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Epigenetic aspects of somaclonal variation in plants

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Abstract

Somaclonal variation is manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, and gene activation and silencing. Activation of quiescent transposable elements and retrotransposons indicate that epigenetic changes occur through the culture process. Epigenetic activation of DNA elements further suggests that epigenetic changes may also be involved in cytogenetic instability through modification of heterochromatin, and as a basis of phenotypic variation through the modulation of gene function. The observation that DNA methylation patterns are highly variable among regenerated plants and their progeny provides evidence that DNA modifications are less stable in culture than in seed-grown plants. Future research will determine the relative importance of epigenetic versus sequence or chromosome variation in conditioning somaclonal variation in plants.

Introduction

Variation in plant phenotype is determined by genetic and epigenetic factors. Phenotypic and DNA variation among putative plant clones is termed somaclonal variation. The purpose of this review is to describe evidence indicating that epigenetic variation is an important mechanistic basis of somaclonal variation in plants.

Somaclonal variation is defined as genetic and phenotypic variation among clonally propagated plants of a single donor clone (reviewed in Sunderland, 1973; D'Amato, 1977, 1985; Bayliss, 1980; Larkin and Scowcroft, 1981, 1983; Orton, 1984; Ahloowahlia, 1986; Larkin, 1987; Lee and Phillips, 1988; Sun and Zheng, 1990; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993a; Duncan, 1997; Kaeppler *et al.*, 1998; Veilleux and Johnson, 1998; Olhoft and Phillips, 1999). Somaclonal variation caused by the process of tissue culture is also called tissue culture-induced variation to more specifically define the inducing environment. Somaclonal variation can be manifested as either somatically or meiotically stable events. Somatically stable variation includes phenotypes such as habituation of cultures and physiologically induced variation observed among primary regenerants. This type of variation is often not transmitted to subsequent generations and is of most impact in situations where the primary regenerant is the end product such as the amplification of ornamental plants or trees for direct use. Meiotically heritable variation also occurs and is important in situations where the end product of the tissue culture is propagated and sold as seed. Mechanisms producing both somatically and meiotically heritable variation also contribute to the decline in vigor and regenerability of cultures over time. The loss of culture health with time is a major detriment to the efficiency of transgenic plant production and much effort has been devoted to avoiding this problem.

Epigenetic control of gene expression can be defined as a somatically or meiotically heritable alteration in gene expression that is potentially reversible and is not due to sequence modification. Epigenetic aspects of somaclonal variation would therefore involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change. These changes might be unstable or reversible somatically or through meiosis, although certain epigenetic systems outside of tissue culture are quite

stable for many generations (Patterson *et al.*, 1993; Cubas *et al.*, 1999). Therefore, epigenetic changes induced by tissue culture could be manifested as the activation of quiescent loci or as epimutation of loci sensitive to chromatin-level control of expression.

Somaclonal variation is manifested as quantitative and qualitative trait mutation, karyotype changes, and sequence modification

Somaclonal mutant phenotypes segregate as qualitatively and quantitatively inherited mutations

Somaclonal variation was first detected by the high frequency of qualitatively segregating phenotypes observed among progeny of plants that were expected to be genetically identical (reviewed in Larkin and Scowcroft, 1981, 1983; Orton, 1984; Ahloowahlia, 1986; Larkin, 1987; Sun and Zheng, 1990; Peschke and Phillips, 1992; Kaeppler and Phillips, 1993a). This was especially true in diploid species such as maize where mutations could be easily observed and were not obscured by genetic buffering that is prevalent in polyploid species. For example, a study of maize grown for 8 months in culture found that, on average, every regenerated plant contained 1.32 mutants that produced a visible phenotype (Lee and Phillips, 1987b). These qualitative mutants were stably inherited for several subsequent seed-derived generations.

Detailed phenotypic analyses in later studies showed that quantitative variation is also frequently found among regenerant-derived progeny (reviewed in Duncan, 1997; Veilleux and Johnson, 1998). Quantitative variation has been described for many phenotypes including plant height, plant biomass, grain yield, and agronomic performance (e.g. Earle and Gracen, 1985; Zehr *et al.*, 1987; Lee *et al.*, 1988; Carver and Johnson, 1989; Dahleen *et al.*, 1991; Bregitzer *et al.*, 1998). A generalization of studies that have assessed quantitative variation is that quantitative variation is frequent and inheritance studies indicate alteration of numerous loci.

Tissue culture variation has also been harnessed in some cases to confer desirable traits to cultivars including desirable morphological traits, disease resistance, insect resistance, acid tolerance, and salt tolerance (Duncan, 1997; Veilleux and Johnson, 1998). While there are a few examples of variants that are unstable such as the variegated Empress tree variety 'Somaclonal Snowstorm' (Marcotrigiano and Jagannathan, 1988) and an unstable flower color mutant of alfalfa (Groose and Bingham, 1986), the general lesson from the analysis of phenotypic variation across species is that mutation is frequent and often inherited stably through sexual generations.

Cytogenetic abnormalities and specific sequence changes are frequently found in regenerated plants and their progeny

Chromosome-level mutations

Chromosomal and sequence changes are prevalent in culture, consistent with the high frequency of observed phenotypic variation. Cytogenetic abnormalities including ploidy changes and chromosome rearrangements have been found among tissue culture regenerants. Hang and Bregitzer (1993) found ploidy changes to be the most prevalent cytological change among barley regenerants, although chromosome breakage events also occurred. A comparative analysis in oat and maize showed that chromosome breakage occurred more frequently than ploidy changes (reviewed in Benzion *et al.*, 1986; Kaeppler *et al.*, 1998). Translocations were the most frequent chromosomal abnormality observed with inversions and insertions/deletions also occurring. The fact that most breakages were either between heterochromatic knobs and the centromere in maize, or within the centromeric heterochromatin in oat, led to the hypothesis that replication of heterochromatin occurred late in tissue culture leading to chromosome bridges and breakage events (Johnson *et al.*, 1987). Chromosome breakage events also involved heterochromatin in the Hang and Bregitzer (1993) study on barley, further supporting this hypothesis.

Sequence variation

Sequence variation arising through the culture process has been detected in several different ways (reviewed in Kaeppler *et al.*, 1998; Olhoft and Phillips, 1999) including random genome scanning using RFLPs or RAPDs, and analysis of specific mutants. Variant storage protein or isozyme electrophoresis profiles suggest sequence change occurs much more frequently than gene deletion since migration profiles are altered, but null alleles are rare. A direct link between variant protein and DNA sequence change has only been established for two events (Brettell *et al.*, 1986; Dennis *et al.*, 1987). In these studies, tissue culture-derived mutant *Adh1* alleles were found to be due to two independent point mutations, both A-to-T transversions.

Methylation pattern instability

DNA methylation variation has been hypothesized as an underlying mechanism of tissue culture-induced mutagenesis due to the high frequency of quantitative phenotypic variation, the activation of transposable elements, heterochromatin-induced chromosome-breakage events, and the high frequency of sequence change. Phillips *et al.* (1994) proposed that DNA methylation variation conditions chromatin changes which ultimately cause quantitative variation by modulating the effects of multiple loci, chromosome breakage by altering timing of replication, and base changes through a process similar to RIP in fungi (Selker and Stevens, 1985). While none of the hypothesized effects of DNA methylation change on tissue culture variation have been confirmed, DNA methylation patterns have been shown to vary among regenerated plants and their progeny in a number of studies.

Methylation patterns were first reported to vary among regenerated plants and their progeny in rice (Brown *et al.*, 1990) and maize (Brown *et al.*, 1991). Kaeppler *et al.* (1993b) reported that DNA methylation patterns varied substantially among maize regenerant-derived families from the same cultured explant, with all families having unique methylation profiles across 20 single-copy probes. In this study, a significant frequency of the methylation changes did not segregate among R_1 progeny, indicating that the changes were homozygous in the primary regenerant. In addition, hypomethylation was much more frequent than hypermethylation. Olhoft (1996) conducted a more extensive analysis of methylation variation among regenerant-derived progeny using 206 single-copy probes. This work corroborated the observation that the majority of methylation changes are decreases, with decreases in methylation occurring three times as frequently as increases. Quantification of global methylation levels on these same plants indicated a significant increase in methylation overall. It is not known if the overall increase in methylation was due to increased methylation of repeated sequences or to another mechanism such as genome expansion due to gene amplification or retrotransposon activity.

Global methylation changes have also been studied in tissue culture. LoSchiavo *et al.* (1989) showed that global methylation levels changed in response to hormone concentration in the media of carrot cultures. Methylation levels decreased with increasing concentration of kinetin, but increased with increasing amounts of the auxin 2,4-D. In this study, methylation levels were also developmentally regulated, being 'reset' as cells were induced to embryogenesis. Arnholt-Schmitt *et al.* (1995) also reported that global methylation levels of carrot culture changed in a growth-phase-dependent manner. A companion study (Arnholt-Schmitt, 1995) found that the copy number of repeated sequences also changed through development, concurrent with the methylation change. Therefore, the combined data suggest that methylation reduction may have occurred due to genome diminution rather than changes in the frequency of methylated target sites.

Studies of both global methylation levels and methylation of specific sites show that variation in DNA methylation occurs frequently in the culture process. Global methylation studies support the idea that developmental timing may play a role in effecting variation in methylation levels and patterns.

Transposon activation and some examples of gene silencing indicate that epigenetic mechanisms contribute to the process of somaclonal variation

The observation of chromosome breakage events in culture supports the contention that tissue culture induces a genomic shock. Genomic shock has been shown to activate transposable elements in other systems (McClintock, 1984), so it was not unexpected that *Ac* (Peschke *et al.*, 1987) and *Spm/En* (Peschke and Phillips, 1991) activity was detected among regenerant-derived progeny. The frequency of independent regenerants with active elements is quite low, but these studies clearly show that element activation occurs through the culture process.

Hirochika *et al.* (1996) showed that retrotransposons also have enhanced transcription in culture. Retrotransposons are another class of DNA elements that are generally quiescent in the genomes of plants. In the Hirochika *et al.* (1996) study, three rice retrotransposon families (*Tos10*, Tos17, and *Tos19*) had increased transcription rates as cell cultures aged. Sequence analysis of flanking sequences indicated that retrotransposon integration occurred into genic regions. No phenotype was specifically correlated with the insertion events.

Quiescent transposons and retrotransposons exist in the genomes of many plant species, and their activation in tissue culture supports the notion that derepression of epigenetically silenced sequences is induced by the culture process. Several interesting observations were made during the study of transpos-

able elements in culture. Brettell and Dennis (1991) showed that a recently inactivated *Ac* element was reactivated at a high frequency in culture, a frequency much higher than observed in seed-derived plants. Reactivation was correlated with reduced methylation. This result indicated that tissue culture does destabilize epigenetically silenced events, and suggests that recently silenced elements can be activated more easily than other quiescent elements in the genome. Molecular analysis of activated *Ac* elements by Peschke *et al.* (1991) found that there was not an exact correlation between activity and methylation state. Therefore, reduction in DNA methylation may not be responsible for the activation, but may occur over time keeping elements in an active, or easily 'activatable', state. Interestingly, none of the maize regenerant-derived lines with transposable element activity had cytogenetic abnormalities that would have supported a chromosome breakage event occurring in their lineage. Therefore, the genomic shock induced by culture may not be initiated by chromosome breakage. Rather the instability of repeated sequences and the late replication of heterochromatin may be due to genome stresses which also activate elements.

Another intriguing mutant putatively resulting from epigenetic silencing is a dwarf mutant in rice identified by Oono (1985). This mutant was first observed as a 'homozygote' in the primary regenerant and could be maintained through sexual self-generations. However, the mutant showed a low frequency of reversion and could not be maintained in crosses of the regenerant-derived progeny to uncultured plants. Interestingly, treatment of dwarf types with the DNA methylation inhibitor 5-deoxyazacytidine resulted in the restoration of the normal phenotype. This result further indicated that the dwarf mutant was the result of a reversible, epigenetic silencing event.

These examples indicate that epigenetic repression and derepression does occur during the tissue culture process. To date, there has not been a mutant phenotype reported, outside of transposable elements, where the basis of the mutation was shown to be epigenetic by both genetic and molecular analysis.

An example in maize of a locus potentially sensitive to silencing induced by the culture process

We have recently analyzed a series of white cob mutants in maize and have evidence to suggest that gene silencing may be responsible for a mutant phenotype in these lines. The genetic pathway conditioning cob color in maize is well defined and the genes in the pathway have been cloned. The *p* locus is the transcription factor that activates the pigment pathway, and the genotype used as an explant source contained the *P-wr* allele at this locus. The *P-wr* allele conditions red pigmentation of the glumes of the cob, but not the pericarp, and is a complex locus with seven repeats (Chopra *et al.*, 1996). The P locus activates three metabolic genes, *a1*, *c2*, and *chi*, which metabolize a red phlobaphene pigment. The *a1*, *c2*, and *chi* loci are single-copy genes, although there are non-complementing orthologues at homeologous positions in the genome. The tissue culture-induced mutants under analysis were all identified among progeny of B73Ht or LH51 plants regenerated after 3 to 5 months in culture.

The first intriguing result in this study was that complementation analysis indicated that six of the nine white cob mutants were allelic to *p*. One of the mutants was allelic to *c2* and the other two were not allelic to *p*, *c2*, or *a1*. A *chi* tester stock was not available. This result is intriguing because of the preponderance of mutations at the *p* locus. The *P-wr* allele has a lower than average mutation frequency in most mutagenesis experiments, a fact attributed to the likely expression of more than one of the copies of this complex locus. Therefore, the frequency of *p* mutants is expected to be less than 1 out of 4, and certainly not the predominant type. This led to the conclusion that the *p* locus was more sensitive than the enzymatic loci to some type of tissue culture-induced mutagenic process.

The second intriguing result was the molecular analysis of the mutants. None of the mutants with known complementation had large deletions as resolved using restriction enzyme analysis. Therefore, the observed mutations could not be explained by mechanisms such as localized transposition or intragenic recombination. However, Southern analysis with methylation-sensitive restriction enzymes indicated that the *p* locus had substantial hypermethylation across the complex in the six *p* mutants, but not in the other mutants (Figure 1). No apparent methylation change was observed in the *c2* mutant when probed with sequences from that locus. Therefore, these results suggest the possibility that mutants at the *p* locus were unexpectedly frequent due to sensitivity to epigenetic silencing.

We are currently exploring whether epigenetic silencing is the molecular basis of the P mutants or if

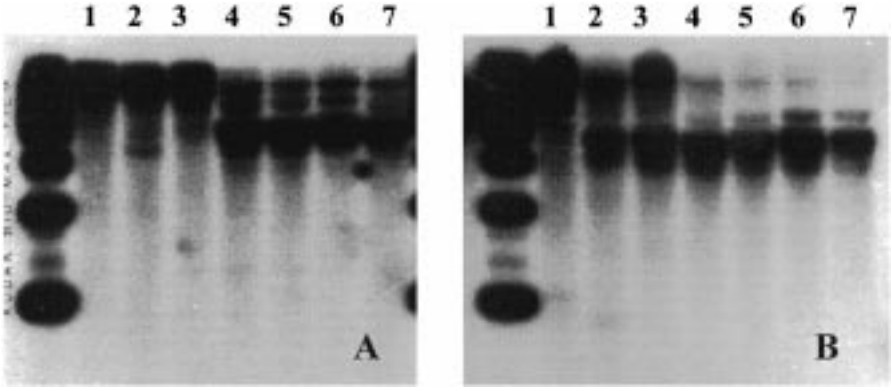

Figure 1. DNA of tissue culture-induced, white-cob mutant and non-cultured control plants restricted with the methylation-sensitive restriction enzymes *Hpa*II (A) and *Hha*I (B) and probed with a sequence specific to the P locus. Lanes 1–3 are independent white cob mutants allelic to P, lanes 4 and 6 are white cob mutants not allelic to P, and lanes 5 and 7 are the uncultured control. Methylation at the P locus is increased in the mutants allelic to P, but not in the other genotypes, relative to the uncultured control.

hypermethylation occurred subsequent to a different mutagenic event. However, these results indicate that epigenetic silencing may be responsible for six independent tissue culture-induced mutants in maize. If so, this result raises the question of why the P locus is apparently more sensitive to modification than other genes. Certain *p* alleles have been shown to be subject to epigenetic control (Lund *et al.*, 1995), so it is possible that this locus is inherently more sensitive to epigenetic control and that tissue culture causes an increased level of epigenetic change at this locus. Note that the methylation change in the *p* mutants is hypermethylation whereas single-copy sequences in regenerant-derived plants generally show hypomethylation. Another intriguing aspect of the *P-wr* allele is its repeated structure. Repeats have been shown to trigger methylation change in several systems (reviewed in Wolffe and Matzke, 1999), so it is possible that the repeated structure of the *P-wr* allele triggers epigenetic silencing. Further analysis of these particular mutants should allow us to determine if epigenetic silencing is the molecular basis of the mutant phenotype. However, additional studies will be necessary to determine if endogenous repeats are inherently less stable in culture, and if genes subject to epigenetic control outside of culture are more likely to be mutated in culture.

How important are epigenetic mechanisms as a basis of somaclonal variation? Models and speculation

Plant regeneration bypasses the normal developmental process of fertilization and plant development potentially resulting in instability of epigenetic patterns

Tissue cultures are initiated from a number of different explant sources, many of which (such as leaf blades) are highly differentiated. Cells from these explants dedifferentiate resulting in totipotent cultures from which plants can be regenerated. Since the process of dedifferentiation and regeneration bypasses the normal fertilization and development events of seed-grown plants, it is possible that the normal epigenetic program is not established in regenerated plants. Imprinting is thought to occur primarily in the endosperm and not in diploid somatic tissue (Messing and Grossniklaus, 1999), so imprinted genes may not be a target for variation. However, epigenetic mechanisms are likely involved in the process of plant development (Richards, 1997), and parts of the process of patterning may be bypassed in the regeneration process. Primary regenerants (R_0) are often more variable than their progeny. Examples of aberrant phenotypes in regenerated plants include abnormal leaf structures and variant floral morphology. One example of a variant phenotype is the observation that many primary maize regenerants have seed development on the tassel. These types of developmental abnormalities in the primary regenerants support the notion that developmental patterns are less tightly followed in plant

regeneration. The deviation from the normal developmental pattern may be important for totipotency, but may also cause phenotypic variation which is not meiotically heritable.

If epigenetic patterns are part of the normal pattern of development, then certain explants which have reached an advanced stage of development produce callus representing cells with different epigenetic patterns. Studies of qualitative mutations from tissue culture indicate that mutations accumulate sequentially with time in culture (Fukui, 1983; Zehr *et al.*, 1987). For example, plants regenerated from a three-month old culture may contain a dwarf mutation, and a subset of plants regenerated from the same culture after six months contain both the dwarf mutation and an additional mutation such as leaf stripes. Sequential accumulation of mutations over time provides evidence that mutations are occurring during the culture process and not pre-existing in the explant.

However, Matzke and Matzke (1996) provide evidence that pre-existing variation in the explant should not be overlooked, especially in the case of epimutations. In this study, the authors implemented a sequential transgenic strategy using hygromycin resistance and kanamycin resistance markers to show that different epigenetic states of expression could be obtained from regenerated plants from the same explant. Specifically, leaves of Hyg^R transgenics contained mosaic sectors for Hyg^R expression. Culture of leaf sectors from these Hyg^R plants, followed by a secondary transformation with selection for kanamycin resistance to generate cultures derived from single cells, showed that double transgenic cell lines with different epiphenotypes were obtained. These epiphenotypes appeared to reflect the mosaicism of the Hyg^R primary regenerant leaf.

Developmentally programmed epigenetic patterns and resetting of those patterns, therefore, may be quite important in somaclonal variation. These patterns may be variable within the explant source, and resetting of the patterns outside the normal process of meiosis and fertilization may be imprecise. The most pronounced effect of variation resulting from pre-existing epigenetic patterns may be the somatically heritable, but meiotically reversible phenotypes observed in T_0 plants. In some cases, these pre-existing patterns may also be heritable as shown in the transgenic experiment by Matzke and Matzke (1996), a study indicating that pre-existing variation may be more frequent than generally thought. The testable hypothesis in this case is that more mature explant sources would produce a higher rate of observed mutation in regenerated plants and their progeny.

An attractive aspect of the hypothesis that developmental epigenetic states are reprogrammed during tissue culture is that it seems consistent with the observation that most methylation changes observed in regenerated plants and their progeny are decreases in methylation. Assume that the process of development involves epigenetic repression of sets of genes once developmental patterns are determined, an assumption consistent with models of patterning and the trend toward increased methylation with age in plants and animals (Razin and Cedar, 1993; Finnegan *et al.*, 1993). By this model, methylation of genes would increase through development. Dedifferentiation would remove the repressive chromatin states at developmental genes, thereby allowing totipotency. However, if epigenetic components of the normal developmental regime require a process of programming that is initiated with gamete formation and fertilization, then the epigenetic re-programming may not occur normally during the process of regeneration. A consequence of this hypothesis is that methylation at specific loci would decrease during dedifferentiation, but not re-establish normally during regeneration. A corollary of this hypothesis is that genes involved in the developmental process would be more subject to epigenetic instability in culture than 'housekeeping' genes.

Can epigenetic mechanisms be responsible for the array of genetic changes occurring through the tissue culture process?

Genetic variation in culture is primarily embodied as cytological changes including ploidy changes and chromosome breakage, single-gene mutations most likely due to base changes, and gene activation/inactivation including transposons (reviewed in Kaeppler *et al.*, 1998). The following discusses the possibility that an epigenetic basis could be responsible for each of the major types of changes seen.

Cytological changes

The primary cytological changes observed among regenerated plants and their progeny include chromosome rearrangements and changes in chromosome number. Data from oat and maize indicate that chromosome rearrangements are primarily due to chromosome breakage events (summarized in Kaeppler and Phillips, 1993a). In maize, chromosome breakages occur between the centromere and the distal hete-

rochromatic blocks called knobs (Lee and Phillips, 1987a). In oat, breakage events occur primarily in the centric heterochromatin (Johnson *et al.*, 1987). The involvement of heterochromatin in the breakage events led to the hypothesis that heterochromatin replicates later than normal in the cell cycle during culture. Replication of the heterochromatic sequences does not complete at division, leading to anaphase bridges and subsequent chromosome breakage. This model is based on the observation that heterochromatic knobs are involved in chromosome breakage events in high-loss stocks of maize, presumably due to incomplete replication before mitosis (Rhoades and Dempsey, 1972, 1973).

Ploidy changes also occur in culture, with polyploidy generally occurring more frequently than aneuploidy. Aneuploidy, specifically monosomy and trisomy, could reasonably be due to the same nondisjunction mechanism giving rise to breakage events, or could result from neocentromeric activity of heterochromatin. Polyploidy, however, is more likely due to an endoreduplication event. Endoreduplication would be unlikely to be caused by late replication.

Possible mechanisms causing late replication of heterochromatin include cell cycle disturbances and metabolic difficulties such as imbalance of nucleotide pools (Peschke and Phillips, 1992). However, it is possible that late replication of heterochromatin has an epigenetic basis since timing of replication is controlled at the level of chromatin. Csink and Henikoff (1998) proposed that centromere function is related to the timing of replication and that functional centromeres replicate last. Late replication of centromeres is determined by the accumulation of heterochromatin-forming repeats, and other noncanonical, late-replicating, repeated sequences have the ability to form active centromeres in some cases. It is therefore possible that the primary cytological aberrations observed in tissue are caused by modification of the chromatin of repeats, perhaps with noncanonical sequences having a centromere function under conditions of genome stress.

Single-gene mutations

Qualitative mutation is frequent among tissue culture regenerants and the summation of protein assays, random DNA studies, and specific mutant analyses suggests that single-base changes, or very small insertions/deletions, are the basis of these changes (reviewed in Kaeppler, 1998). Two instances in which a mutant phenotype was explained by a specific mutation were reported by Brettel *et al.* (1986) and Dennis *et al.* (1987). In these studies, two independent mutations at the *Adh1* locus were the result of A-to-T transversion mutations.

Specific site mutations could be the result of the activity of certain types of transposons (one to a few basepair footprints), infidelity of synthesis or repair mechanisms, or base instability. Culture-induced activation of transposons has been demonstrated, but is likely not frequent enough to account for the number of changes observed. In addition, a much larger number of mutants would show instability and be explained by transposon insertion per se. Synthesis or repair mechanisms may have less stability in culture. Infidelity could result from physiological conditions, disruption in a methyl-directed mismatch repair system, or some other type of cellular stress. A methyl-directed mismatch repair system has not been shown to occur in higher eukaryotes, so it seems more likely that synthesis or repair infidelity would have a physiological basis if it is a common problem.

Phillips *et al.* (1994) suggested that a repeat-induced-point mutation (RIP) or methylation-induced-premeiotically (MIP) type mechanism could explain both the high frequency of base change and of methylation change. This hypothesis suggests that duplicate sequences in the genome, which peacefully coexisted under normal cellular conditions, begin to interact under the stress conditions of culture inducing a mutagenic process. No direct data currently exist to support the notion that duplicated sequences are more often mutated in culture, although increased diminution and amplification of repeated sequences in culture (e.g. Kidwell and Osborn, 1993; Arnholt-Schmitt, 1995), which could occur by intralocus somatic recombination, may indicate that repeated sequences are more available for interaction in cultured cells. As pointed out by Matzke and Matzke (1996), even if mutation is not occurring by a RIP process, it is still conceivable that an increased frequency of deamination of methylated cytosine or its metabolic precursors may raise the mutation rate indirectly through a methylation-based mechanism.

Activation and silencing of genes and transposons

Transposons and retrotransposons are activated by the culture process (Peschke *et al.*, 1987; Peschke and Phillips, 1991; Brettell and Dennis, 1991; Hirochika *et al.*, 1996), and we have described previously in this article a set of mutants putatively silenced through tissue culture. Therefore, heritable gene activation

and silencing are clearly part of the tissue culture process. The basis of the activation and silencing is not completely clear.

Transposable element activation has been shown to be induced by genomic shock (McClintock, 1984) which raises the possibility that element activation is a secondary effect of chromosome breakage. Several cases of transposable element activation in maize had no evidence of breakage events in their genome, indicating that chromosome breakage may not be a prerequisite for activity (Peschke *et al.*, 1987; Peschke and Phillips, 1991).

DNA methylation has previously been shown to correlate with transposable element activity (Chandler and Walbot, 1986; Chomet *et al.*, 1987; Banks *et al.*, 1988) which, in the context of the high frequency of methylation variation in culture, raises the possibility that hypomethylation in culture activates elements. This notion is supported by research on a cycling *Ac* element subjected to the culture process (Brettell and Dennis, 1991). In this study, plants containing a quiescent, unstable *Ac* element were cultured, and regenerated plants had a high frequency of element reactivation. Ac activation was correlated with the expected change in methylation. On the other hand, Peschke *et al.* (1991) analyzed *Ac* elements activated from tissue cultures and found that complete demethylation of diagnostic *Pvu*II sites was not observed in every case, although the trend was toward hypomethylation. These authors suggested that activation of the elements may precede a reduction in methylation, and that hypomethylation at specific sites may subsequently be involved in stabilizing the active state.

Summary

Evidence that epigenetic mechanisms play a role in somaclonal variation include activation of transposable elements and retrotransposons, putative silencing of genes, and a high frequency of methylation pattern variation of single-copy sequences. However, the extent of that role and the mechanistic basis of the process remain unknown. Do common mechanisms result in both chromosome breakage and transposon activation or does chromosome breakage induce activation of repressed genes, perhaps in a cell non-autonomous manner? Is replication of heterochromatin and non-disjunction of chromosomes the result of chromatin modification or due to a physiological or metabolic imbalance? If epigenetic change is a primary event in tissue culture mutagenesis, what cellular stress initiates the epigenetic change? These questions and many more remain to be answered in future experiments.

Study of somaclonal variation is relevant to applications such as *in vitro* plant propagation, plant transformation, and considerations in cloning of all eukaryotes. In addition, somaclonal variation is likely a reflection of response to cellular stress in other situations as well. Therefore, understanding the mechanism of tissue culture variation will be useful in defining cellular mechanisms acting in the process of evolution, and in elucidating the mechanism by which plants respond to stress. Epigenetic processes are likely to play an important role in this mechanism.

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