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REVIEW PAPER

An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond

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Abstract

Epigenetic mechanisms are highly dynamic events that modulate gene expression. As more accurate and powerful tools for epigenetic analysis become available for application in a broader range of plant species, analysis of the epigenetic landscape of plant cell cultures may turn out to be crucial for understanding variant phenotypes. *In vitro* plant cell and tissue culture methodologies are important for many ongoing plant propagation and breeding programmes as well as for cutting-edge research in several plant model species. Although it has long been known that *in vitro* conditions induce variation at several levels, most studies using such conditions rely on the assumption that *in vitro* cultured plant cells/tissues mostly conform genotypically and phenotypically. However, when large-scale clonal propagation is the aim, there has been a concern in confirming true-to-typeness using molecular markers for evaluating stability. While in most reports genetic variation has been found to occur at relatively modest frequencies, variation in DNA methylation patterns seems to be much more frequent and in some cases it has been directly implicated in phenotypic variation. Recent advances in the field of epigenetics have uncovered highly dynamic mechanisms of chromatin remodelling occurring during cell dedifferentiation and differentiation processes on which *in vitro* adventitious plant regeneration systems are based. Here, an overview of recent findings related to developmental switches occurring during *in vitro* culture is presented. Additionally, an update on the detection of epigenetic variation in plant cell cultures will be provided and discussed in the light of recent progress in the plant epigenetics field.

Key words: Cell fate, chromatin, dedifferentiation, DNA methylation, organogenesis, somaclonal variation.

Introduction

Although the ability to regenerate whole plants from cells, tissues, or organs cultured *in vitro* has been long known, the question of how a somatic cell can differentiate into a whole plant has been considered as one of the big questions facing science over the next quarter-century (Kennedy and Norman, 2005; Vogel, 2005), and also one of the major features distinguishing plant from mammalian cells. Very early in the history of plant tissue culture it was observed that clonally propagated plants often exhibited some level of variation, termed somaclonal variation (Larkin and Scowcroft, 1981), which has been defined as a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones, namely plants derived from any form of cell culture involving the use of somatic plant cells (Schaffer, 1990).

How a single plant genotype can result in a variety of phenotypic outcomes under the same *in vitro* culture conditions is still far from being completely understood. While underlying mutations in DNA sequence have been pointed out as a likely cause of phenotypic variation, it is also becoming clear that a major role is played by epigenetic regulation. In this review the broad definition of epigenetic events proposed by Bird (2007) as the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states will be considered.

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A growing number of studies are focusing on the investigation of epigenetic variation to evaluate stability of *in vitro* grown plants, revealing unexpectedly high frequencies of epigenetic variation. Furthermore, cultures are being successfully used as experimental model systems for uncovering dynamic epigenetic mechanisms acting during cell dedifferentiation and subsequent developmental reprogramming (Williams *et al.*, 2003; Avivi *et al.*, 2004; Koukalova *et al.*, 2005; Berdasco *et al.*, 2008). Epigenetic variation *in vitro* reflects the adaptation process of cells to a different environment which includes the response to signals that may trigger switches in the developmental programme. However, potentially undesired effects of such adaptive adjustments may compromise the objectives for which the plant cells or tissues were cultured. Therefore, a deeper knowledge of the epigenetic events likely to occur *in vitro* as well as the potential consequences in the longer term is required.

Chromatin structure is critically affected by the interplay among epigenetic mechanisms such as DNA methylation, histone modifications, and RNA interference (RNAi) (Henderson and Jacobsen, 2007; Huettel *et al.*, 2007) (Fig. 1). Regulation of these mechanisms influences gene expression by modifying the access to the underlying genetic information, ultimately affecting phenotypes. It has been shown that variation in chromatin states is highly abundant in experimental and natural populations (Riddle and Richards, 2005; Zhang *et al.*, 2008) and represents an additional source of phenotypic variation (Peaston and Whitelaw, 2006; Henderson and Jacobsen, 2007).

DNA methylation is perhaps one of the best described epigenetic mechanisms known to play a role in genomic imprinting, X-chromosome inactivation, silencing of transposons and other repetitive DNA sequences, as well as in the expression of endogenous genes. DNA methylation can be categorized into three types according to the sequence context of the cytosines, namely CG, CHG, and CHH (H=A, C, or T) (Fig. 1). While CG methylation predominates in animals, DNA methylation in plants has been found in all three cytosine contexts, with CG sites

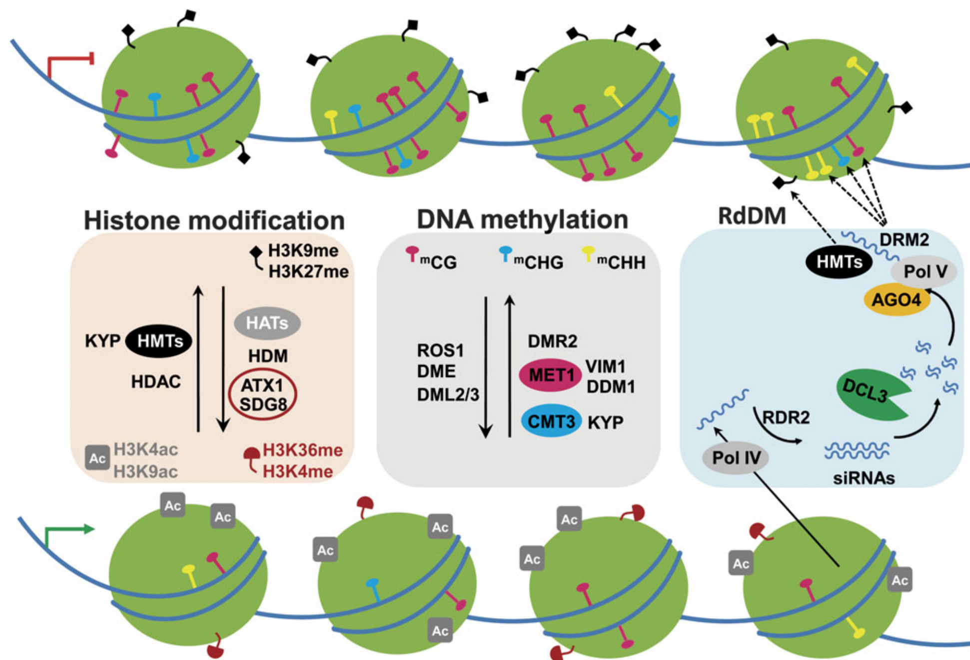

Fig. 1. Schematic illustration of changes in chromatin structure via DNA methylation, histone modification, and small RNA-directed DNA methylation (RdDM). Repression of gene transcription is associated with methylation of DNA by maintenance and *de novo* DNA methyltransferases. CG methylation (^{m}CG) is maintained by MET1 but it is also controlled by VIM1 and the chromatin remodeller DDM1. CHG methylation (^{m}CHG) is maintained by CMT3 and the HMT KYP, and at some loci is redundantly controlled by CMT3 and DRM2. CHH methylation (^{m}CHH) is maintained by the RdDM pathway, and *de novo* methylation of DNA in all of these sequence contexts is generally established by DRM2. In addition, the chromatin repressed state is associated with histone methylation at H3K9 and H3K27 by HMTs. Upon acetylation at H3K4 and H3K9 by HATs and methylation at H3K4 and H3K36 by HMTs (ATX1 and SDG8), as well as DNA demethylation by the glycosylase/lyase proteins ROS1, DME, DML2, and DML3, chromatin is modified and gene transcription may be activated. Transposable elements are kept silent by the RdDM pathway involving the generation of transcripts by PolIV that are converted into dsRNAs by RDR2 and cleaved by DCL3 into 24 nucleotide long siRNAs. Antisense siRNAs are loaded onto AGO4, which guide PolV to homologous DNA, which is then methylated in all cytosine sequence contexts by *de novo* DNA methyltransferases (DRM2). AGO4, ARGONAUTE 4; ATX1, ARABIDOPSIS TRITHORAX-LIKE PROTEIN 1; CMT3, CHROMOMETHYLASE 3; DCL3, DICER-LIKE 3; DDM1, DECREASE IN DNA METHYLATION 1; DME, DEMETER; DML2/3, DEMETER-LIKE 2/3; DRM2, DOMAINS REARRANGED METHYLTRANSFERASE 2; HAT, histone acyltransferase; HDAC, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase; KYP, KRYPTONITE; MET1, DNA METHYLTRANSFERASE 1; RDR2, RNA-DEPENDENT RNA POLYMERASE 2/3; ROS1, REPRESSOR OF SILENCING 1; SDG8, SET DOMAIN GROUP 8; VIM1, VARIANT IN METHYLATION 1.

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methylated at the highest level, CHG sites at a medium level, and CHH sites at the lowest level (Feng *et al.*, 2010*a*).

Both in monocots (rice) and in dicots (*Arabidopsis* and poplar), CG methylation, but not CHG or CHH methylation, exhibits a peak in the body of protein-coding genes (Tran *et al.*, 2005; Zhang *et al.*, 2006; Cokus *et al.*, 2008; Feng *et al.*, 2010*a*). Exons tend to be more highly methylated than introns, and the end of the gene shows a similar drop in methylation to the gene's promoter region (Jeltsch, 2010). Gene body methylation seems to be an ancient property of eukaryotic genomes (Tran *et al.*, 2005; Feng *et al.*, 2010*a*; Zemach *et al.*, 2010) and it might be involved in the prevention of transcriptional initiation within the gene body.

Plants, as well as other highly developed multicellular organisms, show increased DNA methylation when compared with other eukaryotic organisms, probably due to the need for more efficient control of transposons, or the need for additional epigenetic regulation to control the development of many different cell types. In humans it is well demonstrated that DNA methylation patterns vary with cell type and developmental stage (Meissner *et al.*, 2008; Hodges *et al.*, 2009) and among individuals (Zhang *et al.*, 2009; Maegawa *et al.*, 2010). However, increased methylation may pose additional mutagenic risks since 5-methylcytosine (5mC) deamination is repaired less efficiently than deamination of unmethylated cytosine (Jeltsch, 2010). In plants, genome-wide DNA methylation reprogramming occurs in non-germline reproductive cells, which may function to reinforce silencing of transposable elements in germ cells (for a review, see Feng *et al.*, 2010*b*), but, unlike animals, plants are not known to undergo genome-wide waves of demethylation in germ cells. However, reprogramming of the DNA-packaging histone proteins takes place in the zygote.

The N-terminal tails of core histone proteins can be covalently modified by acetylation, methylation, phosphorylation, sumoylation, carbonylation, and glycation (Kouzarides, 2007). The combinatorial set of modifications (histone code) plays an essential role in regulating dynamic changes in chromatin structure, ultimately influencing gene transcription (Berger, 2007) in response to diverse exogenous and endogenous stimuli including stress, pathogen attack, temperature, light, and hormones (Chen and Tian, 2007; Anzola *et al.*, 2010). For instance, hyperacetylation of histones is associated with active gene expression, while hypoacetylation correlates with gene repression (Hebbes *et al.*, 1988); methylation on H3K4 is enriched in the regions of active transcription, whereas methylation on H3K9 is linked to transcriptional repression (Shilatifard, 2006; Li *et al.*, 2007a) (Fig. 1). Global identification of histone modification sites in model plant species such as *Arabidopsis thaliana* and *Oryza sativa* is providing important information to understand how plant epigenomes respond to developmental or environmental cues. By using a high-density whole-genome tiling microarray, Zhang *et al.* (2007) found that H3K27me3 regulates an unexpectedly large number of genes in *Arabidopsis* (~4400), suggesting that this is a major silencing mechanism in plants that acts independently of other epigenetic pathways, such as small RNAs (smRNAs) or DNA methylation.

SmRNAs act not only at the post-transcriptional level by guiding sequence-specific transcript degradation and/or translational repression (reviewed in Chen, 2009), but can also play a role in targeting DNA methylation through RNA-directed DNA methylation (Wassenegger, 2005; Huettel *et al.*, 2007) (Fig. 1). These events lead to chromatin modifications eventually resulting in transcriptional silencing and heterochromatin formation (Bayne *et al.*, 2007). By using grafts between wild-type and mutant roots and shoots of *Arabidopsis,* recent studies have shown that small interfering RNA (siRNA) and microRNA (miRNA) can act as mobile signals that direct epigenetic modifications in the genome of recipient cells (Carlsbecker *et al.*, 2010; Dunoyer *et al.*, 2010; Molnar *et al.*, 2010). The non-cell-autonomous activity of different smRNA species has important implications and opens up new perspectives regarding the control of gene expression during plant development. By targeting DNA methylation, RNAi pathways in plants also play a role in inherited epigenetic variation (Bernstein and Allis, 2005; Matzke and Birchler, 2005; Wassenegger, 2005). Unravelling the interplay between DNA methylation, histone modifications, and smRNA pathways in the establishment of specific epigenetic programmes during development and in response to the environment will be essential to understand the behaviour of plant cells *in vitro* and the variant profiles detected.

In vitro cell fate switch is associated with changes in chromatin structure

The regeneration of whole plants from differentiated cells cultured *in vitro* is a clear demonstration of the plasticity of plant cells. In response to specific environmental signals, cells acquire competence to switch fate which is accomplished by going through a dedifferentiation process followed by the implementation of a new developmental pathway. This sequence of events is accompanied by changes at the chromatin level and reprogramming of gene expression, highlighting the central role of epigenetic regulation in these processes (Fig. 2).

Dedifferentiation

Plant cell dedifferentiation has been mostly studied in protoplast systems. In such experimental systems dedifferentiation can be resolved into acquisition of competence for pluripotentiality and signal-dependent re-entry into S phase, each phase being accompanied by a broad chromatin decondensation (Zhao *et al.*, 2001; Williams *et al.*, 2003). For reactivation of the cell cycle it is usually necessary that plant growth regulators are applied, but this occurs independently from the acquisition of competence for pluripotentiality. In *Arabidopsis* protoplasts acquisition of pluripotentiality is associated with chromatin reorganization at specific domains (Avivi *et al.*, 2004). Condensation of

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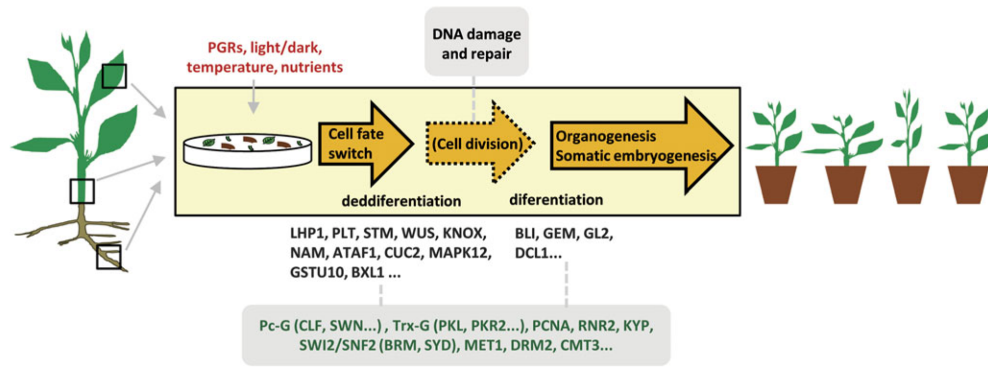

Fig. 2. *In vitro* plant regeneration. The choice of different tissue types and culture conditions including plant growth regulators (PGRs), nutrients, light/dark, and temperature, and the occurrence of extensive cell division before organogenesis or somatic embryogenesis may affect the regeneration of variant plants. Chromatin modifiers (in green) referred to in the text as well as interacting genes (in black) or putative targets with a potential role during cell fate switch/cell division and differentiation of plant cells cultured *in vitro* are represented. Although many key regulators have been identified, it is still not well understood how they function at the molecular level. For a detailed overview of the genes taking part in these plant developmental processes refer to Desvoyes *et al.* (2010). Abbreviations: BRM, BRAHMA; PKL, PICKLE; PKR2, PICKLE RELATED 2; SWN, SWINGER; SYD, SPLAYED.

the nucleolar 18S rDNA gene and decondensation at the pericentromeric and telomeric subdomains of chromosome 1, which may lead to resetting of the gene expression programme, have been described. Competence for fate switch also seems to be associated with disruption of nucleolar domain appearance, as well as modifications of histone H3 and redistribution of like heterochromatin protein 1 (LHP1) (Williams *et al.*, 2003). Indeed, attempts to find mutants defective in proliferation and callus formation resulted in the identification of a *KRYPTONITE (KYP)/SUVH4* gene encoding a histone H3 lysine 9 (H3K9) methyltransferase (Grafi *et al.*, 2007), leading the authors to suggest that histone methylation activity is required for the establishment/maintenance of the dedifferentiated state and/or re-entry into the cell cycle. This may occur, at least partially, through activation of genes whose products are involved in the ubiquitin proteolytic pathway. It is also known that during the cell cycle, extensive chromatin remodelling and histone modifications occur (reviewed in Desvoyes *et al.*, 2010), although some modifications seem to be plant species specific (Fuchs *et al.*, 2006; Sanchez *et al.*, 2008). Furthermore, recent studies have highlighted how cell cycle regulatory mechanisms both control and respond to chromatin modifications (Dominguez and Berger, 2008).

Transcriptional activation of specific genes has been found as cells acquire competence for fate switch and progress into S phase. Williams *et al.* (2003) reported that the retinoblastoma protein Rb/E2F-target genes *RNR2* (small subunit of ribonucleotide reductase) and *PCNA* (proliferating cell nuclear antigen) are condensed and silent in differentiated leaf cells but become decondensed as cells acquire competence for fate switch and turn transcriptionally active during progression into S phase, concomitantly with Rb phosphorylation. Rb has also been shown to bind to a transcription factor that functions in the *Arabidopsis* root stem cell niche. Rb loss, concomitant with the overexpression of the transcription factor PLETHORA (PLT), causes a massive expansion of root stem cells (Wildwater *et al.*, 2005; Grieneisen *et al.*, 2007). More recently, Kornet and Scheres (2009) reported that the histone acetyltransferase GCN5 is also essential for *Arabidopsis* root stem cell niche maintenance, playing an important role in shaping a developmentally instructive gradient in the root which is formed by the PLETHORA (PLT) stem cell transcription factors. In addition to PLT, other pluripotency transcription factors, including WUSCHEL (WUS), SHOOT MERISTEMLESS (STM), KNOX, and GRAS (Abarca and Díaz-Sala, 2009) family transcription factors, having a role in the maintenance of plant stem cell populations in the root and in the shoot apical meristems are likely to be key players during *in vitro* cell fate switch. Pluripotency transcription factors are regulated by and act together with chromatin regulators including SWI2/SNF2 chromatin-remodelling ATPases, histone modification enzymes, and DNA methyltransferases (Sang *et al.*, 2009; Shen and Xu, 2009). It has been reported that the expression of genes involved in hormone perception and signalling, as well as genes encoding DNA methyltransferases and enzymes of glutathione metabolism, is affected in a *Brassica oleracea* SHOOTMERISTEMLESS (STM)-overexpressing line showing a pronounced enhancement of somatic embryo yield (Elhiti *et al.*, 2010). Additional pharmacological experiments performed by the authors showed that *Arabidopsis* somatic embryogenesis is encouraged by a global hypomethylation of the DNA during the induction phase.

The NAC domain-containing genes *NAM*, *ATAF1*, and *CUC2*, whose products have been implicated in meristem functionality (Souer *et al.*, 1996; Duval *et al.*, 2002), have shown a hypomethylation-dependent up-regulation in pluripotent protoplasts (Avivi *et al.*, 2004). The authors suggested that they may act in concert in determining the pluripotent state of the cells. On the other hand, Tessadori *et al.* (2007) reported that dedifferentiation of specialized *Arabidopsis* mesophyll cells into undifferentiated protoplasts is accompanied by the disruption of chromocentre structures but not by changes in DNA methylation or

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H3K9 dimethylation. Although protoplasts showed a dispersed 5mC pattern distinct from the clustering at chromocentres in mesophyll cells, no large changes in the intensity of the 5mC signals per nucleus were detected, suggesting that the overall level of DNA methylation remained unchanged. However, by using *Arabidopsis* cells deficient in different members of the family of plant DNA methyltransferases, and treating undifferentiated *Arabidopsis* cell suspensions with a demethylating drug, Berdasco *et al.* (2008) identified several hypermethylated genes in callus and cell suspensions. Promoters of the *MITOGEN-ACTIVATED PROTEIN KINASE 12* (*MAPK12*), *GLUTATHIONE S-TRANSFERASE TAU 10* (*GSTU10*), and *BETAXYLOSIDASE1* (*BXL1*) genes were densely hypermethylated in callus and cell suspensions, whilst the *TRANSPARENT TESTA GLABRA 1* (*TTG1*), *GLUTATHIONE S-TRANSFERASE* (*CLASS PHI*) *5* (*GSTF5*), and *H3-K9-HMTASE 8* (*SUVH8*) genes occasionally become hypermethylated only in cell suspensions. Promoter hypermethylation primarily occurred at CpG sites and specifically depended on MET1 and DRM2 methyltransferases. It has been proposed that callus and *Arabidopsis* cell suspensions possess a unique epigenetic signature with subsets of genes whose expression is controlled by promoter hypomethylation and hypermethylation (Berdasco *et al.*, 2008), as has been described in mammals (Bibikova *et al.*, 2006). Also in long-term suspension cultures, it has been reported that euchromatin becomes hypermethylated, whereas heterochromatin undergoes DNA hypomethylation resulting in transcriptional activation of specific transposable elements accompanied by production of 21 nucleotide smRNAs (Tanurdzic *et al.*, 2008). Reprogramming of the epigenome of proliferating cells seems therefore to implicate DNA methylation, histone modifications, and RNAi.

Differentiation

In many established *in vitro* culture systems, proliferating cells start to differentiate when specific changes in the balance of growth regulators are introduced in the culture medium, ultimately leading to organogenesis. When dividing cells eventually arrest the cell cycle, cell fate decisions have to be made. At the transition from mitosis to G_1, genes involved in cell fate decisions and differentiation have to be reactivated.

Polycomb-group (Pc-G) proteins as well as the antagonist Trithorax-group (Trx-G) proteins have been pointed out as epigenetic regulators of cell fate in both plants and animals (Schatlowski *et al.*, 2008; Aichinger *et al.*, 2009; Schuettengruber *et al.*, 2009). Pc-G complexes are master regulators that maintain epigenetically repressed states that need to be reprogrammed when cells become committed to differentiation. Pc-G proteins are required to maintain stem cell identity by suppressing key regulators of differentiation pathways in flies and animals (Boyer *et al.*, 2006; Bracken *et al.*, 2006; Lee *et al.*, 2006). Because many of the Pc-G targets are genes involved in transcriptional regulation in *Drosophila*, mammals, and plants, it has been suggested that the evolutionarily conserved main function of Pc-G proteins is the regulation of transcriptional pathways (Schuettengruber *et al.*, 2007; Zhang *et al.*, 2007). In plants, >4000 genes were predicted to be direct target genes of Pc-G complexes (Zhang *et al.*, 2007). Recently, Schatlowski *et al.* (2010) identified a novel, plant-specific protein in *Arabidopsis*, BLISTER (BLI), which prevents premature differentiation by interacting with the Pc-G histone methyltransferase CURLY LEAF (CLF), possibly providing an important link between the plant Pc-G machinery and the control of cell cycle progression.

Cell specification in the *Arabidopsis* root epidermis has been a useful system to study epigenetic regulation of developmental processes. Using this system, Costa and Shaw (2006) provided evidence that the chromatin state can be reset and remodelled in each cell cycle during development. The authors showed that alternative states of chromatin organization around the homoeodomain transcription factor GLABRA2 (GL2) locus are required to control position-dependent hair and non-hair cell specification. Another study clearly demonstrating the role of histone modifications in developmental regulation was reported by Caro *et al.* (2007) in which GEM, a modulator of the *GLABRA2* (*GL2*) gene determining hair/non-hair cell fate, has been found to participate in the maintenance of the repressor histone H3K9 methylation status of root patterning genes, providing a link between cell division, fate, and differentiation during *Arabidopsis* root development. SmRNAs have also emerged as essential regulators of cell fate in both animals and plants (Carlsbecker *et al.*, 2010; Ivey and Srivastava, 2010). Recently, the lack of DICER-LIKE 1 (DCL1), which is required for miRNA biogenesis, has been shown to arrest development of *Arabidopsis* embryos. The requirement of DCL1 for cell differentiation events is perceived in eight-cell stage embryos and thereafter for proper division of the hypophysis and subprotoderm cells (Nodine and Bartel, 2010). The authors suggested that miRNAs enable proper embryonic patterning by preventing precocious expression of differentiation-promoting transcription factors, namely SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL).

It should be pointed out that, although there is no compelling evidence for trans-generational resetting of CG methylation as has been documented in mammals, other epigenetic marks, such as some histone modifications and histone variants, are reset between generations (for a review, see Paszwlaoski and Grossniklaus, 2011). *In vitro* plant cell culture and regeneration systems surpass these epigenetic reprogramming events which may be needed to erase the effects of epigenetic marks caused by external stimuli (Feng *et al.*, 2010*b*). Therefore, an accumulation of epigenetic changes over generations of cell divisions may occur, increasing the risk of perpetuating dangerous epigenetic alleles. This also applies to *ex vitro* clonally propagated plant species such as the long-lived crops oil palm, grapevine, and cocoa.

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Methodologies for genome-wide screening of epigenetic variation

Variation in DNA methylation patterns of plants cultured *in vitro* has been analysed mainly by AFLP (amplified fragment length polymorphism)-based (Vos *et al.*, 1995) techniques. Methylation-sensitive amplified polymorphism (MSAP) is one such technique that makes use of a pair of methylation-sensitive restriction enzymes, *Hpa*II and *Msp*I, which, being a pair of isoschizomers, recognize the same tetranucleotide CCGG but have differential sensitivity to methylation at the inner or outer cytosine (Reyna-López *et al.*, 1997). Selective PCR amplification and comparison between fragments generated by each enzyme of the isoschizomer pair allows the examination of the cytosine methylation status of the restriction site, making it possible to visualize a large number of markers per sample. Thus, MSAP has proven to be an efficient method for detecting alterations in cytosine methylation in fixed genotypes However, this method can only investigate a small proportion of the methylated cytosines in the genome because the detection is restricted to the recognition sites of the isoschizomers used. Furthermore, only the changes between unmethylated sites and internally methylated or hemimethylated sites are detected, excluding other possibilities such as the switch from a fully or externally methylated site to an unmethylated or internally methylated site. Nonetheless this method has been used for surveying CpG methylation at the CCGG site in the genome of several plant species aiming at characterizing variation in methylation patterns associated with *in vitro* culture (Table 1).

Although not providing site-specific information, global quantification of 5mC can also be used to analyse changes in DNA methylation. Quantification has been conducted by high performance separation means, such as HPCE (high performance capillary electrophoresis) and HPLC (high performance liquid chromatography), or by enzymatic/chemical approaches (Fraga and Esteller, 2002). The HPLC- and HPCE-based methods involve the digestion of genomic DNA to nucleotides, nucleosides, or bases, for subsequent separation and analysis of 5mC. The HPCE method has been used in some plant species to detect epigenetic variation in *in vitro* cultures (Table 1).

In the last couple of years, with the development of high-throughput genome technologies, a number of options have become available to profile the epigenome of several organisms including plants such as *Arabidopsis*. These new methods are able to provide a detailed characterization of genomic DNA methylation and histone modifications at an unprecedented resolution, which can be integrated with transcriptomics data including the smRNA transcriptome (Zhang *et al.*, 2007; Lister *et al.*, 2008, 2009; Wang *et al.*, 2009).

The sites of DNA–protein interactions can be mapped through the use of tools such as chromatin immunoprecipitation (ChIP)-chip as well as ChIP-seq involving immunoprecipitation of specific chromatin through its interaction with a protein of interest. While in ChIP-chip hybridization of the immunoprecipitated genomic DNA to arrays enables identification of the genomic sites at which interaction of the protein with the genomic DNA occurs, ChIP-seq takes advantage of new sequencing technologies coupling a chromatin immunoprecipitation technique to shotgun sequencing (reviewed by Lister *et al.*, 2009). A few reports already describe the application of these methods to plant species (Kaufmann *et al.*, 2010; Wang *et al.*, 2010). Single-base resolution analysis of sites of DNA methylation can be achieved by sodium bisulphite (BS) treatment of genomic DNA, which converts cytosines, but not methylcytosines, to uracil (Frommer *et al.*, 1992), followed by deep sequencing. This approach has recently enabled shotgun BS sequencing of the entire *A. thaliana* genome with a technique dubbed BS-seq or methylC-seq, offering an unprecedented view of the DNA methylome (Cokus *et al.*, 2008). Finally, genome-wide expression profiling through microarray analysis or next-generation sequencing focusing on coding and non-coding RNA will be crucial for correlating epigenetic status with transcription and phenotypic data. By directly sequencing the cytosine methylome (methylC-seq), the transcriptome (mRNA-seq), and the smRNA transcriptome (smRNA-seq) Lister *et al.* (2008) have reported a direct relationship between the location of smRNAs and DNA methylation, perturbation of smRNA biogenesis upon loss of CpG DNA methylation, and a tendency for smRNAs to direct strand-specific DNA methylation in regions of RNA–DNA homology. Additionally, the authors stated that strand-specific mRNA-seq detected variation in transcript abundance of hundreds of genes, transposons, and unannotated intergenic transcripts upon modification of the DNA methylation state.

These new technologies are expected to become more cost-efficient in the short term and will certainly contribute to a better understanding of somaclonal variation. However, at present, the useful application of such high-throughput methodologies for studying somaclonal variation is dependent on the availability of genomic resources in target species and the ability to correlate such a large amount of data with plant phenotype/behaviour *in vitro*. Nonetheless, recalcitrant or long-lived species such as forest trees in which somaclonal variation events may become noticed many years later with potentially deleterious consequences are among the species that may benefit the most from these novel technologies, as they may provide clues for developing adequate *in vitro* protocols.

Detection of epigenetic variation in plant cell cultures *in vitro*

The evaluation of epigenetic modifications in plants cultured *in vitro* has up to now been mostly focused on the analysis of DNA methylation because this is one of the best described epigenetic mechanisms and, as described above, several tools for analysis of variation are readily available (Table 1). Nevertheless, a few studies have also reported the detection of modifications in histones and smRNA levels in plant cells cultured *in vitro* (Table 2).

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Table 1. Analysis of DNA methylation in plant *in vitro* cultures for assessment of somaclonal variation

Species	Tissue culture[a] system	DNA methylation detection method[b]	Variation	Reference
Bambusa balcooa	SE and axillary shoot multiplication	MSAP	No	Gillis *et al.* (2007)
Cedrus atlantica and *C. libani*	Axillary bud multiplication	HPLC	Yes	Renau-Morata *et al.* (2005)
Citrus paradisi	SE	MSAP	Yes	Haoa *et al.* (2004)
Codonopsis lanceolata	Adventitious bud regeneration	MSAP	Yes	Guo *et al.* (2007)
Corylus avellana L.	Axillary shoot multiplication	Isoschizomer restriction analysis	Yes	Diaz-Sala *et al.* (1995)
Doritaenopsis	Micropropagation	MSAP	Yes	Park *et al.* (2009)
Elaeis guineensis	SE	HPLC, *Sss*I-MAA, MSAP	Yes	Jaligot *et al.* (2000, 2004)
Freesia hybrida	SE	MSAP	Yes	Gao *et al.* (2010)
Gentiana pannonica	SE	HPLC reversed phase	Yes	Fiuk *et al.* (2010)
Hordeum brevisubulatum	SE	MSAP	Yes	Li *et al.* (2007b)
Hordeum vulgare	SE, androgenesis	MSAP	Yes	Bednarek *et al.* (2007)
Humulus lupulus	Adventitious bud regeneration	MS-AFLP	Yes	Peredo *et al.* (2006)
Malus×domestica	Axillary shoot multiplication	MS-AFLP	Yes	Li *et al.* (2002)
Musa AAA cv. 'Grand Naine'	Micropropagation	MSAP	Yes	Peraza-Echeverria *et al.* (2001)
Myrtus communis L.	Axillary shoot multiplication, SE	HPLC	No	Parra *et al.* (2001)
Oryza sativa	SE from protoplast-derived calli	MS-RFLP	Yes	Brown *et al.* (1990)
Pinus pinaster	SE	HPCE/MSAP	No/Yes	Klimaszewska *et al.* (2009)
Pinus pinaster	SE	HPCE	Yes	Marum (2009)
Pisum sativum	Axillary shoot multiplication	MSAP, HPCE	Yes	Smýkal *et al.* (2007)
Rosa hybrida L.	SE, adventitious bud regeneration	MS-AFLP	Yes	Xu *et al.* (2004)
Solanum tuberosum	SE, microtuberization	MS-AFLP	Yes	Sharma *et al.* (2007)
Solanum tuberosum	Axillary shoot multiplication	MS-AFLP	No	Sharma *et al.* (2007)
Solanum tuberosum	Cryopreserved shoot tip	MSAP	Yes	Kaczmarczyk *et al.* (2010)
Theobroma cacao	SE	MSAP	Yes	López *et al.* (2010)
Vitis vinifera	Axillary shoot multiplication	MSAP	Yes	Baránek *et al.* (2010)
Vitis vinifera	SE	MSAP	Yes	Schellenbaum *et al.* (2008)
Zea mays	SE	MS-RFLP	Yes	Kaeppler and Phillips (1993)

[a] SE, somatic embryogenesis.
[b] HPCE, high performance capillary electrophoresis; HPLC, high performance liquid chromatography; MS, methylation-sensitive; *Sss*I-MAA, *Sss*I-methylase accepting assay.

Table 2. Modifications in histones and small RNA levels detected in plant cells/tissues cultured *in vitro*

Species	*In vitro* cultured cells/tissues	Histone and small RNA level modifications	Reference
Arabidopsis thaliana	Cell suspension cultures	Loss of H3 methylation on Lys9 and, in some cases, gain of H3 trimethylation at Lys4 Increased levels of 21 nucleotide siRNAs	Tanurdzic *et al.* (2008)
Arabidopsis thaliana	Cell suspension cultures	Loss of acetylated H3 and H4 and trimethylated Lys4 H3	Berdasco *et al.* (2008)
Nicotiana tabacum	Protoplasts	Increased levels of acetylated H3; modification of Lys9-methylated H3	Williams *et al.* (2003)
Solanum tuberosum	Cell suspension cultures	Multiacetylation of H3.1, H3.2 and H4	Law and Suttle (2005)
Zea mays	callus cultures	Increase of ubiquitinated H2A in callus derived from root differentiation zone compared with callus derived from other root zones	Alatzas and Foundouli (2006)

One of the most striking examples of plant tissue culture-induced variation is the 'mantled' somaclonal variation of oil palm. This variation affects the formation of floral organs in both male and female flowers in ~5% of the regenerants obtained through somatic embryogenesis (Corley *et al.*, 1986), but its occurrence and severity are highly variable between and among clonal progenies. The detection of genome-wide DNA hypomethylation and sequence-specific methylation changes in mantled palms when compared with their normal counterparts (Jaligot *et al.*, 2000, 2004; Kubis *et al.*, 2003) suggests that epigenetic deregulation of gene expression is the cause of the variant phenotype. However, despite several efforts (Jaligot *et al.*, 2004; Rival *et al.*, 2008; Beulé *et al.*, 2010), the nature of the epigenetic deregulation occurring in mantled palms remains to be determined. In fact, several studies using different plant regeneration systems, including somatic embryogenesis and axillary bud proliferation, have

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reported hypomethylation in tissue culture regenerants as detected either by AFLP-based methods or HPLC (Renau-Morata *et al.*, 2005; Peredo *et al.*, 2006; Schellenbaum *et al.*, 2008) (Table 1). In callus-derived hop plants, 83% of changes of the polymorphic loci detected by MSAP between controls and regenerated hop somaclones were demethylation of the recognition sites (Peredo *et al.*, 2006). In tobacco protoplasts, Koukalova *et al.* (2005) found that hypomethylation of particular rDNA gene families, accompanied by a moderate increase in rRNA gene expression, was initiated as early as 2 weeks after the callus induction, with the established epigenetic patterns being stably maintained for at least 2 years of *in vitro* cultivation. However, remethylation took place upon plant regeneration. On the other hand, Guo *et al.* (2007) reported that in spite of the different kind of methylation changes in individual regenerants of *Codonopsis lanceolata*, overall tissue culture did not cause significant alteration in cytosine methylation levels at CCGG sites. Also in *Pisum sativum* L., tissue culture-derived regenerants from different genotypes even showed evidence of hypermethylation, or no obvious difference in methylation (Cecchini *et al.*, 1992). However, the timing of plant regeneration from callus cultures may be determinant for the detection of variation. López *et al.* (2010) found that leaves of 'late regenerants' exhibited significantly less genetic and epigenetic divergence from source leaves than those exposed to short periods of callus growth, evidencing a progressive erosion of genetic and epigenetic variation in callus-derived plants. The authors suggest that if such findings apply generally, they go against the common practice of minimizing somaclonal variation by limiting time in callus culture.

Cell-specific DNA methylation patterns can also play a role in the methylation status of regenerant plants. In fact, when studying the influence of cell culture stress on epiallelic stability Krizova *et al.* (2009) showed evidence for cell-to-cell methylation diversity of tobacco callus cultures and erasure of parental methylation imprints in callus culture. Regenerated plants showed high interindividual but low intraindividual epigenetic variability, indicating that the callus-induced epiallelic variants were transmitted to plants and became fixed. Moreover, in regeneration systems that do not implicate an intermediate dedifferentiation step, such as axillary shoot proliferation, different explants taken from the same source plant can have a strong influence on the methylation patterns of the derived regenerants. In *Vitis vinifera*, significant differences between woody cuttings have been observed despite originating from a single plant (Baránek *et al.*, 2010). López *et al.* (2010) reported that leaves and staminode explants from cocoa could be separated based on their MSAP profile and, despite an intermediate callus phase, the epigenetic profiles of leaves from the regenerants appeared more similar to those of leaves from the source plant regardless of the type of explant from which they were derived (López *et al.*, 2010). This is in accordance with the work by Luo *et al.* (2009) reporting that, in addition to sequence context and genome position, organ type also affects the targeting of epigenetic regulators. By comparing the *in vivo* bioluminescence in four lines containing an identical T-DNA harbouring the *LUCIFERASE* (*LUC*) and *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPTII*) genes inserted into different loci on *Arabidopsis* chromosome 2, the authors found that the expression level of a reporter gene was different not only among lines, but even in different tissues of the same plant, implying that regulators target the same DNA sequences in a genome locus- and tissue-specific manner. The mobility of smRNAs may originate gradients and differential accumulation of specific molecules across different plant tissues (Carlsbecker *et al.*, 2010; Dunoyer *et al.*, 2010; Molnar *et al.*, 2010). Explants carrying different sets of epigenetic regulators will probably affect the regenerant outcomes by mediating responses to external stimuli and initiating epigenetic changes that influence adaptation to stress (Borsani *et al.*, 2005; Molnar *et al.*, 2010). Such effects might be minimized when isolated cells in suspension culture are used.

The ontogenetic state of the explants is a major factor affecting the ability to regenerate plants *in vitro*, and a decline in the ability to regenerate shoots, roots, or embryos from somatic tissues of woody species has been associated with age and maturation (Day *et al.*, 2002). Additionally, in species such as *Pinus radiata* (Fraga *et al.*, 2002), *Sequoiadendron giganteum* (Monteuuis *et al.*, 2008), *Acacia magnum* (Baurens *et al.*, 2004), and *Castanea sativa* (Hasbún *et al.*, 2007), it has been found that global epigenetic changes are related to phase change. In fact, a recent study in *P. radiata* by Valledor *et al.* (2010) reported that needle maturation, which is associated with a decrease in organogenic capability, is related to an increase in heterochromatin-related epigenetic markers including high DNA methylation and low acetylated histone H4 levels, and the presence of histone H3 methylated at Lys9.

In some species, different results have been obtained depending on the plant regeneration system used. For instance, in *Rosa hybrida*, methylation patterns during somatic embryogenesis appear to be quite different from those during shoot organogenesis (Xu *et al.*, 2004). The authors show that demethylation of outer cytosines in CCGG sequences occurred at a high frequency during somatic embryogenesis, and most alterations in embryogenic callus were passed on to its regenerants. However, most modifications observed during organogenesis were reverted in shoot regenerants derived from organogenic callus, leading the authors to suggest that altered DNA methylation patterns, especially demethylation of outer cytosines, are only related to embryogenic ability. In potato, changes in methylation pattern were analysed in *in vitro* regenerated plants via somatic embryogenesis and axillary bud multiplication, and only in the latter was epigenetic stability confirmed (Sharma *et al.*, 2007*a*). In fact, axillary branching has been usually pointed out as the most faithful way of propagating plants *in vitro* since it does not involve cell dedifferentiation of differentiated cells but rather the development and growth of new shoots from pre-existing meristems. On the other hand, tissue culture systems that involve acquisition of competence for pluripotentiality,

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extensive cell division, and eventually the acquisition of a new cell fate are usually regarded as more risky in what concerns genome and epigenome instability. Nevertheless, the detection of off-types at the DNA level has also been reported for plants propagated by axillary branching (Devarumath *et al.*, 2002), and examples of stable MSAP patterns have been reported using somatic embryogenesis from tissues at different developmental stages (pseudospikelets, embryogenic callus, and regenerated plantlets) (Gillis *et al.*, 2007). Also in *Freesia hybrida* DNA cytosine methylation alterations in both CG and CNG levels and patterns have been obtained at similar rates from either direct or indirect embryogenesis pathways (Gao *et al.*, 2010). In *Pinus pinaster*, Marum (2009) observed that the relative percentages of 5mC as quantified by HPCE both in mature somatic and zygotic embryos, as well as derived plants, were very similar (23–24% 5mC for embryos and 17% 5mC for plants). However, mature somatic embryos with abnormal phenotype presented ~3.5% less 5mC when compared with normal phenotype somatic embryos (Marum, 2009).

In a few cases, variation in DNA methylation has been associated with the addition of specific compounds to the culture medium, as is the case for plant growth regulators. Since 1989 with the work of LoSchiavo *et al.* (1989) with carrot cell cultures, plant growth regulators have been suggested to affect genomic DNA methylation. However, it is not yet clear how the activity of these compounds interferes with DNA methylation. Also antibiotics such as hygromycin, kanamycin, and cefotaxime, which are routinely used as selective agents in the production of transgenic plants, have been found to cause DNA hypermethylation in *Nicotiana tabacum* plantlets grown *in vitro*. Plantlets exposed in culture to antibiotics such as cefotaxime, kanamycin, and hygromycin showed a dose-dependent increase in the 5mC content of their DNA which was not reversible upon the removal of the antibiotics (Schmitt *et al.*, 1997). In *Arabidopsis* calli the use of kanamycin as a selective agent caused dosage-dependent methylation changes in the genome (Bardini *et al.*, 2003). Both hyper- and hypomethylation events were observed but the net result was genome-wide hypomethylation.

Concluding remarks

Although the epigenetic events taking place in plant cells cultured *in vitro* are certainly not specific to *in vitro* culture, the conjunction of stimuli such as exogenously applied growth regulators together with other artificially provided chemical and physical factors, to which the cells are exposed, presents a unique opportunity to study epigenetic regulation of plant development and adaptation. Phenotypic plasticity, as defined by the capacity of a genotype to take different phenotypes for a given trait under different environmental conditions (Bradshaw, 2006), is undoubtedly playing a major role in *in vitro* plant cell cultures. Significant advances in plant epigenetics are providing clues to understand the molecular basis of somaclonal variation, but much progress is still expected, with the wider application of high-throughput technologies allowing a full picture of the plant epigenome under different environmental scenarios. It has become clear that DNA methyltransferases, histone modification enzymes, and other regulatory proteins have essential roles in plant development. Understanding epigenetic regulation will probably have important implications in plant biotechnology while avoiding the negative consequences of variation. Eventually, it will be possible to modulate the regenerant outcomes by selecting conditions leading to diverse epigenetic landscapes. This modulation may include directed manipulation of epigenetic regulators opening the way to epigenetic engineering in plants.

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