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Review

Plant genomic DNA methylation in response to stresses: Potential applications and challenges in plant breeding

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Abstract

The plant genome can respond rapidly and dynamically to stress in a manner that overcomes the restrictions of a highly stable DNA sequence. Abiotic stresses such as chilling, planting density, rubbing, cutting, and successive rounds of subculture generally decrease the levels of DNA methylation. The opposite effect is seen for salt stress, and the effects of heavy-metal stress are species specific. Biotic stresses such as pathogenic infection can lead to two contrasting effects on the levels of methylation in plants: hypermethylation on the genome-wide level and hypomethylation of resistance-related genes. Both phenomena may contribute to the adaptation of plants to stress. Although heritable methylation patterns and phenotypic variations that arise in response to stress are of potential value for plant breeding, their exploitation presents great challenges.

Keywords: DNA methylation; Plant breeding; Stress

1. Introduction

Plant genomes contain the basic genetic information that determines the properties and biological behavior of a species and therefore need to be relatively stable. Although genetic recombination can give rise to new gene combinations that improve resistance to the tolerance of environmental stresses, the rate at which such new gene combinations occur cannot compare with the rate of change in the environment. Under stress, DNA methylation by methyltransferase [1], and related mechanisms such as RNA interference (RNAi) [2], can rapidly and reversibly modify plant genomic DNA, which avoids the need for excessive genetic recombination and population diversity [3]. This type of modification also highlights the adaptability of DNA in a complex environment. After experiencing stress, plant somatic cells can "memorize" this experience by epigenetic mechanisms [4]. Recent studies have shown that methylation of GC-rich sequences acts as the central coordination point for many epigenetic mechanisms, and ensures that the "memory" is transferred faithfully to progeny [5]. This suggests that methylated alleles are of potential value for breeding applications. In this paper, we summarize the responses, features, and biological significance of plant genomic DNA methylation under different types of stress and analyze the possibilities for, and difficulties with, the exploitation of methylated alleles that are dependent on stress in plant breeding.

2. Stress and modification of plant genomic DNA by methylation

2.1. Pathogens and plant genomic DNA methylation

When the type I DNA methyltransferase NtMET1 in tobacco (*Nicotiana tabacum*) is silenced using an anti-sense RNA technique, the expression of approximately 30 genes

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is up-regulated, 62.5% of which are related to responses to biotic or abiotic stress [6]. This suggests that a large number of the genes that can be modified by methylation have a close relationship with the stress response. After artificial inoculation with tobacco mosaic virus (TMV), the methylation pattern of the pathogen-responsive genes *NtAlix1* [6] and *NtGPDL* [7] undergoes rapid (1–24 h) and dynamic changes as the plant hypersensitivity reaction (HR) is established. The levels of gene expression are also altered. The change in the methylation level of *NtGPDL* precedes the alteration in gene expression [7]. This is consistent with the hypothesis that viral infection changes the methylation level of plant genomic DNA and leads to alterations in gene transcription. However, the change in *NtAlix1* methylation lags behind the change in gene expression [6]: transcripts begin to accumulate 12 h after the onset of HR but the change in methylation status is detected 24 h later. This phenomenon can be explained as follows: (1) the method for detection of gene expression may be more sensitive than that for analysis of methylation; (2) slight variations in methylation status may be amplified in the process of gene expression, differences in which are then easier to detect; and (3) the expression of *NtAlix1* might not be related directly to DNA methylation.

Rice (*Oryza sativa* L.) variety Wase Aikoku 3 is not resistant to the blight pathogen *Xanthomonas oryzae* pv. *Oryzae* in the seedling stage but develops resistance in the adult plant. After artificial inoculation of seedlings and adult plants, methylation-sensitive amplified polymorphism (MSAP) analysis revealed that most polymorphic loci have a higher level of methylation in adult plants than in seedlings [8], which may contribute to adult plant resistance (APR) in rice plants. The demethylating reagent 5-azadeoxycytidine can also induce resistance to the blight pathogen in Line-2 rice [9]. The MSAP technique has been employed to screen for DNA fragments that were methylated differently between Line-2 and wild type rice, and one clone, *Xa21G*, which is similar to the rice blight resistance gene *Xa21*, was selected. The promoter of *Xa21G* is not methylated at any cytosine residue in Line-2 rice but is fully methylated in the wild type, which is consistent with the high expression of *Xa21G* in Line-2 [9]. Therefore, we can deduce that demethylation of the *Xa21G* promoter enhances expression of this gene and contributes to the resistance of the plants to the pathogen. As a rule, rice blight pathogen resistance is related to genomic hypermethylation [8] and hypomethylation of resistance-related genes [9]. This rule is exemplified in more detail by TMV-infected tobacco. After infection with the virus, an overall increase in genomic methylation is accompanied by the hypomethylation of resistance-related leucine-rich-repeat (LRR)-containing loci [10], which is consistent with the increased amount of recombination that is observed within the LLR-containing resistance gene (N-gene) [11]. The overall increase in methylation may favor genomic stability when the plant is attacked by the virus, whereas the decreased methylation of the resistance genes may accelerate genetic recombination [12] and thus the development of novel resistance genes. Therefore, both phenomena are important for plant resistance to disease. TMV resistance can be transferred to the progeny [10], and blight pathogen resistance and the related methylation status are inherited for at least nine generations [9]. Classical Mendelian genetics cannot explain the inheritance of stress-derived phenotypic variations [10], which reminds us of the 'acquired' genetics theory that was proposed by Lamarck 200 years ago.

2.2. Chilling and plant genomic DNA methylation

Low-temperature stress decreases the amount of methyltransferase in corn (*Zea mays* L.) [13], which, in turn, might decrease the level of genomic methylation. Such a decrease was documented in corn by HPLC and the degree of reduction was over 10% [14]. It is noteworthy that hypomethylation of approximately 10% of loci in the tobacco genome also occurs under abiotic stress [7]. Under cold stress, the modification of methylation in corn seedlings seems to be organ- and site-selective: methylation is decreased in roots but there is no change in shoots, and demethylation only occurs in Ac/Ds transposons, and not in other genes [14]. In general, a decrease in methylation level can activate chromatin and gene expression [7,14]. Therefore, demethylation under stress may be the cause of activation of Ac/Ds transposons. However, this logic is not applicable to Tam3 transposons in *Antirrhinum majus*. A low temperature of 15 °C decreases the level of Tam3 methylation and activates its transposition activity, whereas a normal temperature of 25 °C reverses these effects [15,16]. However, Hashida et al. [16] have shown that at low temperature, transposase binds to Tam3 immediately after DNA replication and cell division, and this results in a decrease in methylation level caused by decreased access of the methyltransferase to the DNA. Therefore, the activation of Tam3 transposition is the reason for the decrease in methylation, rather than the result of this change. Methylation mapping by Steward et al. [14] has revealed that cold stress induces severe demethylation in core regions but leaves linker regions relatively intact. The methylation of DNA cytosine residues and histone proteins is closely related to gene expression; therefore, this periodic genomic methylation pattern should have an effect on the expression of the transcriptome [14]. This conclusion is confirmed by a 1.8-kb fragment that is designated *ZmMI1*. This fragment, which contains part of a retrotransposon-like sequence, is expressed only at low temperature, under which conditions its methylation level is decreased [14]. It has been deduced that the level of methylation in coding regions decreases under stress, which loosens the chromatin structure and ultimately promotes transcription [17].

2.3. Metal ions and plant genomic DNA methylation

High levels of methylation are considered to be capable of protecting DNA against endonuclease cutting and

multi-copy transposition [18], which favors resistance to heavy metals. For example, a hamster cell line with a higher methyltransferase activity than normal also has a better resistance to heavy metals [19]. Clover (*Trifolium repens* L.) is sensitive to heavy metals, whereas hemp (*Cannabis sativa* L.) is partially resistant. The DNA methylation level in the root of clover is only 30% of that in hemp, as revealed by the technique of immunolabelling with a monoclonal antibody [20]. The authors deduced that the higher methylation level contributed to the tolerance of heavy metals in hemp. Therefore, plants may resist heavy-metal ion stress by increasing their methylation levels. This point has been supported by the results of other studies. Cadmium ($CdCl_2$) can change the methylation status of both oilseed rape [21] and radish (*Raphanus sativus* L.) DNA [22]: MSAP revealed that the MSAP ratio of radish genomic DNA increased in proportion to the intensity of the stress [22]. In addition to cadmium, the methylation level of rape genomic DNA can also be altered by chromium. Using MSAP and immunolabelling techniques, the level of hypermethylation in the rape genome was shown to correlate positively with the stress dosage of chromium [23], which suggested *de novo* synthesis of methylated cytosine. However, some reports show a contrary trend. For example, nickel, cadmium and chromium stress can reduce cytosine methylation levels in clover and hemp by 20–40%, and the decrease is proportional to the stress dosage of the heavy-metal ions [20]. Chromium stress can therefore promote methylation in rape [23] but induces a decrease in methylation in clover and hemp [20], which suggests that different methylation mechanisms for heavy-metal resistance exist in different plant species. MSAP has revealed that the methylation patterns of different individual plants within CCGG sites are similar before and after heavy-metal stress, which suggests that the changes in methylation that are induced by stress are not distributed randomly [20].

Although there are quite a number of reports on variations in DNA methylation under heavy-metal stress, most of them employed methods such as MSAP to perform an overall investigation at the whole-genome level. With the exception of a few genes such as *NtGPDL*, we still know very little about methylation modification at specific genomic loci under heavy-metal stress. Bisulfite mapping has revealed that CG sites in the *NtGPDL* coding region were demethylated rapidly, within 1 h, after aluminum stress in detached leaves, but the methylation pattern of the promoter did not change. *NtGPDL* transcripts appeared within 6 h [7], which is consistent with the theory that DNA methylation levels are inversely related to the levels of gene expression [24]. The results of this study also show that the regulation of gene expression under stress is not limited to the promoter region, but can be achieved by changing the methylation status of the coding region (body methylation). This is consistent with the observation by Zhang et al. [25] that genes that are methylated in their coding and promoter regions are generally expressed at high and low levels, respectively. In addition to aluminum, both salt and low temperature can induce demethylation of *NtGPDL* and up-regulate its expression [7]. Furthermore, both aluminum and herbicides can cause overproduction of reactive oxygen species (ROS) [26,27]. Therefore, it can be deduced that oxidative damage as a result of stress might cause variations in DNA methylation patterns [28]. Cadmium stress does activate superoxide dismutase and catalase and alter the methylation status of genomic DNA. Selenium prevents the change in methylation levels and reduces the amount of ROS. The authors have also suggested that this is one of the causes of the change in methylation status [21]. It is interesting that pathogens do not affect the levels of *NtGPDL* methylation and expression [7], which suggests that DNA methylation is affected differently by biotic and abiotic stresses.

2.4. Salt/water stress and plant genomic DNA methylation

High concentrations of salt cause osmotic pressure and restrict water absorption by plants. Therefore, salt stress is often accompanied by a water deficit. When Crassulacean acid metabolism (CAM) plants such as *Mesembryanthemum crystallinum* encounter a water deficit that is caused by salt stress, they can switch from C_3-photosynthesis to the CAM pathway [29], in which stomata are closed during the daytime but open at night, to reduce water loss and increase resistance to stress. Switchover to the CAM pathway upon salt stress is coupled to the promotion of genomic methylation and hypermethylation of satellite DNA [30]. Further analysis has revealed that the methylation pattern of CCWGG sequences in the nuclear ribosomal DNA and of the promoter region of the phosphoenolpyruvate carboxylase gene, which is the key enzyme for C_4-photosynthesis and CAM, is not changed. Thus, hypermethylation of satellite DNA is probably associated with the formation of a specialized chromatin structure that can regulate simultaneously the expression of a large number of genes, to enable the switchover to CAM upon adaptation to salt stress [30]. The results of this study also show that the promotion of plant genome methylation might be one of the mechanisms that is used for developing tolerance to salt stress. Differences in salt tolerance among cultivars are also related to methylation levels. For example, 10 days after salt stress, the salt-tolerant wheat variety has a higher level of methylation than salt-sensitive wheat [31]. Hypermethylation is also detected in the root tip DNA of pea (*Pisum sativum* L.) under conditions of water deficit. MSAP has revealed an increase in the methylation level of both cytosine residues in CCGG motifs, especially for the inner cytosine [32]. Against a background of overall elevation of methylation level, salt stress can also cause demethylation at specific loci. Guangyuan et al. [33] have performed MSAP in oilseed rape plants that had been subjected to salt stress, and defined three types of bands. Type I bands were observed with both of the isoschizomers MspI (methylation insensitive) and HpaII (methylation sensi-

tive), whereas type II and type III bands were observed only with HpaII and MspI, respectively. Extensive changes in the types of MSAP bands were observed after treatment with 10–200 mmol/l NaCl; these included the appearance and disappearance of all types of band, as well as exchange between band types. An increase of 0.2–17.6% in cytosine methylation at CCGG sites was detected in different plants, which included both *de novo* methylation and demethylation events. They also observed that 10–1000 mmol/l NaCl can induce not only changes in the methylation status, but also mutations in the DNA sequence. These genetic variations were also dose-dependent and showed polymorphism between individuals [33]. Similar phenomena have also been observed in rape that is stressed with potassium dichromate [23], which suggests that genetic variations can be accompanied by epigenetic mutations.

As with the analysis of heavy-metal stress, very limited information is available about the effects of salt stress on the methylation of specific genes. The DNA methylation status of two heterochromatic loci (HRS60 and GRS) was inspected in tobacco suspension tissue culture cells that had been exposed to osmotic stress with NaCl and D-mannitol. Digestion of the DNA with the isoschizomers MboI and *Sau*3AI, which are differentially sensitive to methylation, in combination with Southern hybridization revealed a reversible hypermethylation of the external cytosine at CCG trinucleotides. However, there was no change in the methylation of the cytosine of CG dinucleotides, as revealed by digestion with MspI and HpaII [34], which may be because the loci that were examined are located in a heterochromatic region and, therefore, have a high background level of methylation.

2.5. Other stresses and plant genomic DNA methylation

As breeders of plants, we are highly concerned with the effects of DNA methylation on important agricultural traits such as yield. However, yield is often controlled by multiple genes. It is difficult to study even classical genetic effects on yield-related genes. Therefore, we cannot expect a direct study on the methylation of multiple yield-related genes. The yield of hybrid maize in the state of Iowa in the United States has remained almost the same since 1940, at a low planting density, but has increased continuously at a high planting density [35]. Tani et al. [36] have revealed that at a high planting density, the methylation level was lower in hybrid lines with a high yield than in those with a low yield. In addition, the density-tolerant varieties express more genes than the sensitive ones [37]. These observations tempt us to deduce that some maize hybrids have a lower level of methylation at a high planting density, which results in higher levels of gene expression and more active metabolism and, in turn, produces a higher yield.

One or two Cm^5CGG sites in the tobacco genome are demethylated upon viral infection, but to a much lower extent than that observed upon mechanical stress [6]. Rubbing young internodes of *Bryonia dioica* can cause a rapid and transient decrease in DNA cytosine methylation, from approximately 25% to nearly 0% in less than 1 h [38]. The results of this study clearly demonstrate a rapid and flexible epigenetic response of plant genomic DNA to stress, which contrasts sharply with the conservation of the DNA sequence itself. This flexibility in combination with the conservation of the genetic information embodies the perfection of DNA as genetic material. Changes in ethylene metabolism and DNA methylation occur simultaneously under rubbing stress. These changes may influence a wide range of developmental processes and fitness responses, such as seed germination, leaf and flower senescence, fruit ripening, programmed cell death, and biotic and abiotic stress responses [39]. Polyploidization in *Arabidopsis* can also result in changes in the DNA methylation status [40] and suppress the expression of ethylene-related genes [41], which implies that the stresses of both endogenous genome redundancy and external mechanical pressure have a similar effect.

Rounds of subculture, which involve deviation from normal growth environments, can also be regarded as a stress. In barley (*Hordeum brevisubulatum* (Trin.) Link) [42] and *Codonopsis lanceolata* [43], subculture can induce not only variations in the type and level of methylation, but also population genetic diversity. Although subculture does not change the genetic structure of hop (*Humulus lupulus* L.), genomic demethylation is detected in all generations. Cluster analysis has revealed further that the genetic distance from the control is greater for seedlings that have been subcultured for a greater number of generations [44]. However, it is noteworthy that the correlation between MSAP and inter-simple sequence repeat (ISSR)/randomly amplified polymorphic DNA (RAPD) marker polymorphisms is significant in *Codonopsis lanceolata* seedlings that have been subcultured [43], but not in barley [42]. In rice [45], *Arabidopsis* [46] and *Brassica oleracea* [47], MSAP polymorphism cannot reflect fully the genetic relationships, geographic distributions or phenotypic variations. Therefore, the genetic and epigenetic distances should be interpreted differentially.

3. Features and implications of methylation variations under stress

Biotic stresses such as pathogen infection generally increase the overall level of genomic methylation. Against this background, the methylation levels of pathogen response or resistance genes are reduced. The increase in genomic DNA methylation may repress expression of the transcriptome. We deduce that this slows down the metabolism of the plant, which enables it to conserve energy for the sake of pathogen resistance, and helps the plant to overcome the temporary challenge, by a mechanism that is similar to dormancy. In contrast, the decreased methylation of resistance-related genes favors chromatin activation and the evolution of novel resistance genes, which results in long-term or permanent resistance to pathogens. In this

sense, epigenetic mechanisms such as DNA methylation not only have a short-term effect on plant survival in response to stress, but also change the structure of chromatin and influence gene evolution, and therefore enable the combination of genetics and epigenetics under conditions of stress.

The DNA methylation responses of plants to abiotic stress depend on the type of stress. Most of the existing data support the conclusion that cold stress induces genomic demethylation. This finding has been documented extensively in many plant species such as rape, corn, wheat, Arabidopsis, and *Antirrhinum majus*. Other abiotic stresses such as plant density, rubbing, wounding, and subculture also generally decrease methylation levels on the genomic scale. In contrast, a high level of methylation is observed in the satellite sequences of *Mesembryanthemum crystallinum*, salt-tolerant wheat varieties, pea root tips, and heterochromatin loci (HRS60 and GRS) under abiotic stress. The DNA methylation responses of plants to heavy-metal stress are much more complex. Hypermethylation of rape and radish genomic DNA occurs under cadmium or chromium stress. However, cadmium, chromium, and nickel can also decrease the methylation levels of clover and hemp. Therefore, even when stressed with the same metal, for example chromium, different plant species may have totally different methylation responses, which suggests that complex interactions occur between heavy-metal stresses and plant species. Therefore, these data should be interpreted carefully. Completely opposite results may be obtained with different metal stresses or plant species.

4. Potential breeding applications of modification of DNA methylation in response to stress

4.1. Polymorphism and inheritability of DNA methylation make breeding applications possible

Plant cells can sense environmental variations or stresses, which induce epigenetic variations such as the modification of methylation patterns. Germ cells are derived from somatic tissues at a late developmental stage. Therefore, epigenetic variations, such as changes in methylation pattern, that are caused by environmental changes or stress can be transferred to subsequent generations [2,48]. This is important for the understanding of phenotypic variations in nature and biological adaption to stress [49] and also suggests that variations in methylation are of potential value in breeding. This potential value is embodied directly by the influence of variations in DNA methylation on important agricultural traits such as flowering time and plant height [50,51], pathogen resistance [9,10], and yield [36]. Methylation polymorphisms have been observed in different varieties, biotypes or intra-species of rice [45], *Arabidopsis* [46], *Brassica oleracea* [47] and cotton (*Gossypium hirsutum* L.) [52]. This diversity of methylation may lead to phenotypic variations [53,54], which provide the raw materials for plant breeding. However, breeding selection can be effective only when the methylated alleles are heritable. DNA from different batches of seeds shows considerable variation in the extent of total and strand-specific methylation. This variation is thought to be induced during seed formation, perhaps as a result of different environmental conditions. Seeds that are produced in a more stressful greenhouse give rise to seedlings that have enhanced strand-specific methylation. Such patterns are likely to be established during the early stages of development and are heritable over several days of seedling growth [55]. The recent data on tobacco infected with TMV [10], rice infected with blight pathogen [9], and MSAP polymorphism among *Brassica oleracea* populations [47] have substantiated further the inheritability of methylation variations that are derived from environmental pressures. The breeding of crops such as rice and corn aims mainly to exploit heterosis, which emphasizes the importance of inherited patterns of methylation diversity in F_1 hybrids. Compared to their parents, rice hybrids have reduced levels of methylation at some loci but increased levels at others [56]. Although most of the methylated loci in corn can be inherited by its hybrid progeny, 6.59–11.92% of loci in the hybrids are different from those of their parents, and these loci represent many types of functional proteins or ESTs. These variations can be directed or stochastic [57], which suggests that the inheritance of methylated loci is complex and that methylation variations have a potential application in plant hybrid breeding.

4.2. Applications and potential value of different methylation variations in breeding

Type I: stably inherited methylated alleles. In contrast to animals, some methylated loci in plant species can be inherited for several generations [58,59]. For example, treatment with 5-azacytidine decreases the level of methylation in flax (*Linum usitatissimum*) and induces the traits of dwarfism and early maturity [51]. The F_2 and F_3 progenies that are derived from crosses between wild type and mutant plants that are selected for the phenotype of early maturity also have a lower level of methylation [50]. This suggests that the hypomethylation that is induced by 5-azacytidine can be inherited for at least three generations. Resistance to the blight pathogen, which arises from methylation variation in rice, can be inherited for at least nine generations [9]. This is suitable for the commercial development of new rice varieties because their market vitality is generally less than 5 years. Some methylation variations can even be inherited for hundreds of years, and thus exert an influence on evolution. A toadflax (*Linaria vulgaris*) mutant in which the fundamental symmetry of the flower has been changed was identified 250 years ago by Linnaeus. A gene that is involved in flower development, *Lcyc*, has been found to be heavily methylated and silenced in this mutant [53]. These stably inherited modifications of DNA methylation are similar to DNA sequence mutations, which implies that

they show similar genetic behavior to that defined by classical Mendelian genetics [60]. For example, classical hybridization and segregation procedures proved that the phenotype of early maturity in flax is controlled by three methylated alleles, two of which are recessive and one dominant or codominant [61]. The similarity to classical genetics means that the classical breeding procedures of hybridization, selection and purification can also be applied to the development of new plant varieties with novel methylated alleles.

Type II: methylation variations that are reversed upon the removal of stress. During eukaryotic life cycles [24] and the various stages of cancer development [62], methylation patterns are dynamic and reversible, which is sometimes also the case in plants [63]. Between individual clover and flax plants, the distribution patterns of methylated loci are generally similar before and after metal stress [20], which suggests that variations in methylation pattern may occur with stress and then disappear after the stress has been removed. This situation was also documented in *Bryonia dioica* and *Antirrhinum majus*. The decreased level of methylation that occurs upon exposure of *Bryonia dioica* to rubbing stress is restored to the normal level after removal of the stress [38]. Even within the same generation in *Antirrhinum majus*, methylation variations that are induced by low temperature are reversed upon return to a normal environmental temperature [15]. Lower levels of methylation generally contribute to higher levels of gene expression. If a plant cannot recover its methylation level after removal of the stress, gene over-expression and energy waste are unavoidable. Just as the resistance response to pathogens only occurs under pathogen attack, we deduce that variations in methylation that are reversed after the removal of the stress may be more common in nature, because they enable the more rational utilization of biological energy. This highlights the potential importance of this type of methylated allele in breeding. We can analyze this type of methylation in the same way as pathogen resistance. Stress is first created by natural or artificial means, and then the resulting methylation modifications in the plant genome are identified. The methylation pattern must be analyzed at several time points, and the amount of methylation should also be taken into account. As for disease resistance responses, a numerical system can be employed to represent the level of methylation variation. The methylation variations that are shown to be induced reversibly in response to stress by several rounds of multi-drop experiments can be identified with the assistance of molecular markers. If the loci are closely related to stress resistance, classical hybrid breeding procedures can be employed to develop new varieties.

Type III: stochastic methylation variations. For example, a few methylation changes that arose at random during their transmission to the next generation were discovered [57]. It is difficult to research this type of methylated allele. They may also not be important for breeding.

5. Challenges for the exploitation of stress-induced methylation variations in breeding applications

Modification of plant genomic methylation can respond rapidly to stress. For example, rubbing stress can decrease the level of methylation to zero within 1 h [38]. However, some other variations in methylation can only be observed after relatively longer periods of stress, for example, 12 h for wounding and chilling stresses [6]. On the other hand, the variation in methylation is dynamic [63]. The flexibility and dynamic features of DNA methylation modifications require us to accurately determine the time points at which observations are made. Contrary conclusions may result from errors of just a few hours. Observation at multiple time points and repeated analysis under several rounds of stress are both needed to determine whether the variations in methylation are reversed after removal of the stress. For example, the lower level of methylation and early maturity phenotype that are induced by treatment of flax with 5-azacytidine are reversed slightly in the first generation and totally in the fourth generation of one line [61]. Quantitative differences in methylation variation under stress can also be detected. For example, the degree of the decrease in DNA methylation, between 20% and 40%, in stressed clover and hemp obviously depends on the concentration of metal ions to which they are exposed [20]. A similar phenomenon was also observed in *Stellaria longipes* that was exposed to red/far red light [64]. Quantitative analysis is much more complex than qualitative analysis. The main techniques of bisulfite sequencing and Southern blotting are tedious and expensive procedures at present, which makes their application to breeding practice difficult.

The use of methylated alleles in the area of breeding is still uncommon. All the existing studies have involved treatment with 5-azacytidine to induce a decrease in plant methylation levels and produce novel phenotypes that are explored in subsequent breeding studies. Typical examples are the traits of early maturity and dwarfism in flax [50,51] and resistance to blight pathogen in rice [9]. We think that there are several limitations of 5-azacytidine-induced methylation variation in breeding. First, similar to breeding with radiation-induced mutations, although the changes in methylation may not be stochastic [50], the process does not allow the variation to be directed. Secondly, the whole genome is affected. Therefore, favorable phenotypes are inevitably linked to abundant unfavorable ones. Most plants cannot even survive the treatment [9], which greatly weakens its value in breeding practice. For example, the early maturity that is induced by 5-azacytidine has multiple effects [65] and is accompanied by dwarfism [51]. For such variations in methylation pattern, a strategy that is similar to molecular marker-assisted breeding in classical genetics can be developed to allow breeding selection with a clear target. Therefore, classical genetics and breeding techniques should be reviewed in the search for strategies that can be adapted to these purposes.

Classical genetics has progressed from DNA sequencing to the post-genomicera, in which the biological functions of genes are of great interest. Molecular linkage maps have been constructed for important crops such as rice, corn and wheat and provide a platform for molecular-assisted selection and the analysis of gene function. These linkage maps can be used in gene mapping and cloning, which allows association of the DNA sequence, gene function and phenotype. The construction of genomic methylation maps is equivalent to the process of DNA sequencing in classical genetics. However, this work is just at the initial stages. The only example that is available at present is the *Arabidopsis* high-resolution methylation map that was constructed by Zhang et al. [25] and Cokus et al. [66]. No similar map has been reported for other plant species, including crops. One reason for this situation is that too much of our attention is focused on the DNA sequence, which results in ignorance of the important influence of DNA methylation on genetics and evolution. The other reason is the lack of suitable methods for genomic methylation mapping. We turned first to bisulfite sequencing. However, it is too time- and labor-consuming to be applied to a large-scale genomic analysis. In 2006, Zhang et al. first combined chromatin immunoprecipitation with a tiling microarray technique to detect the distribution of methylated cytosines in the *Arabidopsis* genome. They achieved a resolution as high as 35 bp [25]. However, commercial tiling microarrays are not available for most plant species, including crops, which greatly limits the potential applications of this technique. To address this situation and the limitations of microarrays, Peng et al. [67] have proposed to substitute chip hybridization with Solexa sequencing to construct genomic methylation maps with a high resolution. This design, which involves high-throughput sequencing, theoretically improves the precision and detail of the constructed map [68]. Compared to the use of microarrays, the main advantages of Solexa sequencing are the digital signal, the lack of interference from hybridization, and the absence of a requirement for PCR amplification [69].

Even before the completion of genome sequencing, molecular marker-assisted selection was applied extensively in breeding practice. Theoretically, it is possible that it can also be used for the selection of methylated alleles. However, difficulties still remain in breeding practice. At present, the most frequently used type of molecular marker for methylated alleles is the MSAP marker, which is derived from the amplified fragment length polymorphism (AFLP) marker. The main difference between them is that, for MSAP, restriction enzymes are used that are differentially sensitive to methylation. There are several limitations to the use of MSAP markers in assisted selection. First, a single enzyme can only detect one type of methylation in a particular sequence context. However, different methylation modifications may arise from different stresses. For example, salt stress induces hypermethylation in CCWGG sequences in *Mesembryanthemum crystallinum* but leaves CCGG sequences untouched [30]. Secondly, the AFLP procedure is tedious and complex and is thus only suitable for theoretical research, which suggests a gloomy prospect for the application of MSAP in breeding practice. Thirdly, it is difficult to quantitate the variation in methylation using the MSAP technique. Here, we propose an alternative method. Methylated and unmethylated DNA is fractionated into two pools by methylcytosine immunoprecipitation (mCIP). Primers are designed that will allow amplification of the target gene and are used to amplify the target gene in the two pools. The ratio of the amount of PCR product that is obtained from each pool represents the methylation level of the target gene. For qualitative determination, the detection procedure of this design is similar to that of microsatellite (SSR: simple sequence repeat) markers in classical genetics. For quantitative analysis, it is similar to RT-PCR (reverse-transcription PCR). Compared to MSAP, the merits of this design are the production of a single band, the ability to analyze any locus, and straightforward detection by agarose gel electrophoresis. Only one round of mCIP is needed to allow the detection of multiple genes in a single sample. However, several rounds of mCIP are needed for the detection of a single gene in multiple samples, which results in a high cost at present.

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