SI : TISSUE CULTURE

Epigenetics in plant tissue culture

M. J. M. Smulders · G. J. de Klerk

Abstract Plants produced vegetatively in tissue culture may differ from the plants from which they have been derived. Two major classes of off-types occur: genetic ones and epigenetic ones. This review is about epigenetic aberrations. We discuss recent studies that have uncovered epigenetic modifications at the molecular level, *viz.*, changes in DNA methylation and alterations of histone methylation or acetylation. Various studies have been carried out with animals, and with plant cells or tissues that have grown in tissue culture but only little work has been done with shoots generated by axillary branching. We present various molecular methods that are being used to measure epigenetic variation. In micropropagated plants mostly differences in DNA methylation have been examined. Epigenetic changes are thought to underlie various well-known tissue-culture phenomena including rejuvenation, habituation, and morphological changes such as flower abnormalities, bushiness, and tumorous outgrowths in, among others, oil palm, gerbera, *Zantedeschia* and rhododendron.

Keywords Methylation · Micropropagation · Off-types · Rejuvenation · Habituation

Introduction

In tissue culture, new plants may be generated by outgrowth of axillary buds or by adventitious regeneration (De

M. J. M. Smulders (✉) · G. J. de Klerk
Wageningen UR Plant Breeding, P.O. Box 16,
6700 AA Wageningen, The Netherlands
e-mail: rene.smulders@wur.nl

Klerk 2009). Researchers expected initially that these clonally propagated plants would be exact copies of the parent plant, but frequently aberrant plants were observed. Various causes have been established:

- Genetic changes (also referred to as somaclonal variation): changes in the DNA sequence;
- Epigenetic variation: long-lasting changes in the expression of the information in the genome;
- And—less relevant for this review—chimeral segregation and loss of pathogens, in particular viruses.

Various types of genetic changes have been observed including polyploidy, aneuploidy, (point) mutations, and new insertions of (retro) transposons. In crosses, mutations behave as Mendelian traits, with the notable exception of plants that become 'normal' when an inserted transposon jumps out again. Epigenetic changes are caused by changes in the expression of the information in the DNA brought about by alterations in DNA methylation, in histones, or in both. These modifications may influence gene transcription. Epigenetic changes are often temporary and plants may revert to the normal phenotype relatively easily, but some can be long lasting and may even be transferred during sexual propagation (Brettell and Dennis 1991) (Fig. 1). The latter occurs regularly in plants as epigenetic marks are not systematically erased in embryogenesis, as they are in mammals, and this leads to inheritable epimutations (epialleles) (Jullien and Berger 2010). Johannes et al. (2009) detected epigenetic changes in *Arabidopsis thaliana* that were stable for at least eight generations.

It is characteristic for epigenetic changes that in a population of generated plants the same aberration occurs at high frequency and can be 'reproduced' when the same conditions are imposed during the production of another population (Smulders et al. 1995). In contrast, genetic

Fig. 1 Schematic representation of the differences between genetic and epigenetic variation. Note that during the various indicated tissue culture processes, the extent of genetic variation remains the same, but the extent of epigenetic variation is sharply reduced. In plants some epigenetic changes may survive sexual propagation
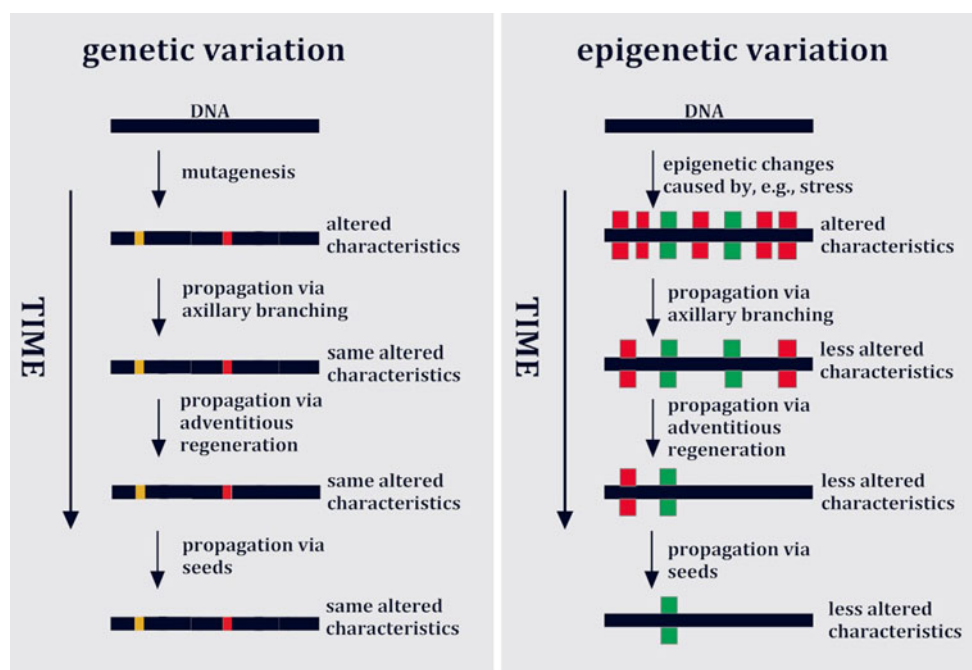
changes occur at random. It should be noted that in a somaclonal population the same genetic change may be found in many individuals but then the mutational event happened early during the tissue culture period and was clonally multiplied into a number of descendants (Brettell and Dennis 1991; Kohli et al. 2004).

Characteristics of epigenetic changes

Epigenetic mechanisms include DNA methylation and modifications of amino acids in the tails of histones. Histones are proteins that package the DNA into nucleosomes and make up the chromatin in nuclei. DNA methylation and histone modifications interact and reinforce one another. In this way, transcription can be enhanced, for example, by acetylation of the lysine on position 9 of histone 3 ('H3K9'), which is found in promotor regions of actively transcribed genes. Transcription can also be decreased, for example, by di- and trimethylation of H3K9, which is a histone mark of repeat elements in the DNA (Bernatavichute et al. 2008; Tessadori et al. 2007). Repeat elements are also characterized by methylation of cytosines in the DNA.

This regulatory system has several functions. On the one hand, it is a defence mechanism against retrotransposons. Silencing of repeats is thought to occur in all cells, regardless of their location or developmental state. It is targeted by 21- and 24-mer short RNAs. Intriguingly, the same mechanism contributes to sustain the centromere through silencing and compacting the centromeric repeats

(Tessadori et al. 2007). On the other hand, epigenetic mechanisms regulate gene expression during development: genes may be methylated and switched off in some tissues, but demethylated and active in others (see Matzke et al. 2009 for a description of the mechanism and the genes involved). The clearest examples of this type of epigenetic regulation are found in mammalian embryogenesis: imprinting (where the activity of a gene in early embryo development depends on whether it is inherited from the father or the mother), X-inactivation (to achieve the proper gene dosage, one of the two X chromosomes in females is heavily methylated and transcription is largely switched off), and reprogramming (of genome-wide DNA methylation patterns in the gastrula stage). Imprinting also occurs during plant endosperm development (Hsieh et al. 2009). When 'genes' are said to be methylated and transcriptionally silenced, it is actually the promotor region that becomes methylated, as even actively transcribed genes may be methylated in the 'body' of the coding region, i.e., at some distance from the 5′ and 3′ end (Roudier et al. 2009; Tanurdzic et al. 2008).

During plant development, the epigenetic imprints provide a transient 'memory' of the developmental program and influence the cell's reactions and its future development (Roudier et al. 2009). A notable example of the memory function is vernalization, the acquisition or acceleration of the ability to flower by a chilling treatment. In *Arabidopsis*, vernalization involves the silencing of a group of flowering repressor genes, including *Flowering Locus C* (FLC; Bastow et al. 2004; Roudier et al. 2009). Silencing occurs during a cold period through an epigenetic

change, *viz.*, through histone modifications (Bastow et al. 2004; Kim et al. 2009). Interestingly, in barley it is mediated by different histone modifications at the floral activator gene VERNALIZATION1 (VRN1), allowing it to become active (Oliver et al. 2009) Another example is the phase change from juvenile to adult, which is paralleled by changes in gross DNA methylation (Valledor et al. 2007).

De Klerk (1990) and Vázquez (2001), amongst others, noted that the distinction between genetic and epigenetic changes is an oversimplification. Molecular evidence has accumulated showing that epigenetic changes may lead to many genetic abnormalities. The transposable element Activator (Ac) in maize looses DNA methylation and becomes activated upon dedifferentiation (Brettell and Dennis 1991). The same occurs in transgenic rice plants containing Ac from maize (Kohli et al. 2004). In maize, the activation is passed on for two sexual generations (Brettell and Dennis 1991). The miniature inverted repeat transposable element (MITE) mPing becomes activated in rice, e.g., in callus culture, which is correlated with alterations in cytosine methylation (Ngezahayo et al. 2009). The retroelement Tos17 becomes active in rice suspension cultures, supposedly as a consequence of changes in the epigenetic modifications that silence it. As a result, plants regenerated from these cultures have many new copies inserted in their genome (Hirochika et al. 1996). The endogenous chromovirus LORE1a looses DNA methylation in dedifferentiated cells of *Lotus japonicus*, so that it can become active and transpose after regeneration. Strikingly, it only does so during pollen formation (Fukai et al. 2010). Insertions of transposable elements and retrotransposons can serve as insertional mutagens of plant genomes, whereas widespread activation can lead to a wide array of chromosomal rearrangements (Tanurdzic et al. 2008). These rearrangements can, in turn, lead to misregulation of genes, aneuploidy and new transposon insertions (Lee and Phillips 1988).

Detection of epigenetic status

For an accurate evaluation of the epigenetic status, both DNA methylation and chromatin modifications should be considered, and this should be done for particular genomic regions. Differences in genome-wide levels of DNA methylation are not very informative, as there can be massive local changes whereas overall levels are very similar. Measurement of the methylation of randomly chosen cytosines is inadequate since many changes may occur in DNA with no specific function. In addition, the methylation pattern of a stretch of DNA is important, usually not that of any particular cytosine by itself. As a consequence, changes in random sequences do not correlate with particular morphological or physiological abnormalities observed in regenerants (Jaligot et al. 2002; Smulders et al. 1995). Possible correlations between the amount of changes at the molecular level and the extent of abnormalities may be established by studying many more sites. Technically, such research is becoming possible. However, if specific stretches of DNA, e.g., in a particular promotor, affect the expression of a specific gene that leads to one type of change, an estimation of the overall level of variation would not necessarily provide a strong correlation with the specific abnormality under study.

It may be easier to study changes that occur repeatedly as the correlation of certain changes in DNA and histone modifications with the phenotypic changes can be determined and used as a selection criterion. In cancer research, this yields interesting results (certain aberrantly methylated CpG islands in the genome are biomarkers for several types of cancer). The progression of a cancer goes through several specific stages, including an initial step of massive DNA demethylation and subsequent selection for cell lines that manage to establish themselves. The changes induced by adventitious regeneration may not be so predictable, and the selection for abnormal lines not so severe, although examples exist where certain types of abnormalities occur at a relatively high level.

The methods to assess epigenetic status, either DNA methylation or histone modifications, are quickly developing (Fraga and Esteller 2002; Laird 2010; Vogel et al. 2009). For DNA methylation detection, various methods and protocols exist, including bisulfite treatment of DNA changes, which changes unmethylated cytosines into uracil but does not change methylated cytosines, and amplification after digestion of DNA with methylation-sensitive restriction enzymes (Gonzalgo and Jones 1997; Xiong and Laird 1997). Some researchers use antibodies against 5-methylcytosine (Barton et al. 2001). The methylation-dependent restriction enzyme McrBC can be used to separate methylated from non-methylated DNA, followed by detection using whole genome microarray hybridisation (Vaughn et al. 2007). Bisulfite-treated DNA can be sequenced and compared to untreated DNA, thus producing detailed information on the methylation of cytosines on both strands of the DNA (Krizova et al. 2009; Zhang et al. 2009). Histone modifications are studied using chromatin immunoprecipitation (ChIP) of associated DNA followed by polymerase chain reaction (PCR) of cDNA or whole genome microarray hybridisation (Matarazzo et al. 2004; Roh et al. 2004; Shi et al. 2003). In this way, many known sites can be probed simultaneously.

Using a combination of these approaches a whole genome analysis of chromosome 4 of *Arabidopsis thaliana* was done on tiling microarrays by Tanurdzic et al. (2008). They generated detailed patterns of both DNA methylation

and various modifications of the associated chromatin. They compared the epigenetic patterns of an immortalised cell suspension of *Arabidopsis thaliana* with that of the leaf cells from which the culture had been started. The heterochromatin had undergone dramatic DNA hypomethylation, which led to the transcriptional activation of certain transposable elements in culture, while other types of elements remained silent. The elements activated in culture had increased levels of 21-nucleotide small interfering RNAs, indicating interplay of RNA interference and histone modifications in epigenetic restructuring of the genome. Euchromatin, in which active genes reside, was hypermethylated in culture, but only a small percentage of genes obtained heterochromatic marks, which might lead to long-term silencing.

Krizova et al. (2009) studied the fate of an epigenetically silenced LO1 transgenic allele in tobacco taken through cell culture and regeneration from callus. They observed partial loss of Lo1E 35S promoter methylation compared with leaf tissue in the parent plant. In comparison, when starting with plants in which the promotor was not silenced, they observed an increase in promotor methylation in some regenerated plants. The data indicate that in the cell culture process the parental imprinting state was gradually lost. Bisulfite sequencing of the promoter in callus revealed molecules with no, intermediate, and high levels of methylation, demonstrating cell-to-cell methylation diversity in callus. Among regenerated plants the DNA methylation pattern was highly variable, but within a regenerated plant most cells were very similar in DNA methylation, indicating that the callus-induced epiallelic variants were transmitted to plants and had become fixed (Peredo et al. 2006). Dedifferentiation in cell suspensions and calluses of *Arabidopsis thaliana* led to hypermethylation of promoters of various genes. This hypermethylation in undifferentiated cells was associated with histone hypoacetylation (Berdasco et al. 2008). These changes were associated with transcriptional silencing of linked genes.

In the near future, next-generation sequencing will enable studying the detailed pattern of DNA methylation by sequencing bisulfite-treated DNA (Hsieh et al. 2009) or studying the effect of a treatment on histone modifications by comparing the DNA sequences associated with immunoprecipitated histones. Flusberg et al. (2010) managed to detect DNA methylation directly using single-molecule, real-time sequencing. These methods will be used to produce detailed overviews of epigenetic modifications among tissues and between developmental stages, in a variety of species. For a better understanding of the tissue culture process, the challenge will be to focus on relevant regions in the genome and to link the changes to the processes of dedifferentiation and differentiation. This may require sampling and studying time series of plant material while they proceed through the tissue culture process, as mature plants, and into future generations.

In tissue culture, adventitious regeneration is one of the main developmental pathways to obtain new plants. It is based on the ability of plants to redirect development (De Klerk 2009). To regain totipotency (the capability to generate a complete new plant) or pluripotency (the capability to generate an organ such as a root or a shoot), cells erase at least a part of the previous epigenetic marks. This process is called dedifferentiation. Accordingly, differences in regeneration capacity between genotypes may be brought about by differences in how fast and how easily epigenetic marks can be erased or reprogrammed. It has been hypothesised that cell type, developmental age, and physical age may affect the speed of reprogramming, and the accuracy of the process (Grafi 2004). Thus, epigenetic changes in adventitiously regenerated plants may be caused by incorrect, possibly incomplete, resetting of the epigenetic developmental program, much like what causes abnormalities in cloned mammals. Unfortunately, the dedifferentiation process is still not well understood. Dedifferentiation of specialized mesophyll cells of *Arabidopsis thaliana* into undifferentiated protoplasts leads to a dramatic reduction of heterochromatin with decondensation of all major repeats regions except the 45s rDNA repeat (Tessadori et al. 2007). DNA methylation and histone H3K9 dimethylation do not change during the decondensation. The decondensation process is reversed in a stepwise way upon prolonged culturing. Tanurdzic et al. (2008) observed hypermethylation of euchromatic DNA in a long-term cell suspension of *Arabidopsis thaliana* that had lost the capacity to readily regenerate shoots. Law and Suttle (2005) observed rapid and reversible changes in DNA methylation and histone modifications in the course of 15 days after establishment of a potato cell suspension.

Tanurdzic et al. (2008) proposed that dedifferentiation initially involves the loss of epigenetic marks. Accordingly, the expression of maintenance DNA methylases, such as MET1 in *Arabidopsis thaliana*, is lower during the initial phase of a cell suspension culture. Subsequently, new epigenetic marks need to be established. Indeed, the expression of *de novo* DNA methylases and various histone modifying enzymes such as histone deacetylases (HDACs) is generally increased in tissue culture, while that of DNA demethylase ROS1 is lowered (Law and Suttle 2005; Tanurdzic et al. 2008). Mistargeted *de novo* methylation would allow the establishment and perpetuation of DNA hypermethylation of promoters, which would create epialleles, and hypomethylation of repetitive DNA, which may lead to activation of mobile elements. Krizova et al. (2009) examined the development of a tobacco cell culture into callus followed by plant regeneration. They observed the loss of parental imprinting in 25% of the regenerated tobacco plants, regardless of whether the epiallele was originally methylated or unmethylated.

In micropropagation companies, the most frequently used tissue culture procedure involves inducing outgrowth of existing axillary buds by exogenous application of cytokinin or removal of the apical bud. It is generally believed that in this way very little or no genetic variation is induced (De Klerk 1990). Peredo et al. (2009) evaluated hop cultures with molecular techniques namely random amplified DNA polymorphism (RAPD), retrotransposon microsatellite amplified polymorphism (REMAP), and methylation-sensitive amplification polymorphism (MSAP). They did not detect any genetic variation between the original plants and propagated plants even after 12 cycles (2 years) of micropropagation. It should be noted that with molecular methods only a very small portion of the genome is screened, so there may actually still be abundant genetic changes. Epigenetic changes, though, were easily detected in these plants with molecular methods. Nearly 30% of the changes present in the polymorphic loci were shared by all in vitro plants. Lower levels of epigenetic variation were detected among plants from the different subcultures. The most common difference between field plants and in vitro plants was demethylation of cytosines in some restriction targets, representing 63% of the observed changes. Smýkal et al. (2007) used various molecular methods to assess the genetic stability of long-term (24 years) propagated shoot cultures of pea. No differences were found using microsatellite, inter-simple sequence repeat (ISSR) or inter-retrotransposon amplified polymorphism (IRAP) markers. However, amplification fragment length polymorphism (AFLP) and particularly methylation-sensitive amplification polymorphism (MSAP) detected 11 and 18% polymorphism, respectively. An examination of the global cytosine methylation status revealed no significant differences. Zeng et al. (2009) examined integration and expression level of transgenes during long-term micropropagation of transgenic birch. There was a stable integration of transgenes into regenerated plants, but expression levels of the transgenes among shoots subcultured 4, 7, 9 or 15 times were significantly different: the transcriptional expression level of transgenes decreased with increasing subculture number. The silencing rate of beta-glucuronidase (GUS) in the fifth subculture plants was 22–65%. 5-Azacytidine, a drug that causes global hypomethylation, reactivated transgene expression at 50–200 μM, indicating that the decrease in expression level with increasing number of subcultures was associated with DNA methylation.

Epigenetic changes of plants propagated in vitro: rejuvenation and habituation

Rejuvenation

During their lifetime, plants pass through a developmental process referred to as 'phase change'. Immediately after germination plants are juvenile. They are unable to flower, even when the conditions are optimal. After some time, depending upon the species ranging from a few weeks to decades, plants become adult and capable of flowering. This phase change may be accompanied by morphological alterations such as changes in leaf shape and direction of stem growth. From the horticultural point of view, the change in the capability to root is important. Cuttings from the juvenile phase are usually easy-to-root while cuttings from the adult phase are recalcitrant. When adult buds are cultured in vitro, though, they often undergo the reverse change (rejuvenation) within a few subculture cycles. Rejuvenation becomes apparent as improved rooting (Webster and Jones 1989). The change in the ontogenetic phase is long-lasting: Cuttings taken from micropropagated plants that have been grown in the field, still have the improved capacity to root (Jones and Webster 1989). In birch, Jones et al. (1996) found that micropropagated trees flowered sooner than seedlings indicating that not all features of the micropropagated trees had become juvenile. The ontogenetic state (juvenile vs. adult) has been related to the methylation status of DNA. In Pinus radiata, for example, the extent of DNA methylation is higher in mature meristematic regions when compared to juvenile meristematic regions (Fraga et al. 2002). In addition, histone modifications (acetylation and methylation) play a major role (Valledor et al. 2010). Irish and McMurray (2006) observed that the cytosine methylation pattern of Pl-Blotched, an allele of purple plant1 (pl1), which encodes a myb-like transcription factor that regulates anthocyanin production in maize, in culture-rejuvenated plants resembled the hypomethylated state of seedlings, rather than the hypermethylated state in adult maize organs they were derived from.

Habituation

Plant tissues usually require auxin and cytokinin for continuous proliferation in tissue culture. In 1942, Gautheret reported that strains of carrot tissue gradually lost the requirement for exogenous auxin. This is referred to as auxin habituation. It was found later that habituation for cytokinin also occurs. Habituation is now defined more generally as a stable heritable loss in the requirement of cultured plant cells for growth factors (Meins 1989). The epigenetic nature of habituation is shown by the observation that plants regenerated from habituated cell cultures have the original requirement for hormones when cell cultures are established from them. The habituation shift occurs at rates that are orders of magnitude higher than mutations rates in plants (Meins 1989). One approach towards the identification of the mechanism of habituation is a comparison of gene expression in habituated and

non-habituated cell cultures. A transcriptome-based analysis of habituated and non-habituated *Arabidopsis thaliana* plant cell cultures revealed the differential expression of more than 800 genes, including a 19-fold up-regulation of the transcript encoding the cytokinin receptor CRE1. The alterations in gene expression found among habituated and nonhabituated callus cultures do not result simply from global hypomethylation of DNA (Pischke et al. 2006). Nevertheless it has been reported that treatment of in vitro cultured cells of a nontumorous *Nicotiana glauca* × *N. langsdorffii* hybrid with 5-azacytidine, a drug causing hypomethylation, leads to the induction of a habituated phenotype (Durante et al. 1989).

Epigenetic aberrations observed in micropropagated plants

Commercial micropropagation operates since the 1970s–1980s. The main advantages include high speed of propagation and production of disease-free, superior, clonal starting-material. During tissue culture, existing epigenetic differences may become visible, e.g., as the differential performance of buds taken from several positions on the same plant. Webster and Jones (1989) reported that two apple lines established from different buds at the same plant remained different with respect to propagation and rooting after a large number of subcultures. Similar observations were made with four lines of *Helleborus sp.* (G. de Klerk, unpublished observations). Baránek et al. (2010) reported differences in DNA methylation between lines established from different nodes on the same parent plant. In several crops unwanted epigenetic variation caused in tissue culture is a major drawback. Below we describe cases that have been examined in some detail.

Bushiness in Zantedeschia and gerbera

Zantedeschia is a relatively 'new' ornamental crop that is propagated in vitro on a large scale. The propagation factor is low, for many cultivars less than two per subculture cycle of 4 weeks (C. Randag, pers. comm.). To increase propagation, micropropagation companies have been using high cytokinin concentrations but in sensitive genotypes this resulted in bushiness after transfer to soil. Figure 2 shows bushy *Zantedeschia* plants. Bushiness is a long-lasting disorder and tubers that generate bushy plants in the first years after tissue culture also generate bushy plants during the following years. A cultivar that is sensitive to bushiness, Florex Gold, was most sensitive to cytokinin compared to other cultivars in a test in which the effect of cytokinin on root growth was determined (D'Arth et al. 2002), but there were no significant differences in cytokinin content between

bushy and nonbushy plants (D'Arth et al. 2007). In this regard it should be noted that the conventional concept of hormonal control of apical dominance, involving only auxin as inhibitor and cytokinin as stimulator of outgrowth of axillary buds, is an oversimplification (Ongaro and Leyser 2008; Werbrouck and Debergh 1995). *Zantedeschia* is relatively prone to the induction of aberrations in tissue culture and various other aberrations occur such as large leaves (called "elephant ears") and genetic mosaic disease (Fig. 2).

Another case that has been studied in some detail is bushiness in gerbera. Anatomical analysis of longitudinal serial sections of bushy shoots shows that the apical meristem and the axillary buds have the same potential for further development, i.e., axillary bud primordia are not inhibited and develop instantaneously (Topoonyanont et al. 1999). The type of explant has a major effect on bushiness, the main shoot axis being the most sensitive. Higher cytokinin concentrations also enhance bushiness (Topoonyanont and Debergh 2001). Interestingly, addition of imazalil reduces bushiness. Imazalil is a fungicide that interferes with cytochrome P450 enzymes. These enzymes are involved in the metabolism of many xenobiotics and plant hormones.

Tissue proliferation in rhododendron

An abnormal phenotype has been observed in container-grown rhododendron cultivars initially propagated by tissue culture. This aberration has been named "tissue proliferation" and has also been found in azaleas and *Kalmia latifolia*. The plants display an abnormal tumour-like growth at or near the crown of the plant, and in some genotypes occasionally on aerial plant parts. The abnormal growth appears similar in morphology to crown galls, but tissue proliferation is not equal to crown gall development as *Agrobacterium tumefaciens*, the causal agent of crown gall, has not been detected. In some cultivars, the crown tumours produce shoots that are smaller than normal, with short internodes and a whorled arrangement of small leaves. In many affected cultivars the disorder is only observed during the first 1–3 years after initial propagation, resulting in significant financial losses for nurseries. Brand (2000) and Brand et al. (2000) reported that plants displaying tissue proliferation have an altered hormone metabolism or sensitivity. This change is apparently stable over many years but has disappeared after some 6 years. Higher cytokinin levels in vitro lead to a greater incidence of tissue proliferation in the field (Brand 2000).

Flower malformation in oil palm

Oil palm can be propagated *via* callus and somatic embryogenesis. Plants that have been generated in this

Fig. 2 Various *Zantedeschia* off-types. The plants have been grown from T1 (D'Arth et al 2002) tubers. Normal (**a**); genetic mosaic (**b**); bushy plants (**c**); and "elephant ears" (**d**). (Photos were kindly provided by C. Randag MSc, 't Zand, The Netherlands.)
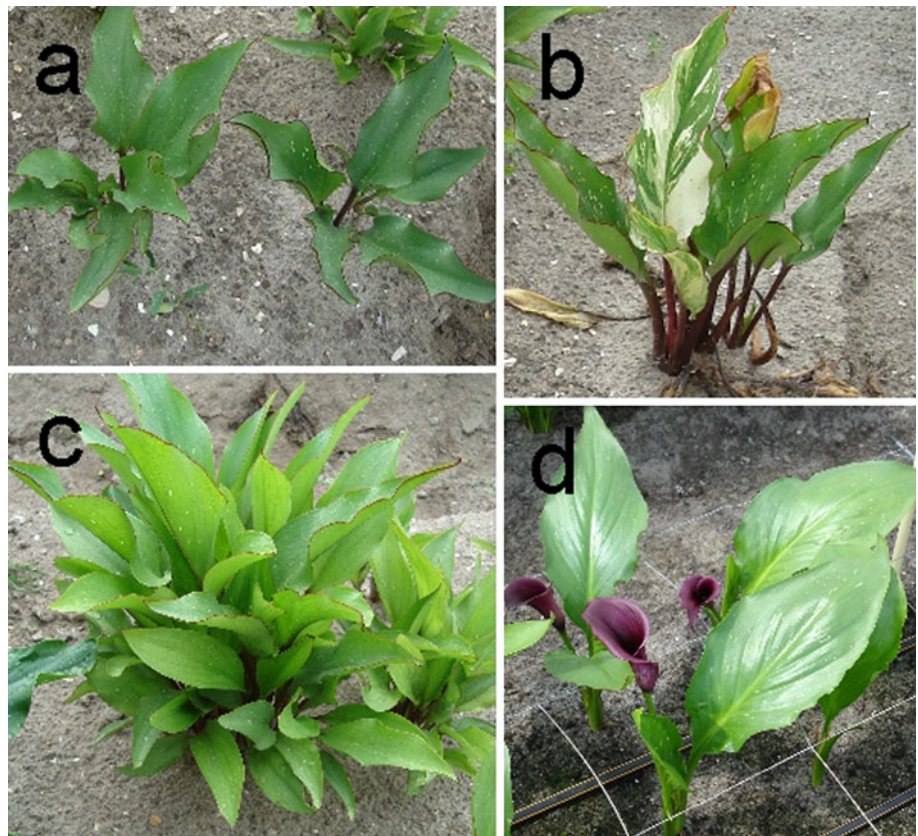
way, may show an aberration in their flowers, referred to as 'mantled'. These flowers develop a second whorl of carpels instead of stamens (Alwee et al. 2006). Most often the fruit does not develop properly (Corley et al. 1986) (Fig. 3). This causes a major financial loss, in particular as this abnormality is only detectable once palms start flowering, i.e., after 2 to 3 years in the plantation. Eeuwens et al. (2002) performed a careful study to find out which tissue culture conditions caused the malformed flowers and found that the disorder occurred more frequently in sensitive genotypes, when short subculture periods were used and when high cytokinin/low auxin was added. The disorder did not seem to be related with endogenous cytokinin levels (Jones et al. 1995). As this is typically a homeotic change, alterations in the homeotic genes that participate in the determination of floral organ identity should be considered (Alwee et al. 2006). Almost all of these are members of the MADS box transcription factor family. As the change occurs regularly, one would expect an epigenetic rather than a genetic change to underlie the abnormality. Indeed, although some genetic changes were found, these did not correlate with the occurrence of the morphological aberration (Matthes et al. 2001). Random DNA methylation changes were found among regenerated palms, and these did not correlate with the occurrence of the abnormality either (Jaligot et al. 2002).

Fig. 3 A fruit bunch from a normal oil palm (**a**) and from a palm with aberrant, 'mantled' flowers (**b**)

Leaf shape in begonia

Searching for a method to quantify the extent of variation, De Klerk et al. (1990) determined the standard deviation of the leaf shape (measured as the natural logarithm of the ratio of length and width) in populations of regenerated plants. Plants had been regenerated either directly from leaves or after an intermediate callus phase at a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D). The leaf shape itself was the same in both populations but

the standard variation of the leaf shape was far larger in the 2,4-D population. Addition of the chemical mutagens *N*-methylnitrosourea (NMU) during direct regeneration also increased the standard variation significantly. As the variation was random—in some plants the width of the leaves was increased and in others the length—, the detected variation was expected to be genetic. However, when new plants were produced by direct regeneration from the leaves of plants produced *via* callus, the standard variation of the leaf shape almost dropped to the value of the control. The plants produced after NMU treatment, though, displayed the same high level of the standard variation after adventitious regeneration. This indicates that the larger part of the variation in 2,4-D plants was epigenetic (Bouman and De Klerk 2001).

Is epigenetic variation caused by stress?

It was suggested above that epigenetic variation may be caused by incomplete resetting of epigenetic marks during adventitious regeneration, but there are likely additional origins. When plants experience stress, they show a multitude of responses, which may include epigenetic variation (Molinier et al. 2006). Oh et al. (2007) hypothesised that labile portions of the genome are affected by stress during micropropagation of *Musa*. Alterations in methylation have been reported in response to water deficiency (Labra et al. 2002), osmotic stress (Kovařik et al. 1997), heavy metals (Aina et al. 2004), and—in tissue culture—heat (Baránek et al. 2010). Some loci acquired epigenetic silencing, while sometimes silenced loci increased expression. It seems evident that 'extreme' procedures such as protoplast culture and also callus formation impose stress. However, for many species shoot culture may very well be stressful (De Klerk 2007; Desjardins et al. 2009; Van Staden et al. 2006). In this respect, it should be noted that in vitro, the tissues grow under very unnatural conditions: Plantlets have been wounded, they are incomplete (protoplasts being an extreme example), they receive sugar from the nutrient medium as a replacement of photosynthesis in the leaves, they receive high doses of auxins and cytokinins, and their water balance is disturbed by the very high humidity in tissue culture containers. Important questions needing answers are: To what extent can epigenetic variation be ascribed to 'stress'? Which of the tissue culture factors are most stressful? Answers to these questions may lead to procedures that result in a reduction of epigenetic variation. One possible improvement of the procedures is in the choice of cytokinin. In micropropagation usually benzyl-aminopurine (BAP) is added but another cytokinin, meta-topolin, is often just as effective with respect to the breaking of apical dominance (so resulting in a similar

propagation factor) but has less negative side-effects (Bogaert et al. 2006; Valero-Aracama et al. 2009; but see Bairu et al. 2008).

Conclusions

Long lasting changes in the expression of DNA are increasingly documented in plant development. They have been studied in relation to the ability to flower (vernalization) and the ontogenetic development from juvenile to adult. In these cases both methylation of DNA and alterations in histones have been shown to be involved. In plant tissue culture, changes in DNA methylation and in histone modifications have also been reported in many cases, ranging from protoplast formation to axillary shoot proliferation. Such changes are believed to underlie the generation of off-types, e.g., the mantled phenotype in oil palm and bushiness in *Zantedeschia*. Molecular research on well-defined off-types has been limited with the exception of oil palm. In oil palm, in spite of extensive research, molecular characterization of the aberration is still unsuccessful. This lack of insight in the molecular basis of epigenetic off-types impedes progress in the development of methods to avoid or counteract these aberrations.

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