 Fatty acid percent composition in wheat samples at different stages.

Fatty acid	Retention time	Control-1	Drought-stressed	Control-2	Post-drought recovery
Lauric acid (C12:0)	16.93	ND	ND	ND	55.42
Myristic acid (C14:0)	20.87	ND	ND	ND	0.13
Palmitic acid (C16:0)	25.52	33.71	39.91	42.93	23.25
Stearic acid (C18:0)	31.43	1.44	ND	ND	1.50
Oleic acid (C18:1)	31.95	0.95	2.11	2.60	1.57
Linoleic acid (C18:2)	33.45	7.52	6.85	5.41	7.01
Linolenic acid (C18:3)	35.80	55.23	51.18	49.06	40.08
Behenic acid (C22:0)	43.35	1.14	ND	ND	ND

*ND: Not Detected.

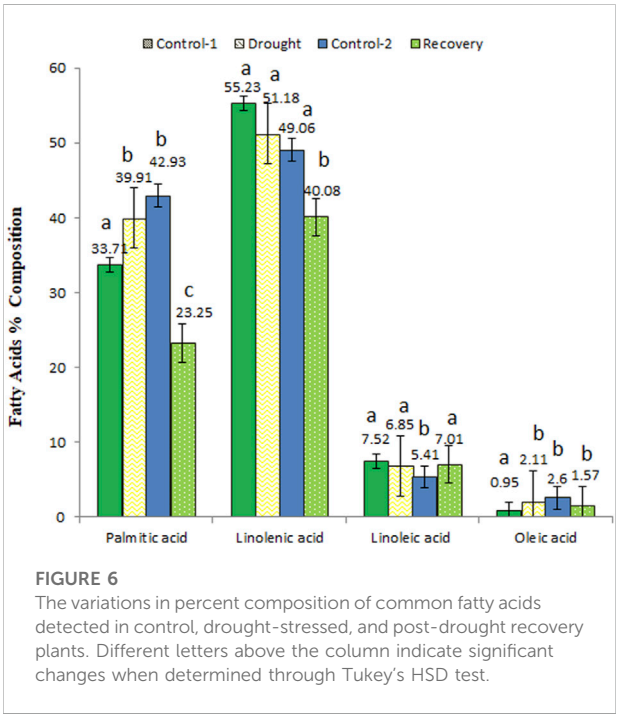

FIGURE 6
The variations in percent composition of common fatty acids detected in control, drought-stressed, and post-drought recovery plants. Different letters above the column indicate significant changes when determined through Tukey's HSD test.

Discussion

Drought is the major stress factor restricting reproductive growth, plant development, and basically survival (Flexas and Medrano, 2002). More than 70% of fertile land around the globe is affected by drought, and the yield loss related to drought stress has gained much attention in recent years as the activities of agriculture have been increased to less arable-friendly or more infertile lands to meet the requirements for growing food (Budak et al., 2013). The present study was executed to test the effect of drought stress and post-drought recovery on the morphological, enzymatic, and metabolite levels of the selected wheat variety, Atta Habib.

The current study has shown that the leaves of the wheat cultivar Atta Habib in the control plants were green in color. The leaves turned to pale yellow when they were exposed to drought stress. After recovery from drought stress, the leaves' color changed to light green. Similar results were reported by Zaharieva et al. (2001), who asserted that the pale yellow color is likely to be a passive adaptation to drought-stress conditions as the green color of *Aegilops geniculata* was affected by drought stress and changed to pale yellow. Our results are also in agreement with Azenas et al. (2018), who studied the effect of water-limiting conditions on the leaf color of five Mediterranean species. The present and previous studies have suggested changes in plant pigmentation and color under water-limiting/drought conditions. The shoot and root weight and shoot length decreased when the wheat was exposed to drought stress for 7 days, although they recovered to some extent after the 7-day recovery phase, while the root length increased during the drought period as they searched for water. Similar findings were reported in soybean (Khan and Komatsu, 2016), in which the root length of soybean increased after 4 days of drought conditions, while recovering to normal after 4 days of recovery, whereas root weight decreased after exposure to stress, while in the recovery stage the root weight increased. The results of the current study are also in agreement with the study of Jaleel et al. (2009), in which the growth of roots and shoots was affected by drought stress.

In the present study, APX activity increased in the wheat under drought-stress conditions, while it decreased in the recovery phase. These results confirm the findings of Hameed et al. (2011), in which different wheat genotypes showed elevated APX activities under drought stress as a tool to minimize oxidative damage. Wang et al. (2016) reported an increased level of APX activity in the shoots of Alfalfa under drought conditions. Increased APX activity was also revealed in drought-stressed soybean (Kausar et al., 2012). These findings suggest the crucial role of APX in scavenging peroxides as an effort to

reduce/repair the oxidative damage caused by drought, and when the plant moves towards the post-drought recovery phase, the enzyme activity is normalized.

The current study has shown that the fatty acid composition of Atta Habib is affected under drought stress and during post-drought recovery conditions. In the present study, the percent composition of palmitic acid and oleic acid increased under drought conditions, while linoleic acid and linolenic acid decreased under stress. In the recovery phase, the percent composition of palmitic and oleic acid decreased, while linoleic and linoleic acid increased. Our results are in agreement with those of Laribi et al. (2009), who reported an increase in the quantities of palmitic acid and oleic acid under drought stress, while a reduction was observed in the levels of linoleic and linolenic acid. Similar results were reported by Baldini et al. (2002), who suggested that drought stress caused accelerated and earlier embryo development and stimulated the enzymatic activities of fatty acid biosynthesis due to which elevated levels of fatty acids were observed under drought-stress conditions. In a more recent study on safflower genotypes (Joshan et al., 2019), drought induced an increase in palmitic, stearic, and oleic acids. Palmitic acid either forms background storage fats and oils or the hydrophobic matrix of cell membranes and the components of cuticle waxes. Palmitic acid is the primary fatty acid formed in the cell that gives rise to higher fatty acids by modifications such as the elongation, desaturation, and insertion of various functional groups (Sidorov et al., 2014). Oleic acid stimulates signaling enzyme phospholipase D that has an anti-cell death function (Zhang et al., 2003). Oleic acid also regulates levels of nitric-oxide-associated protein, thus regulating nitric-oxide-mediated defense signaling in Arabidopsis (Mandal et al., 2012). Drought stress increases the fatty acid saturation of plasma membrane lipids, which leads to membrane rigidification (Lopez-Perez et al., 2009). Saturated fatty acids are involved in shifting membrane fluidity under adverse environmental conditions. Keeping in view the diverse functions of palmitic acid and oleic acid, the rise in their quantities accounts for their involvement in membrane fluidity as well as the synthesis of complex lipids as a way to cope with the stress.

Abiotic stresses have been shown to significantly decrease the amount of linoleic and linolenic fatty acids, while the amount of palmitic and oleic acids increases, suggesting that the plant membrane unsaturation decreases upon suffering stresses (Singer et al., 2016). The study reported that the decrease of membrane lipids in response to different abiotic stresses was mainly due the critical decrease in mono-galactosyldiacyl-glycerol content, compared with the reduction in di-galactosyldiacyl-glycerol and phosphatidyl-glycerol. The reduction in mono-galactosyldiacyl-glycerol content has been considered as a common adaptation strategy for plants when coping with drought, salinity, low temperature, and aluminum stresses (Wang et al., 2016). The degree of fatty acid desaturation has been found to decrease in plants under drought stress (Upchurch, 2008). The signaling roles of lipids or the intermediates of lipid biosynthesis and metabolism have been shown to play a crucial role in plants' environmental stress response (Hou et al., 2016). Linoleic acid and linolenic acids have also been shown to be oxidized spontaneously or during the stress response, resulting in the synthesis of important signaling molecules, such as jasmonic acid, etc., that are involved in multiple signaling pathways (Savchenko et al., 2014). Linolenic acid has been found to participate in the synthesis of jasmonic acid during seedling drought and to alleviate drought stress through jasmonic acid signaling. Maize has been shown to tend to use free fatty acids as antioxidant substances to reduce drought-induced damage (Zi et al., 2022). Linolenic acid is also part of photosynthetic membranes, along with other cellular membranes. Non-tolerant plants subjected to drought and salt stresses commonly show decreased levels of linolenic acid in their membranes, which suggests that the decrease in linolenic acid points to the damage caused by the stress (Liu et al., 2019). In safflower, linoleic acid content has been found to decrease under drought stress (Joshan et al., 2019). Based on the results of current study, it was found that the abundances of palmitic acid and oleic acid increased, while linoleic acid and linolenic acid decreased under drought stress, indicating that the unsaturation of membranes decreases under drought stress. Similarly, unsaturation increased during the post-drought recovery period to restore the integrity of the membranes to enable normal cell functioning.

Conclusion

It can be concluded from the results of the study that drought stress has a negative impact on morphological parameters such as root weight, shoot weight, and shoot length, while they tend to recover in the post-drought recovery wheat in the post-drought stage. The activity of ascorbate peroxidase increased in the drought-stressed plants, while it decreased in the recovery phase. Predominantly, palmitic acid and oleic acid increased, while linoleic and linolenic acid decreased in the drought-stressed plants, whereas in recovery phase, palmitic and oleic acid decreased. These results suggest that wheat copes with the oxidative damage induced by drought stress through scavenging toxic oxides through increased APX activity and adjusts fatty acid abundances in an effort to maintain membranes' integrity and a suitable fat metabolism route, thus helping in drought response and recovery in the post-drought phase. As palmitic, oleic, linoleic, and linolenic aids have physiologically diverse roles in normal growth and development, as well as stress responses through increasing or decreasing membranes' unsaturation and transport across, these fats' metabolic re-adjustments under drought stress and

during the post-drought recovery are beneficial for plants' survival. The findings of the current study are important in revealing the drought stress and post-drought recovery mechanism and related adjustments in the fatty acid metabolism. Post-drought recovery is a relatively little studied area, and this study will provide a helpful basis to explore this avenue further.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material; further inquiries can be directed to the corresponding author.

Author contributions

SU did the morphological and metabolomics analysis; MK planned and supervised the experiment, wrote the manuscript; SSL edited the manuscript draft; IA did statistical analysis and edited the manuscript; MT, TM, ID, MK, and QS helped in manuscript formatting and editing; MA helped in GC-MS data analysis.

Acknowledgments

The authors are thankful to the Institute of Biotechnology and Genetic Engineering, University of Agriculture Peshawar, Pakistan, for providing the chemicals and equipment to successfully execute this research.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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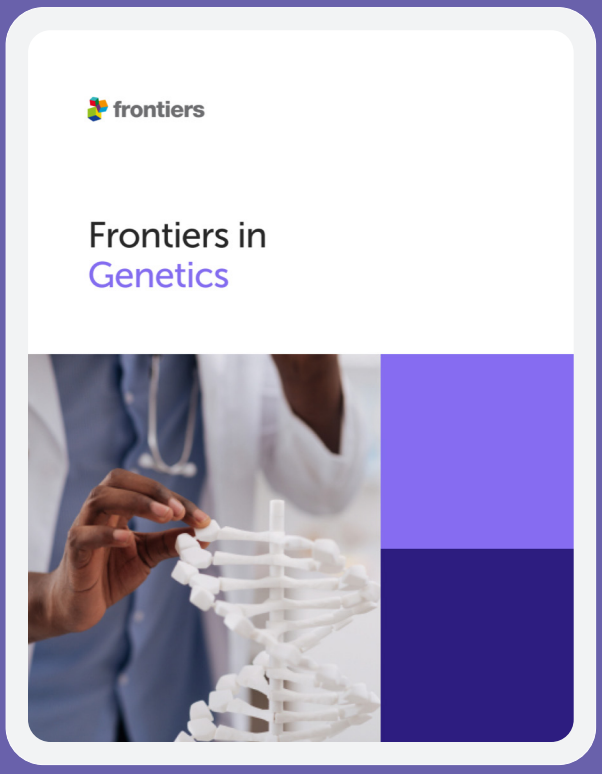

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