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Review

Epigenomics of Plant's Responses to Environmental Stress

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Abstract

Genome-wide epigenetic changes in plants are being reported during the development and environmental stresses, which are often correlated with gene expression at the transcriptional level. Sum total of the biochemical changes in nuclear DNA, post-translational modifications in histone proteins and variations in the biogenesis of non-coding RNAs in a cell is known as epigenome. These changes are often responsible for variation in expression of the gene without any change in the underlying nucleotide sequence. The changes might also cause variation in chromatin structure resulting into the changes in function/activity of the genome. The epigenomic changes are dynamic with respect to the endogenous and/or environmental stimuli which affect phenotypic plasticity of the organism. Both, the epigenetic changes and variation in gene expression might return to the pre-stress state soon after withdrawal of the stress. However, a part of the epigenetic changes may be retained which is reported to play role in acclimatization, adaptation as well as in the evolutionary processes. Understanding epigenome-engineering for improved stress tolerance in plants has become essential for better utilization of the genetic factors. This review delineates the importance of epigenomics towards possible improvement of plant's responses to environmental stresses for climate resilient agriculture.

Keywords: Epigenome; DNA modification; cytosine methylation; gene regulation; histone modification; 5-methylcytosine; stress response

1. Introduction

The epigenome is defined as the sum total of all the biochemical changes in nuclear DNA, histone proteins and non-coding RNAs (ncRNAs) biogenesis of a cell. Studies on the epigenetic changes in and around DNA that regulate genome activity have been defined as epigenetics, and the branch of genomics which deals with epigenomic studies is called epigenomics. A prefix *epi* (means over, outside of, around) implies that the features are "*in*

addition to" or "*from outside of*" the classical genetic basis of inheritance. The area of epigenomics is broadening continuously because of the identification of newer epigenetic marks. With the identification of two additional epigenetic DNA modifications [namely 5-hydroxymethylcytosine (5-hmC) and N^6-methyladenine (6-mA)] having the known epigenetic regulatory functions in the animal system, the significance of epigenomic studies has increased considerably. While DNA allows relatively fewer modifications of its bases, more than 150 modifications have been identified in different types of RNAs [1]. Among the modified nucleosides in DNA, 5-methylcytosine (5-mC) is a well-studied epigenetic mark. However, occurrence and function of 5-mC in RNA is either not completely explored (in tRNA and rRNA) or being noticed (in mRNA and other non-coding regulatory RNAs) [2]. Bases in transfer RNA (tRNA) are heavily modified, and 5-mC has been identified in the variable region and anticodon loop of the archaeal and eukaryotic tRNAs. The modification has been shown to stabilize tRNA secondary structure, affect aminoacylation, codon recognition and confer metabolic stability [3–5]. Emerging evidences indicate that post-transcriptional modifications of nucleotides (e.g. N^6-methyladenosine, 5-methylcytidine, 5-hydroxylmethylcytidine etc.) in RNA are promising players in the area of post-transcriptional regulation of gene expression. This is leading to the emergence of a newer branch of functional genomics known as epitranscriptomics.

Epigenomic changes are continuously being reported to be involved in gene regulation during the developmental processes, tissue differentiation, and suppression of transposable elements (TEs) in both animals and plants. Unlike the genome, which is largely invariable within an individual throughout its life, the epigenome is dynamically altered by the environmental factors. As yet, the concept of evolution has been based on the law of genetics which considers the random mutations in DNA sequence to be responsible for the creation of genetic variability that impacts phenotypic plasticity and adaptability. Most of the proposed models in evolutionary biology have been based on the changes in DNA nucleotide sequence as a primary molecular mechanism underlying heritable variation in the phenotype [6]. However, one of the mysteries of evolutionary theory had been the extremely low frequency of the favorable mutations. Recent studies suggest that the genetic variations may be sufficient for the evolution process, but the genetic theory alone fails to explain some aspects of the evolutionary process [7]. Correlating genotypic variations with the rapid evolutionary changes under environmental pressure has been difficult using the classic genetic approaches because the rate of genetic mutations and the observed phenotypic variations do not match. Additional mechanisms such as epigenetics may help to explain this enigma [8]. If epigenetics is considered as an additional molecular mechanism for regulation of gene expression, many of the phenotypic variations (e.g. dissimilarity between the clones) can be explained easily [9].

Advanced studies in epigenetics, particularly in the area of cancer research, are being reported in the animal system [10–14], while the basic epigenomic study on plant is still in the infancy and only little is understood about the functional consequences of epigenetic/epigenomic changes in plants [15]. Epigenetic changes may also cause variation in the structure of chromatin and function of the genome. The epigenetic mechanisms instigate variation in gene expression

with no change in the underlying DNA sequence and the same may be inherited through mitosis or meiosis [16,17]. The epigenetic changes may lead to chromatin modifications which may cause a stable alteration in transcriptional activity even after withdrawal of the triggering stress/signal [18]. Epigenetic regulation of gene expression is mediated by a complex interplay among different molecular factors which include DNA methylation/demethylation, the enzymes involved in post-translational modifications of histone proteins, chromatin remodelers and ncRNAs [19–21]. Methylated cytosine has been observed to be involved in silencing of TEs, regulation of important developmental processes, genome imprinting and stress responses in both plants and animals [22–24]. Most of the proteins involved in DNA (de)methylation in *Arabidopsis thaliana* have been identified. The components that regulate targeting as well as enzymatic activation of DNA methyltransferase/glycosylases have been discovered, and DNA (de)methylation has been recognized to play crucial roles in several developmental processes in different plant species. However, interaction between DNA (de)methylation and other epigenetic or chromatin features remains unknown. The role of epigenetic regulatory mechanisms in affecting growth, reproductive development, and stress responses have been reported in animals and plants, which can be exploited in crop improvement for climate resilient agriculture [25]. The focus of the present review is the epigenetic modifications of DNA bases, the mechanisms regulating chromatin structure, gene expression and genome stability, transgenerational inheritance of the epigenetic marks followed by the future perspectives of the epigenetic studies.

2. Epigenetics of DNA base modification

Chemical modification of nitrogenous bases of DNA plays important roles in epigenetic regulation of gene regulation. DNA base modification is a tissue-specific, dynamic, sequence-context dependent process, and unraveling the complex patterns of the modifications may answer several biological questions. Methylcytosine (5-mC), which is also known as the 5th base, was reported long before the DNA was accepted as the genetic material [17,26]. In addition to the 5-mC, DNA has also been found to contain 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), 5-carboxycytosine (5-caC) and N^6-methyladenine (6-mA) in small amounts. About 4% of the cytosines present in the human genome are methylated, which reflects its abundance. However, the 5-mC level may vary greatly among the animal and plant genomes. Therefore, the significance of 5-mC cannot be delineated by its abundance. Rather, the importance of 5-mC lies in its positioning (in CG, CHG symmetric; CHH, asymmetric contexts; where H= A, T, or C) or even enrichment in different parts of the gene [27]. In animals, DNA methylation occurs predominantly in CG context [28,29], but it may occur in all three cytosine contexts: CG, CHG CHH in plants. In the human genome, more than 80% of the cytosine in CG context is methylated, which presents a scenario of ubiquitous methylation. However, local gaps are common at regulatory elements like promoters and enhancers of the actively transcribed genes. In plants, symmetric (CG and CHG) methylation is maintained by methyltransferase 1 (MET1) and chromomethylase 3 (CMT3), respectively, whereas asymmetric methylation (CHH) is maintained by RNA-dependent DNA methylation (RdDM) or the chromatin remodeler DDM1-

dependent chromomethylase 2 (CMT2) pathway [30]. Whole-genome bisulfite sequencing of *A. thaliana* revealed that gene-body methylation is mainly associated with symmetric CG methylation, while CHG and CHH methylation is common in TEs and repeats-enriched heterochromatic regions, which are also densely methylated at CG context [31]. Methylation at non-CG sites plays key roles in plants by silencing the activity of the foreign DNA via an RdDM pathway [32]. Therefore, it would be reasonable to assume that the default state of the plant genomes is "methylated" and that specific mechanisms are required to make/maintain the specific regions free of methylation by DNA demethylation processes which may take place by the active or passive method. The active DNA demethylation requires enzymatic removal of methylated cytosine. This process is initiated by a family of DNA glycosylases including DME, ROS1, DML2, and DML3 in plant [33,34], and completed by a base excision repair mechanism. Active DNA demethylation is important for genome-wide epigenetic reprogramming and mediates activation of the genes during the developmental process [35] and environmental stresses [36–38]. On the other hand, passive DNA demethylation refers to the removal of methylcytosine during DNA replication if the maintainer methyltransferases are repressed/inactivated [34]. Transcriptional repression of the maintenance DNA methyltransferase MET1 is associated with the genome-wide DNA demethylation [39].

Although much attention has been focused on the classical modified base 5-methylcytosine, the recent discoveries of additional modifications have resulted in increased interest in the field of epigenomics. Modifications of DNA bases have been found in all the kingdoms of living organisms, including viruses, prokaryotic and eukaryotic cells. However, the purposes of DNA modifications in eukaryotic cells have been less clear. More importantly, the dynamic epigenetic regulation needs removal of the epigenetic marks. Discovery of Ten-eleven translocation (Tet) proteins emphasize that 5-hmC and the Tet-dependent oxidation products (5-formylcytosine, 5-carboxycytosine, 5-hydroxymethyluracil) are the demethylation intermediates of 5-mC, and the potentially stable epigenetic marks in animals [40,41]. Though 5-hydroxymethylcytosine (5-hmC) was identified in mammalian DNA in 1972 [42], its biological implication was investigated lately in 2009 [43]. In mammalian tissues, often the 5-hmC content is about 0.1%, but it can vary significantly with the highest content in the brain, where it can go up to 1% [44]. In mouse embryonic stem cells, about 30000 5-mC, 1300 5-hmC, 20 5-fC, and only three 5-caC per million C residues were reported [45,46], which indicates the sporadic presence of 5-fC and 5-caC. Both these unusual modified bases are removed by base-excision repair mechanisms involving thymine-DNA-glycosylases [45,46]. Erdmann et al. [47] investigated the presence of 5-hmC in Arabidopsis and other plant species using a range of sensitive methods and failed to detect 5-hmC in different tissues and genetic backgrounds. This suggests that 5-hmC is not present in biologically significant quantity in plant genome. Even then, it does not mean that 5-hmC has no role to play in plant. The emerging leap in nucleotide detection/sequencing technology, particularly the high-throughput sequencing, may lead to the identification of such modified bases and their epigenetic functions in plants in the near future.

Methylation of adenine in GATC sequence has been known to be essential for the survival of several bacteria, as Dam methylase creates specific methylation marks important for DNA replication, mismatch repair, segregation, and regulation of gene expression [48,49]. Though N^6-methyladenine (6-mA) is known to play an important regulatory role in RNA, several earlier studies suggested the presence of 6-mA in eukaryotic genomes. Interestingly, many unicellular eukaryotes, such as *Chlamydomonas reinhardtii*, showed comparably higher level of 6-mA [49]. The subsequent discovery of N^6-methyladenine (6mA) in *C. elegans* and *D. melanogaster* (having negligible 5-mC/5-hmC levels) showed low but significant levels of 6-mA in the genome. Experimental data from *C. elegans* suggested a functional interplay of 6-mA with H3K4me2, an established active histone mark [50]. However, mutations in 6-mA–demethylase (DMAD, a Tet-homologue) caused increased transposon activity in Drosophila [51]. In both the organisms, mutations in 6-mA–specific enzymes resulted in significant phenotypic aberrations (developmental defects, infertility), suggesting an epigenetic role of 6-mA in the developmental process. The algal adenine-methylome consists of about 85,000 fully methylated 6-mA (global adenine methylation ≈0.4%), in AT sequence context, enriched in promoter and in the linker regions between adjacent nucleosomes. It was proposed to restrict/mark the positions of nucleosomes near transcriptional start sites [52]. Moreover, the Chlamydomonas genome is characterized by low level of CG methylation, containing CHG and CHH methylation in gene bodies which corroborate with the methylation pattern in plants [29]. A study on *C. elegans* also revealed the presence of adenine methylation in DNA (0.3%) in a strand-specific GAGG and AGAA consensus sequences. Interestingly, accumulation of 6-mA was observed in the worms deficient for spr-5 (coding for an H3K4me2 demethylase) [50]. While 5-mC causes increase in helix stability, 6-mA behaves opposite of it and destabilizes the DNA as measured by denaturing gradient gel electrophoresis. 5-mC is believed to be a repressor of gene transcription when it is found in the promoter region, while 6-mA is hypothesized to be an activator of transcription depending on its location in the genome. Additional insight into the function of 6-mA came from a recent study in Drosophila. Deletions and overexpression of DNA adenine demethylase resulted in lethality, demonstrating an important developmental function associated with 6-mA in Drosophila [51]. However, there is a limited report on the identification of 6-mA in *Oryza sativa* and *Zea mays* using more sensitive detection techniques like high-performance liquid chromatography (HPLC) coupled with mass spectrometry (HPLC-MS/MS) [53]. Generally, organisms with higher levels of 6-mA (such as bacteria and single-celled eukaryotes) tend to have a lower level of 5-mC, while organisms with higher levels of 5-mC (such as plants and mammals) tend to have a lower level of 6-mA. Thus, if 6-mA is also found in significant quantities in eukaryotic genomes, it might turn out to be an important epigenetic mark playing important roles in regulation of gene expression and complementing 5-mC at least at certain loci or during specific stages of development. Finding that 6-mA demethylation can be mediated by a Tet-like enzyme in Drosophila [51], it appears that cytosine and adenine (de)methylation are coordinated process. Hence, it will be interesting to examine the potential interplay between different base modifications to understand the complexity of the epigenetic code.

Though DNA may contain different modifications, yet it is modestly modified compared to the modifications characterized so far in RNA. The newly discovered diversity in DNA base modifications and their combinatorial interactions, if any, indicate that the (epi)genetic DNA code is substantially more complex than it is considered today. Methylated cytosine has mostly been associated with repression of gene, particularly at the enhancer and promoter regions of genes. However, it might also play important role in enhancing transcription, either by recruiting transcription factors [54,55] or by yet to be understood mechanisms when it is present in the coding region of active genes [56]. Epigenetic DNA modifications affect the accessibility of genomic regions to the regulatory proteins or protein complexes, which influence chromatin structure and/or regulate transcriptional activity.

3. Epigenetic regulation of chromatin structure

In eukaryotes, DNA is tightly packaged in a chromatin structure composed of nucleosomal arrays. The nucleosome is composed of protein octamer consisting of pairs of histones H2A, H2B, H3 and H4. N-terminal tail of the histone proteins projects out from the nucleosome core which is subjected to various post-translational modifications. Histone methylation has been reported to be associated with repression or activation of genomic regions depending on the level of methylation of the amino acid residue at the tail of histone proteins, which is dynamically regulated by the actions of histone methyltransferases and histone demethylases. Some of the well-known core histone modifications include methylation of Lys and Arg, acetylation of Lys, phosphorylation of Ser and Thr, and mono- or poly-ubiquitylation of Lys [57]. These post-translational modifications can take place or removed by chromatin modifiers, like histone—methyltransferases, -demethylases, -acetyltransferases and -deacetylases. Histone acetylation influences interaction between the histone proteins and the core DNA, and thus the chromatin structure. Histone acetylation has been reported to be a key conserved epigenetic mark in stress responses, and evidences suggest variation in its pattern change to be associated with the environmental perturbation. These modifications regulate several important DNA-associated processes like chromosome condensation/segregation, replication, and DNA repair. These modifications also regulate transcription process by providing/withholding access to transcription factors, coactivators, and the transcription machinery. Thus, manipulation of histone-methyltransferases and -demethylases can modulate chromatin structure targeted towards improving responses of the plant to environmental stress. As these modifications can be reversible (depending on the environmental conditions) involving cellular/enzymatic machinery, they are considered as epigenetic regulators of phenotypic plasticity. Chromatin structure is well-known to affect transcription of genes in the euchromatic and heterochromatic regions. When chromatin structure becomes dynamic/reversible in response to the environment and/or developmental process it is considered as one of the components of epigenetic machinery. The relationship between chromatin and DNA methylation is still less understood. The two different functional states of chromatin viz. euchromatin and heterochromatin are transcriptionally active and inactive areas of the chromosomes, respectively.

Euchromatin represents an open conformation due to the relaxed state of nucleosome, and contains the genes in active or inactive transcriptional states. On the other hand, heterochromatin is an area where DNA is tightly packaged into condensed form which is largely inaccessible to the transcriptional machinery. The heterochromatic genomic regions primarily consist of repetitive sequences/elements and the repressed genes involved in morphogenesis/differentiation (e.g. imprinting, X chromosome inactivation etc.).

Growing evidence indicates that chromatin modifications are affected by different abiotic and biotic factors and play important role in regulation of gene expression at transcriptional as well as post-transcriptional levels. Chromatin structure is also regulated by the position of nucleosome in the regulatory parts of a gene as well as compactness of the chromatin. ATP-dependent chromatin remodellers (e.g. SWI/SNF complex) were found to influence chromatin structure and its transcriptional activity by modulating nucleosome positioning and the overall nuclear organization [54]. Thus, chromatin structure is influenced by environmental factors, and it acts as an interface because of which environmental factors interact with the genetic components [58]. Moreover, the stable changes in chromatin landscape could be preserved as memory of the mechanisms adopted during environmental exposure leading to the long-lasting phenotypic effects [59]. In general, the plasticity of chromatin during environmental perturbation suggests that chromatin regulators/enzymes may be the important targets in our pursuit to epigenetically engineer the crop plants for climate resilient agriculture.

4. Regulation of gene expression and genome stability

Covalent modification of DNA bases along with histone proteins constitutes an important epigenetic mechanism to control gene expression. Growing evidences indicate that cytosine methylation and ncRNAs are involved in controlling gene expression at transcriptional as well as post-transcriptional levels influenced by various abiotic and biotic factors [17]. Though many epigenetic modifications are known to be reversible, they have been found to be associated with activation as well as inactivation of genes [60]. Thus, gene expression is affected by RNA-directed DNA methylation of genes as well as through histone modifications. Our understanding of the dynamics and functions of epigenetic marks in plants has improved with the recent developments in epigenome profiling. The nuclear genome of plants may contain more than 50% methylcytosine in all the three nucleotide contexts, and it was observed to be concentrated in the centromeric region of the chromosomes and in the repetitive sequences in the *Arabidopsis thaliana* genome [61]. RNAi silencing and knockout mutation of stress-inducible histone deacetylase in maize and Arabidopsis resulted in increased histone acetylation leading to the derepression of silenced genes [62,63]. Thus, one type of epigenetic (histone modification) mark can be converted into another (DNA methylation) more stable mark [64]. Histone proteins have numerous conserved lysine (K) residues that are subjected to acetylation (ac), methylation (me), ubiquitylation (ub) etc. [65]. Methylation of lysine in the histone tail may have differential effects on transcription of the gene depending on the site (K4, K9, K27) and mode (me1, me2, me3) of the modification [66]. Lysine can be either monomethylated (me1),

dimethylated (me2) or trimethylated (me3) which may have different functional consequences [67]. Various histone modifications and their combinations (such as H3K4me3 & H3K27Ac: activation marks, and H3K9me3 & H3K27me3: repressive marks) regulate transcriptional potential of a gene [68]. Modifications of H3 and H4 histones are best understood with respect to their effects on expression of the gene. H3 acetylation and methylation are associated with gene activation, and modifications of lysine residues are well studied. Cytosine methylation further strengthens the histone modification patterns contributing to gene silencing. The level of histone acetylation is controlled by the activities of histone acetyltransferases (HAT) which acetylates, and the histone deacetylases (HDAC) which removes acetylation from the histone [69]. Methylation of histone lysine (K) is catalyzed by the SET domain of the enzyme histone lysine methyltransferases (HKMT) [70]. Certain histone modifications, for example acetylation, phosphorylation, and ubiquitination, are known to enhance transcription of the gene [71], while other modification such as biotinylation and sumoylation repress the gene expression [72]. Lysine methylation can get reverted by the action of two different histone demethylases; while lysine-specific demethylase 1 (LSD1) acts on mono- and di-methylated lysines, the Jumonji-C domain-containing proteins demethylates mono-, di- as well as tri-methylated lysines. Sani et al. [73] reported osmotic priming to influence the epigenomic landscape of the repressive epimark H3K27me3. The stress-priming caused fractionation of H3K27me3 islands, and the effect could be seen even after 10 days of growth under control conditions. However, it got diminished over the time. Interestingly, several genes showing priming-induced changes in H3K27me3) depicted altered transcription level on the next stress treatment. Recently, Wang et al. [74] reported an increase in phosphorylated histone-3 threonine3 (H3T3ph) at pericentromeric regions which were proposed to be involved in maintaining the heterochromatin structure. However, H3T3ph was also found in actively transcribed genes where it emerged to antagonize the effects of H3K4me3 [74], suggesting that H3T3ph might repress the genes required to be down-regulated under osmotic stress. Zheng et al. [75] suggested that histone deacetylase (HDA9) might be involved in negatively regulating Arabidopsis response to abiotic (drought and salt) stresses by controlling the level of histone acetylation in a large number of stress-associated genes.

Variation in ncRNAs biogenesis is another important epigenetic mechanism involved in controlling gene expression. Analysis of Arabidopsis mutants for the genes involved in small interfering RNA (siRNA) biogenesis revealed the role of siRNAs in RdDM pathway which mediate *de novo* DNA methylation in plants [32]. The plant-specific RNA-dependent RNA polymerase 2 (RDR2), RNA polymerases IV and Dicer-Like 3 (DCL3) produce the required 24-nt siRNAs. The siRNAs and Argonaute 4 (AGO4) form a complex in cytoplasm and get imported into the nucleus. A plant-specific RNA polymerase V produces long scaffold transcripts which help in recruiting siRNA–AGO4 complex and DRM2 to the RdDM target loci. In Arabidopsis, a 24-nt siRNA was found to down-regulate the expression of *P5CDH* by mRNA cleavage leading to reduced proline degradation during salt stress [76]. Recent studies show differential expression of the genes encoding epigenetic regulatory proteins [77–79]. Local chromatin changes and DNA methylation in response to abiotic stresses including cold, drought,

salinity, or mineral nutrition are being observed which emphasize the significance of epigenetic regulation during environmental stresses [80–85]. Dijk et al. [86] reported H3 lysine-4 trimethylation (H3K4me3) to be positively-correlated with the transcription level of drought-responsive genes in Arabidopsis under drought stress. Similar findings were reported in rice [87] and in moss [88]. Thus, a better understanding of epigenetic machinery of gene regulation might not only provide the basic information for regulation of genes, but it may also facilitate possible epigenetic engineering of crop plants towards enhanced tolerance to environmental stresses [17].

Considerable (30–80%) portion of eukaryotic genome is comprised of TEs, which are actively transcribed and take part in the regulation of the expression of nearby genes. TEs fraction in plant genomes is variable. It may be as low as ~3% in small genomes and as high as ~85% in large genomes. Of the two classes of TEs, the long terminal repeat (LTR) retrotransposons is considered as a major contributor to the C value differences among the plants. Interestingly, the activity of LTR retrotransposons is under the control of epigenetic mechanisms. Movement of TEs and copy number increases are potentially detrimental to the genome stability. The active transposable elements may induce extensive genomic instability, and they are normally kept under check especially in the germline cells by heterochromatic epigenetic marks like H3K9me3 [89]. Epigenetic modifications play important role in silencing of TEs, gene expression, chromosome stability, and several other cellular processes. Therefore, eukaryotic genomes deploy epigenetic surveillance systems to control TEs movement. LTRs near a gene are targeted for DNA methylation by RdDM pathway which results in the silencing of LTRs as well as the nearby genes. Transcription of an Onsen family of Copia retrotransposons was reported to increase under extreme temperatures, and the effect persisted for seven days which supported the involvement of epigenetics in the process [90]. Activation of the Onsen retrotransposon resulted in frequent transpositions in the progeny of the stressed plant mutated for siRNA production [90], which may affect stability of the genome.

5. Salt-induced epigenetic changes in crop plant: a case study

Evidences implicate epigenetic mechanisms to modulate gene expression in plants under abiotic stresses, epigenetic changes under salt stress and their functional consequences in crop plants are underexplored. Analysis of the stress-associated genes and their regulation of expression in response to the abiotic stresses are commonly employed for enhanced understanding of the plants ability to adapt under changing climatic conditions. Due to the unpredictable climate change, crop plants are frequently exposed to a variety of abiotic stresses including salt stress resulting in reduced crop productivity. Promoter and gene-body methylation play important roles in regulating gene expression in genotype- and organ-specific manner under salt stress. Natural genetic variations for salt tolerance observed in crop plants may be independent of the extent and pattern of DNA methylation which might have been induced by the stress followed by accumulation through the natural selection. Association between the stress tolerance and the variation in methylation observed in some cases suggested that several methylation changes are not ‘‘directed’’. The responses of contrasting wheat genotypes under

salt stress could be explained by the expression level of high-affinity potassium transporters (HKTs) regulated through genetic and epigenetic mechanisms [38]. Coding region of *TaHKT2;1* (second quarter of the gene-body) was found to have variation in 5-mC content in the contrasting wheat genotypes. Salt stress significantly increased the methylation level in the wheat genotypes. With all the cytosine found to be methylated in the CG context, increase in 5-mC was observed in CHG and CHH contexts in shoot of salt-sensitive wheat genotype under the stress. While increase in 5-mC content was observed in salt-tolerant wheat genotype in all the three contexts under the stress, the maximum increase was observed in the CG context. Coding region of *TaHKT2;3* (in the first quarter of the gene-body) showed variations in 5-mC content with respect to the genotypes, tissues, and salt treatments. An increase in 5-mC content was observed in CHG and CHH contexts in shoot of the salt-sensitive genotype under the stress. Significant variations in 5-mC content and differentially-methylated regions (DMRs) were observed in *TaHKT2;1* and *TaHKT2;3* genes of the contrasting genotypes. Increase in methylation due to salt stress was correlated with down-regulated expression of *HKT2* genes. However, the effects of cytosine methylation in different contexts and gene expression level have not yet been fully understood. DNA methylation and/or histone modifications are influenced by abiotic/biotic factors, resulting into better adaptability of the plants to the adverse environmental conditions.

In contrast, only a minor variation in 5-mC content was observed in the coding region (last quarter of the gene-body) of *TaHKT1;4* [15]. Increase in 5-mC content in CG and CHH contexts was observed in the shoot of salt-sensitive genotype under the stress, but no change in 5-mC was observed in salt-tolerant genotype. On the other hand, a decrease in 5-mC content in CHG and CHH contexts was observed in root of salt-sensitive genotype, but increase in 5-mC content was observed in CG context in root of salt-tolerant genotype. However, no considerable variation was observed in cytosine methylation/DMR for the *TaHKT1;4* gene. The variation in 5-mC content could not be correlated with the differential expression level of *TaHKT1;4* and the salt tolerance level of the wheat genotypes. Thus, better understanding about the structural, functional, and regulatory control of HKTs may help improving salt tolerance of crop plants in future.

6. Transgenerational inheritance of epimarks

Epigenetic mechanisms are continuously being reported to be important mediators of plant's responses to environmental perturbations, but their role in long-term adaptation and stress memory is still debatable. Genome-wide epigenetic changes have been correlated with variation in gene expression during the developmental processes and stress exposures. The epigenetic changes, as well as the level of gene expression, may revert back to the pre-stress state once the stress is withdrawn. Some of these epigenetic modifications are retained, and they could be carried forward over the generation as stress memory. In *Taraxacum officinale*, the pattern of genome-wide DNA methylation was found to be changed when the parental plants were imposed with environmental stress. The progenies showed changes in leaf morphology, root/shoot biomass ratio, and stress tolerance compared to that observed in the control plant [91]. In another

example, the tissue culture regenerated rice plants (subjected to the stress experienced during tissue culture procedure) showed changes in the genome-wide pattern of DNA methylation. The changes were predominantly the loss rather than the gain in DNA methylation, and the changes persisted in the regenerated plants as well as in their progenies [92]. These are considered as the indicators rather than the proof of transgenerational inheritance of epigenetic changes affecting adaptive phenotypes. An example related to the defense priming presents a good evidence for a transgenerational epigenetic effect. Progeny of the Arabidopsis plants infected with bacteria was found to be more resistant to secondary infection with oomycete compared to that of the progeny of unprimed/control plants [93]. Chromatin analysis of the defense genes confirmed that inherited priming was because of the epigenetic mechanisms. The up-regulated expression of defense genes was found to be linked with histone acetylation, a known transcriptional activation mark, in the promoter region. On the other hand, down-regulated expression of the genes was found to be associated with the higher level of a repressive epimark H3K27me3. However, the plants defective in DNA methylation at CHG sites mimicked the effects of transgenerational priming [94]. Therefore, it would be appropriate to assume that transgenerational priming might be mediated by demethylation of DNA at the CHG sites; hence, this may not be a simple mechanism but a series of epigenetic changes must be involved wherein the biotic stress causes loss of repressive epimark that, in turn, triggers activating epimark.

Analysis of 30 generations of Arabidopsis showed spontaneous gain or loss in epigenetic marks [95,96]. Although the reason behind some loci being more prone to spontaneous epigenetic changes is not obvious, the existence of overlapping and diverging transcripts might be responsible for these gain or loss in epigenetic marks [97]. Such configuration might affect chromatin structure because of which the epigenetic marks are lost or gained more easily than it may occur in any other region of the genome. In allotetraploids of Arabidopsis, up-regulation of 130 genes was observed due to the loss of repressive histone marks from the circadian clock regulators (CCA1 and LHY) [98]. Evidences for alteration in the biogenesis of siRNAs and changes in the methylation level at a number of associated loci in the hybrids of cultivated- and wild-tomato indicated that wide-hybridization causes a genome-shock in the hybrid leading to induced epigenetic changes [99]. Therefore, priority of the future research on heterosis should be to find out the contribution of various epigenetic mechanisms in providing hybrid vigor.

Zheng et al. [100] reported that drought adaptability of rice plant improved because of multi-generational drought exposure. They identified appearance of non-random drought-induced epimutations, and a higher proportion of the induced epimutations could maintain the altered DNA methylation level in the subsequent generations. Analysis of the drought-associated genes revealed that the DNA methylation level of the genes was affected by the multi-generational drought stress. These results again suggest that epigenetic mechanisms play important roles in plant's adaptations to environmental stresses. Thus, the heritable epigenetic variations having morphological, physiological and ecological consequences can be considered important resources in plant improvement which may help improving adaptation in crop plants for the adverse environments.

7. Future Perspectives of Epigenomic Studies

Epigenetic regulation is considered to be another layer of genetic regulation of the complex traits that are influenced by environmental stimulus. Moreover, unlike other regulatory mechanisms, many of the epigenetic changes may be remembered/inherited over the time/generations as epigenetic memory. The epigenetic memory is viewed as a part of "soft inheritance" wherein the term 'soft' refers to the ability of environmental stimulus in the development of heritable phenotypic changes [101]. The conventional "hard inheritance" in genetics is relatively insensitive to such external influences. One of the interesting examples of soft inheritance was presented by Hauben et al. [102] in double haploid (genetically identical) lineages of oilseed rape selected either for high- or low-respiration rate. Merely four rounds of selection for the trait resulted in the lineages with heritable differences in the energy use efficiencies and the yielding potential. Such a rapid heritable change is unlikely to be explained based on genetic principles; therefore, an epigenetic explanation of this event would be most appropriate. Thus, there is great potential for generation of environment-mediated heritable epigenetic variations, which actually drive/influence the evolution process in living organisms. Another example of environment-induced evolutionary change may be apomictic seed development (apomixis) in plants linked with a dynamic pattern of transcriptional activity in ovule probably regulated through epigenetic mechanisms [8]. In many apomictic species, the embryonic developmental program is not conserved; and the differences in the initiation of apomixis in response to the environmental conditions/stresses provide evidence to support the view that apomixis is epigenetically regulated. Cytosine methylation has been associated with regulation of gene repression either through recruitment of the methylation-specific transcription factors [103] or by yet to be discovered mechanism [56]. Recent developments in the ultra-high-throughput techniques have revolutionized identification of epigenetic changes and improved our knowledge of epigenetic marks as well as their effects on regulation of gene expression. However, further studies need to be focused on revealing the coordination among the known epigenetic marks, which may provide clues on their biological relevance and evolutionary roles. CRISPR–Cas, one of the recent genome-editing systems, needs only two components to edit the target locus: (i) a guide RNA (gRNA), and (ii) a Cas nuclease (Cas9 being the most common). The gRNA (which forms a complex with Cas9) helps in identification/determination of the specific genomic target sequence followed by enabling the nuclease to cleave the DNA, causing a double-stranded break [104]. This double-stranded break activates one of the two pathways for repair of the break caused in the DNA. It may take the non-homologous end joining (NHEJ) pathway which repairs the double-stranded break by randomly adding/deleting nucleotides, or the homology-directed repair (HDR) pathway which uses homologous sequence to repair the break. The NHEJ pathway results in the changes in DNA sequence at the targeted locus, making it suitable for gene-silencing purposes. On the other hand, the HDR pathway makes this technology suitable for tailored repair or gene-correction/editing purpose. The modified versions of this technique like CRISPR–dCas9 would be helpful in RNA-guided dCas9 (de)methylation at targeted loci in the plant genome too in the near future.

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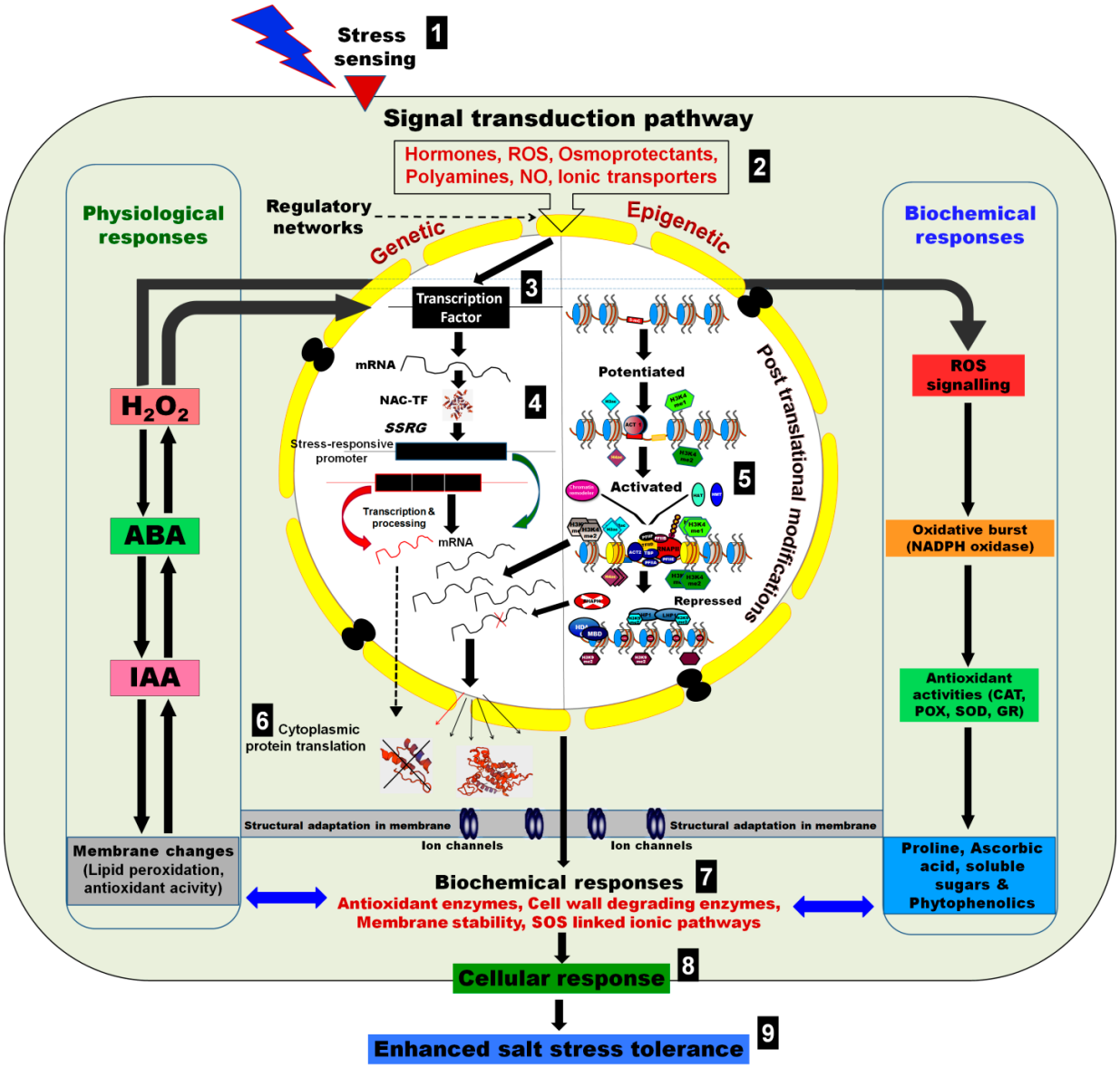

Figure 1. Various biochemical, physiological, genetic and epigenetic mechanisms associated with defense responses of plant under abiotic (e.g. salt) stress. (**1**) Stress sensing, (**2**) Signal transduction through various inducers (e.g. reactive oxygen species, nitric oxide etc.), (**3**) Induction of transcription factor genes, (**4**) Expression of stress-responsive genes, (**5**) Activation/repression of epigenetic (DNA methylation/demethylation, histone modifications and ncRNA biogenesis) factors involved in the regulation of stress-associated gene expression, (**6**) Transcriptional and translational reprogramming to combat the stress, (**7**) biochemical, and (**8**) cellular responses leading to (**9**) the enhanced stress tolerance.

Furthermore, they may also help understanding the mechanistic aspects of DNA (de)methylation and in possible use of epigenetic manipulation for crop improvement [105]. In view of the biosafety concerns of genetic manipulation technology currently being adopted for improving stress tolerance in crop plants [106,107], the targeted epigenetic engineering utilizing

genome-editing technology (which is supposed to have limited biosafety issues, if any) would be a preferred approach. Moreover, the genome-editing techniques are improving very fast and might reach to the point that would enable plant epigenome engineering to be realized soon. This would allow functional interrogation of epigenetic marks and their usage towards stable improvement in the agriculturally important traits [25,108]. Manipulation of DNA (de)methylation level at specific loci may allow us to regulate gene expression and the neighboring chromatin states, impacting cell physiology and biochemistry. A model depicting the mechanisms associated with abiotic (e.g. salt) stress tolerance in plant has been presented in Figure 1. Generally the stress is sensed by the sensor(s) present in cell membrane, transduced to the various inducers to initiate structural and molecular responses like accumulation of reactive oxygen species (e.g. H_2O_2), induction of various transcription factors for the stress-associated genes, genetic and epigenetic (DNA methylation/demethylation, histone modifications and alteration in ncRNAs biogenesis) regulation of the gene expression through transcriptional and/or translational reprogramming for protective defense mechanisms.

These result into biochemical and cellular responses leading to the enhanced stress tolerance. Thus, deciphering the epigenetic machineries to better manage the problems in crop husbandry arising because of the climatic changes has become an important area of research for sustainable agricultural production and global food security even with the diminishing natural resources like cultivable lands and good quality irrigation water.

Author Contributions:
SK conceived the review, prepared and revised the manuscript. The views expressed herein are those of the author only, and these may not necessarily be the views of the institution/organization the author is associated with.

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