REVIEW

Genomic methylation in plant cell cultures: A barrier to the development of commercial long-term biofactories

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Plant cell biofactories offer great advantages for the production of plant compounds of interest, although certain limitations still need to be overcome before their maximum potential is reached. One obstacle is the gradual loss of secondary metabolite production during in vitro culture maintenance, which is an important impediment in the development of large-scale production systems. The relationship between in vitro maintenance and epigenetic changes has been demonstrated in several plant species; in particular, methylation levels have been found to increase in in vitro cultures over time. Higher DNA methylation levels have been correlated with a low yield of secondary metabolites in in vitro plant cell cultures. The longer the period of subculturing, the more methylated cytosines were found throughout the genome, and secondary metabolism decreased significantly. This review summarizes different studies on epigenetic changes during the maintenance of in vitro cell cultures and the insights they provide on the mechanisms involved. It concludes by looking at the perspectives for new approaches designed to avoid declines in metabolite production.

KEYWORDS

cell cultures, epigenetic, methylation, natural products, plant biofactories

1 | INTRODUCTION

Plants produce a wide range of secondary metabolites to protect themselves from pathogens, predators, and environmental stresses. These compounds have also been used by humankind for thousands of years in multiple applications. To date, more than 200 000 natural products derived from plant secondary metabolism have been characterized [1]. They are used extensively in the food industry as dietary supplements or additives (for example, ginseng from *Panax ginseng* or anthocyanins from *Euphorbia milii* and *Aralia cordata*) [2], in the pharmaceutical industry as drugs (for example, the anticancer

agents paclitaxel from *Taxus* spp. and podophyllotoxins from *Podophyllum* spp.) [3,4], and in the cosmetic industry as cosmetics or fragrances (for example, centellosides from *Centella asiatica* and essential oils from *Lavandula* spp.) [5,6]. Research on secondary metabolites continues to look for novel valuable compounds from the Earth's vast plant biodiversity.

Interest in natural products is growing, whether as a source of new drugs for the pharmaceutical industry, or as alternatives to synthetic food additives. However, the low concentrations of secondary metabolites in their natural source, and the fact that the over-harvesting of wild plants has left several species endangered are impediments to meeting this increasing demand [7]. There is, therefore, a need to develop new production systems that guarantee high production rates. For simple chemical structures, total synthesis is an attractive approach, although the overall complexity of natural products often rules this out as a viable procedure [8]. Semi-synthesis

Abbreviations: AzaC, 5-azacytidine; CMT3, chromomethylase 3 protein; DME, DEMETER DNA demethylase; DRM, domains rearranged methylase protein; MET1, methyltransferase 1 protein; siRNAs, small interfering RNAs.

can be a good alternative, using available intermediate metabolites to achieve the target product. However, it remains a costly procedure, often generates toxic derivative products, and can be detrimental to the environment. Furthermore, the intermediates have to be extracted from their natural source, which can lead to the reduction of land available for food crops and the destruction of endangered plant species.

An alternative system for the production of secondary metabolites that avoids these drawbacks is the use of in vitro plant cell cultures. Their advantages include: (i) controlled production conditions that ensure the quality of the target products; (ii) a guaranteed absence of contamination, pesticides and herbicides; (iii) independence of climatic factors; (iv) minimal ecological impact because of lower water requirements and no pressure on endangered plant species, and (v) faster growth cycles [9].

Plant cell cultures, based on environmentally friendly and economical procedures, can be employed as biofactories to produce a great diversity of compounds. To establish a plant biofactory, an in vitro culture of the species of interest is first established, and when callus tissue is obtained, a suspension cell culture is generated by switching cells to a liquid medium. In order for the biofactory to be industrially viable, the process needs to be optimized, which involves the selection of a highly productive cell line, the optimization of the growth and production conditions, the addition of precursors, permeabilizing agents or elicitors, and finally, the scaling up of the process to large-volume bioreactors [10]. Examples of companies that are using plant cell or organ cultures to obtain natural products on an industrial level include Mitsui Chemicals Inc. (arbutin, berberine and shikonin), Phyton Biotech (paclitaxel in *Taxus* spp.), Nitto Denko Corporation, and CBN Biotech Company (ginseng) [11].

Several reviews highlight the suitability of in vitro plant cell cultures for the production of secondary metabolite targets, but some limitations have also been revealed. One of the most relevant is the instability of selected highly productive cell lines during maintenance in in vitro conditions. This instability, which has been reported in only a few species, such as the indole alkaloid producer *Catharanthus roseus* or the diterpene producer *Taxus* spp., shows a gradual loss of secondary metabolite production when cell cultures are maintained in in vitro conditions [12,13]. This loss of production over time is a significant drawback for the development of large-scale and commercial systems to obtain products of interest.

This review summarizes reports of production loss during in vitro culture maintenance and its correlation with epigenetic modifications. Epigenetic mechanisms are known to be related to environmental adaptations and therefore might also be closely involved in the changes taking place in in vitro cell cultures, as indicated by recent studies. Finally, some perspectives for the development of approaches designed to avoid yield loss are discussed.

2 | YIELD LOSS DURING LONG-TERM IN VITRO MAINTENANCE

As mentioned previously, a common obstacle in the use of plant cell cultures as biofactories is maintaining the productivity of the selected cell lines [12]. The instability generated by in vitro conditions is reflected in a gradual loss of secondary metabolism and thus of the target product yield. These inconsistent patterns of production hinder the design of profitable large-scale production systems.

In two of the few studies to focus on this problem, in vitro callus and cell cultures of *Nicotiana rustica* and *N. tabacum* were established in flask conditions to produce secondary metabolites [14,15]. Genetically identical cell lines showed different phenotypes, including variable secondary metabolite production capacities. The yields of selected optimal cultures became unstable, apparently due to changes associated with in vitro maintenance. In a study of *C. roseus*, the capacity of several cell lines to produce indole alkaloids was monitored for 8 years. As in the previous studies, the yield of initially highly productive lines declined significantly over time. After the assay, a clonal selection of cell colonies showed that some cells in the low productive culture still had a highly productive phenotype [13] and thereafter, a heterogeneous culture with cells with different productive capacities was observed.

A study of cultures with variable production capacities revealed no mutations or chromosomal aberrations in the cell lines that had become low-yielding after several subcultures [16]. Based on these results, the loss of production during in vitro maintenance was not associated with genetic factors, and was found to be reversible, opening the possibility of developing strategies to avoid it.

Years later, with the emergence of new insights into the role of epigenetic mechanisms, a correlation was observed between the low-yielding phenotypes arising from long-term in vitro maintenance and epigenetic changes, especially in methylation levels [17]. The longer the in vitro cultures had been subcultured, the greater the number of methylated cytosines was found throughout the genome, accompanied by a significant decrease in secondary metabolism [18].

2.1 | DNA methylation mechanisms and their regulation

In plants, DNA methylation is a more complex and sophisticated system than in other organisms, and data indicate that it plays a key role in diverse plant physiological processes such as transcription regulation, vernalization, or long-term adaptation to the environment. Furthermore, it is essential for the suppression of transgenes, repeated endogenous sequences, viral sequences and transposable elements [19].

FIGURE 1 Cytosine methylation mechanisms in plant genomes. (A) De novo. (B) Maintenance. The different cytosine contexts are CG, CHG, and CHH (H: any nucleotide except G). DRM2 corresponds to the methyltransferase involved in de novo methylation, depending on the RdDM pathway in a complex with siRNAs and AGO proteins (mainly AGO4 and AGO6). MET1 and CMT3 are the methyltransferases involved in the maintenance of CG and CHG methylation, respectively. Passive DNA methylation occurs during DNA replication in CHH context, as it does not have maintenance mechanisms [20–24]. DRM2: domains rearranged methylase 2; CMT3: chromomethylase 3; MET1: methyltransferase 1; siRNA: small interfering RNAs; RdDM: RNA-directed DNA methylation; AGO: Argonaute

DNA methylation involves the addition of a methyl group to a cytosine to form 5-methylcytosine. It occurs through two methylation mechanisms: *de novo*, which establishes new patterns of methylation, and maintenance, which conserves methylation patterns in the newly generated strands of DNA during cell division (Figure 1). In contrast with mammals, where DNA methylation is restricted to CG dinucleotides, in plant genomes it can be found in three cytosine contexts: CG, CHG, and CHH (H being any nucleotide except G). Regarding epigenetic mechanisms, three types of methyltransferases involved in the DNA methylation process have been characterized in plants: methyltransferase 1 protein (MET1), chromomethylase 3 protein (CMT3), and domains rearranged methylase protein (DRM). Whereas, MET1 and CMT3 are involved in the maintenance of CG and CHG methylation, respectively, DRM methyltransferases control de novo methylation in all three contexts [20]. Notably, there are no methyltransferases to maintain methylation in CHH contexts, which therefore depends only on the de novo mechanism. The specificity of DRM de novo methylation is generated by an RNA-directed mechanism, highly conserved in plants. In fact, RNA-directed DNA methylation catalyzed by DRM methyltransferases has been defined as the major transcriptional silencing pathway in plants [19]. The extensively described RNA-directed DNA methylation pathway involves the

production and modification of several small interfering RNAs and protein complexes such as Argonaute. Argonaute proteins are responsible for the direct interaction with DRM methyltransferases, catalyzing the context-independent methylation reaction [21,22]. Although the three types of methyltransferases have been identified in *Arabidopsis thaliana*, *Oryza sativa*, *Prunus persica*, *Zea mays*, or *Brassica rapa* among others, the number of enzymes of each type is variable and the biological function of these copies is still unknown [23].

Genomic DNA demethylation in plant cells is regulated by a group of enzymes actively involved in this function, besides the passive demethylation caused by DNA replication during cell division. Active demethylation, an indispensable mechanism for the activation of silent genes, can explain the rapid changes in methylation patterns. In plants, the processes behind active DNA demethylation are well understood. Four 5-methylcytosine glycosylases (repressor of silencing 1, DEMETER DNA demethylase [DME], DEMETER-like protein 2 and DEMETER-like protein 3) prevent the accumulation of DNA methylation through the expression of certain genes, including transposons, and are also required for gene imprinting [24,25]. Both repressor of silencing 1 and DME enzymes have a strong preference for the demethylation of CG cytosines, but also show significant activity in CHG and

CHH contexts. Similarly, the enzymes of the DME family (DEMETER-like protein 2 and DEMETER-like protein 3) are specific DNA glycosylases involved in the establishment of methylation patterns [26,27].

Although DNA methylation in plants plays a vital role in the suppression of transgenes, transposons, and viral sequences, it also regulates the expression of some genes. The accumulation of DNA methylation can affect the binding of both enhancer and repressor elements, and thus the transcription of some genes is activated when methylated cytosines avoid the binding of repressor proteins [28,29]. This epigenetic mechanism is closely related with several physiological processes, such as paternal imprinting, embryo development, tissue differentiation, vernalization, and flowering [30–32]. It is also associated with adaptive responses to the environment, a key factor in the development of plant organisms. Several studies have reported variable patterns in genomic methylation among genetically identical individual plants kept under different stress conditions. Interestingly, these specific patterns, known as epigenetic alleles or epialleles, can remain stable for several generations by inheritance [33–35].

All these modifications have also been observed during *in vitro* culture establishment, being the major source of somaclonal variation between cultures [34]. It is therefore not surprising that cell lines subcultured over long periods of time in optimal conditions can adapt their physiology to long-term cultivation in *in vitro* conditions, similar to the response of complete plants to environmental changes.

2.2 | Methylation accumulation during *in vitro* culturing

Even when it became known that methylation mechanisms affect secondary metabolism in several plant species during the long-term maintenance of *in vitro* cell cultures, there were few clues about the specific target of this methylation or how the secondary metabolism was altered. In a recent study on the paclitaxel-producing specie *Taxus x media*, the correlation between the methylation accumulation during *in vitro* cell culturing and different phenotypical traits was demonstrated [36] (Figure 2).

Paclitaxel, marketed under the brand name Taxol, is a taxane with a complex structure currently being commercially produced by *Taxus* spp. cell cultures, due to the impossibility of developing a viable chemical synthesis. The taxane tricyclic skeleton is constructed in a multi-enzyme biosynthetic pathway in which the substrate geranyl-geranyl diphosphate undergoes several chemical modifications by cytochrome-P450 hydroxylases, acyl transferases, and other enzymes, leading to the intermediate baccatin III. The late steps of the pathway include the binding of the side chain and its hydroxylation and the benzoylation of the taxane skeleton [37]. Although the final product, paclitaxel, is the main

bioactive compound, baccatin III is also of interest as the source material for semi-synthetic procedures. As both compounds are synthesized at very low concentrations, the development of highly productive *Taxus* spp. cell cultures remains an open research field.

Once a highly productive cell line is generated, a method is required to maintain its productivity. In this context, alterations in the production, transcriptome, and methylation rates were compared in a recently induced callus tissue and 10-year old calli. In the old, highly methylated cell line, the flux-limiting steps of the paclitaxel biosynthetic pathway were partially inhibited, which was associated with the declining production of the target compound and an accumulation of intermediate compounds. These results suggest that the recovery of the transcriptional levels of the inhibited biosynthetic steps would enable the accumulated intermediates to be rapidly converted into the products of interest.

In the promoter, the CHH context underwent a significant increase in methylation levels, and furthermore, a specific region accumulated the methylated CHH. *In silico* predictions identified the target of the methylated cytosines as the Y-patch element (Figure 3), which is characterized by an unusual content of pyrimidines (cytosines and thymine residues), although its specific function was not previously known [38]. The identification of the Y-patch element as a methylation target during *in vitro* culturing indicates its involvement in the epigenetic regulation of plant genes. It is possibly a homologue of the CpG islands in mammals, as elements with this regulatory function have not been defined in plant genomes [39]. However, it is relevant that the methylation accumulation in the promoter only involved the CHH context, which depends on the *de novo* methylation mechanism, indicating a greater dynamism than the other two contexts [19]. Thus, our study confirmed the correlation between methylation accumulation, especially in the Y-patch element of the promoter, and the decline in secondary metabolite production during long-term *in vitro* maintenance.

Stressing environmental factors are known to alter methylation patterns [40]. In the *T. x media* cell cultures, adaptation to *in vitro* conditions generated a methylation accumulation in secondary metabolism-related genes, thus highlighting the role of epigenetic mechanisms in secondary metabolite production. The evidence for the involvement of the *de novo* methylation mechanism in the decline of *in vitro* productivity could contribute to the development of new strategies that avoid the adaptation of cell cultures to long-term maintenance.

The same decline in secondary metabolism has been observed in transgenic cell lines in which the expression of the transgene is silenced over time such as *Brassica oleracea*, *Betula platyphylla*, *Nicotiana tabacum*, or *Oryza sativa* [41–43]. The mechanism responsible is similar

FIGURE 2 Effect of aging on the callus and cell suspension morphology of *T. x media*. (A) Callus tissue; (B) Cell culture; (C) Cell morphology; (D) Total taxane production in young and old cell lines under control or elicited conditions. Samples were harvested at 8, 16 and 24 days. YC: Young cell line under control conditions, YE: Young cell line under elicited conditions, OC: Old cell line under control conditions, OE: Old cell line under elicited conditions. Modified from ref. 36

FIGURE 3 General structure of plant promoters. The main elements of plant core promoters involved in gene expression regulation are shown: CAAT-box, TATA-box, TSS (transcription start site), INR (initiation region), DPEs (downstream promoter elements) and Y-patch [33]

to that described above and restoring treatments have been developed. Transgenic cell lines of *Vitis amurensis* overproducing resveratrol suffered production decay after 2.5 years of cultivation and the expression of the transgene rolB decreased [17]. When this cell line was cultured with the demethylation agent 5-azacytidine (AzaC), the transgene transcription rate was restored without affecting the biomass growth, and the initial concentrations of accumulated resveratrol were almost recovered. This result, linked with the evidence obtained in *T. x media* cell cultures, highlights the potential of demethylating agents such as AzaC to avoid methylation accumulation during in vitro cell culture maintenance.

The treatment of the *V. amurensis* transgenic cell line with AzaC also allowed the role of several DNA methyltransferases in transgene silencing to be studied. A general increase in expression levels of the MET, CMT, and above all the DRM genes after the treatment was possibly due to the necessity of restoring the methylation patterns in order to stabilize the genome. On the other hand, the significantly higher levels of DRM compared to the other two methyltransferases points to its implication in the silencing of transgenes in modified plant genomes.

Interest in the secondary metabolite *trans*-resveratrol has motivated the development of novel strategies to boost its production, including the use of transgenic cell lines. This polyphenol plays a key role in the pathogenic resistance of the grapevine and has several interesting pharmacological properties. It is reported to contribute to the prevention and slowdown of several widespread pathologies such as obesity, cardiovascular diseases, and cancer [44–46]. *Trans*-resveratrol derives from the general phenylpropanoid pathway and is synthesized by the condensation of three malonyl-CoA molecules with one molecule of coumaryl-CoA catalyzed by the enzyme stilbene synthase (STS). Several glycosylated *trans*-resveratrol derivatives, including piceatannol and pterostilbene, are synthesized at lower concentrations but exhibit higher oral availability than *trans*-resveratrol, prompting the development of bio-conversion strategies [47]. Nevertheless, the production of the source material *trans*-resveratrol remains vital.

3 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The high interest of natural products for the cosmetics, pharmaceutical, and food industries has generated a growing demand for these plant-derived compounds. Plant cell cultures are being successfully used as green biofactories by several companies, replacing traditional sources of target compounds. Even so, the optimization and scale-up of plant cell cultures remain an open research field. A particular

limitation in their use as biofactories is the instability of production. The loss of yields during the maintenance of originally highly productive cell lines constitutes an important barrier in their commercial application.

Epigenetic modifications contribute to the regulation of gene expression during plant development in response to environmental stresses and to the silencing of transposons, transgenes, or viral sequences. Although epigenetic modifications have been related to in vitro cultures somaclonal variations, the specific mechanisms involved are still unknown [34]. The involvement of the genes MET1, CMT3, and DRM2 has been demonstrated in several in vitro processes, their expression correlating with the methylation levels of the samples studied [48]. However, the exact relationship between methylation and the decline in secondary metabolism has been scarcely reported. The alteration of methylation patterns seems to be the main mechanism involved in this loss of production over time, which is the principle limitation in the development of commercially viable plant biofactories. Similar to the alteration of DNA methylation in complete plants in response to environmental changes, long-term in vitro cultivation seems to specifically affect secondary and not primary metabolism, transforming what were highly productive cell lines into low producers of secondary metabolites.

Studies in *Taxus* spp. cell lines have revealed the implication of de novo methylation mechanisms, identifying them as a target for corrective strategies. Among the three possible methylation contexts, CHH was one mainly accumulating methylation during in vitro maintenance. In genetically modified lines of *Vitis amurensis*, the initial transcription levels of the methylation-silenced transgene were recovered by treatment with the demethylation agent 5-azacytidine. Not only was the reversibility of the silencing process demonstrated, but the DRM enzymes, responsible for the de novo methylation mechanisms, were found to be involved.

Taking in consideration the results of both studies, the de novo methylation process involving the DRM enzymes may be responsible for the decline in secondary metabolism during in vitro culture maintenance. Demethylation agents show promising strategy to reverse this decline and allow the recovery of the original production ratios. Notably, the use of AzaC in *V. amurensis* did not cause a loss of biomass, indicating its suitability for use in long-term biofactories to avoid methylation accumulation. Alternatively, the generation of genetically modified cell lines specifically blocking the DRM pathway could provide resistance to silencing and ensure stable highly productive bioreactor cultures.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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