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An Analysis on Somaclonal Variation: A Tool for Crop Improvement and Tissue Culture

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Abstract – Somaclonal variation is an important phenomenon that can be observed at high levels in plant tissue culture. Although known to science since plant cell culture techniques were first developed, its origins remain mysterious. Here, we propose that misregulation of microRNAs and small RNA pathways can make a significant contribution to the phenomenon. For many reasons, micro-RNAs and related small RNAs appear ideal candidates.

Their mode of action gives them disproportionate influence over the transcriptome, proteome and epigenome. They regulate important developmental and physiological events such as meristem formation, phase changes and hormone responses. However, the genomic locations of microRNA genes and their unique biogenesis might make them unusually susceptible to aberrant regulation in vitro.

Fifteen somaclonal variants developed from adventitious shootderived callus from the cotyledonary explants of pigeonpea [Cajanus cajan (L.) Millsp.] var. 'ICPL 87' were assessed across two seasons for some agronomic and cooking quality characters. The mutant inbreds showed significant variation for days to maturity, plant height, seed size, seed colour and grain yield. Some of the somaclonal variants such as 'ICPL 99073', 'ICPL 99072' and 'ICPL 99070' were found promising and displayed significant positive changes for some important agronomic traits. Those include a change from small seed size and brown seed coat colour to more preferred large white seeds endowed with more seed yield. For grain yield, 'ICPL 99073' showed 25.3% yield advantage over the parent variety. The studies demonstrated the scope of genetic improvement in pigeonpea through deployment of somatic culture and exploitation of somaclonal variation.

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INTRODUCTION

The phenomenon of somaclonal variation has been recognized in plant tissue culture for decades, from its inception as a technique. Somaclonal variation can be briefly defined as changes in the somatic cells that occur during the mitotic divisions of a single clonal plant that result in variation in derived clonal individuals. However, although in vitro culture systems exhibit much higher rates of somaclonal variation, its origin largely remains enigmatic, despite the important scientific and commercial effects of this variation.Somaclonal variationcanbea negative element in the rapid production of homogenous end-product plant material, for example introducing deleterious traits into the resulting plants. Equally, it can be a positive force in the induction of novel variation and a source of useful newtraits.With increasingly powerful molecular tools available, it is time to propose, and test experimentally, new hypotheses to explain the generation of somaclonal variation.

Explanations for somaclonal variation: a shift from genetic to epigenetic The extent to which somaclonal variation is generated by genetic and epigenetic changes remains the subject of much research. Whilst there is clear evidence for genetic changes being important, from point mutations to cytological aberrations such as polyploidy, an epigenetic contribution to somaclonal variation is now apparent. Early work pointed to the genome-wide alterations downtoDNA(cytosine)methylation levels in plant material exposed to tissue culture conditions. Other molecular changes in tissue culture that are linked unambiguously to epigenetic systems result fromthe reactivation of previously 'silent' transposable elements (TEs) in the genome. For example, the elevated activity of retrotransposons in rice (Oryza sativa) tissue culture has been shown to result in mutations to the DNA sequence. However, the reactivation of TEs in tissue culture can have other subtler effects, such as influencing the transcriptional activity of local coding genes. The epigenetic regulatory system that governs TE silencing is now known to be complex, involving small RNAs, chromatin and DNA methylation. The uncovering of this sophisticated, multipartite 'epigenetic code' that

operates in the genomes of plants and other organisms has been hugely important progress in biology. Within the genome, particular locations can be targeted for combinations of 'repressive' epigenetic marks, for example DNA methylation and particular histones and their post-translational modifications that confer heterochromatic identity and hence suppress normal transcriptional activity. However, by altering these sets of epigenetic marks, the repression can be lifted and euchromatic identity that permits transcription takes over.

Micropropagation of woody plants and fruit crops constitutes a major success in the commercial application of in vitro cultures. An important aspect to be considered when deriving perennial plants from micropropagation is the maintenance of genetic integrity with regard to the mother plant. In this regard, somaclonal variation has been reported at different levels (morphological, cytological, cytochemical, biochemical, and molecular) in micropropagated plants. The economic consequence of somaclonal variation among regenerated plants is enormous in fruit crops and woody plants, because they have long life cycles. In consequence, the behaviour of micropropagated plants should be assessed after their long juvenile stage in field conditions. The occurrence of somaclonal variation is a matter of great concern for any micropropagation system. In order to evaluate its presence several strategies were used to detect somaclonal variants, based on one or more determinants from among morphological traits, cytogenetic analysis (numerical and structural variation in the chromosomes), and molecular and biochemical markers. In addition, studies on somaclonal variation are important for its control and possible suppression with the aim of producing genetically identical plants, and for its use as a tool to produce genetic variability, which will enable breeders the genetic improvement.

Somaclonal variation has been studied extensively in herbaceous plants, whereas few studies have focused on temperate perennial fruit crops. Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue cultures.

Somaclonal variation is also called tissue or culture-induced variation.

(Kaeppler, *et al*., 2000). Because the goal of synthetic seed production is to obtain clonal identity, controlling the somaclonal variation is a challenge.

Many causes have been identified or proposed for each type of variation; these, however, may vary from species to species and determining the genetic nature of the observed variation is difficult (Maraschin *et al*., 2002). These variation causes include: changes in the structure and/or chromosome number, noticeable point mutations, changes in the expression of a gene as a result of structural changes in the chromosome (heterochromatin and effects of position) or activation of transposable elements, chromatin loss, DNA amplification, somatic crossing over, somatic reduction and structural changes in the cytoplasmatic organelle DNA (Rao *et al*., 1992; Kaeppler, *et al*., 2000).

Evans & Sharp (1988) reported four critical variables for Somaclonal variation: genotype, explant origin, cultivation period and the cultural condition in which the culture is made. Plant genotype may have important effects on somaclone regeneration and frequency. These effects are very evident on potatoes: differences are observed in the number of regenerated plants of distinct cultivars grown under identical conditions (Gunn & Shepard, 1981). It is possible to identify cultivars prone to somaclonal variation, which suggests the involvement of a genetic component on the susceptibility to somaclonal variation (Karp & Bright, 1985).

Explant source is considered the most frequent critical variable for somaclonal variation. Since explants may present dissimilar regeneration rates, selection procedures can differ among different explants types. Plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex-induced calli (De Jong & Custers, 1986).

IDENTIFICATION of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants. Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions. Any genetic change induced by *in vitro* conditions of tissue culture is expected to generate stable plants carrying interesting heritable traits. However, such random changes are not desirable in plant transformation experiments. Therefore, their early detection is considered to be very useful in plant tissue culture and transformation studies. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism has found successful application in describing somaclonal variability in regenerated individuals of several plant species. In the present paper, we report successful induction of regenerative calli from tomato leaf explants, cultured on Murashige and Skoog's (MS) medium supplemented with picloram (4-amino-3-5-6-trichloropicolinic acid, a common herbicide) (as an auxin) and benzyladenine (BA; as cytokinin), and the extent of genetic variability in the plants regenerated from one of these calli as examined through RAPD analyses.

Considerable research has been done and substantial amount of literature have been published on somaclonal variation in various crops where in most cases, the *in-vitro* cell cultures displayed significant abnormalities in the form of chromosomal aberrations, chlorophyll deficiency, fertility alterations, and many other unwanted morphological characters.

However, some somaclonal variants derived from single gene mutation with large recognizable effects may be useful in the genetic improvement programmes. Although such variants for plant height, seed colour, and herbicide resistance (Chaleff and Ray 1984) have been reported, but their exploitation in genetic enhancement of yield has been rather limited. Studies have also been conducted to ascertain the use of somaclonal variants in plant breeding. In pigeonpea, Reddy and Rao (1975) reported significant *in-vivo* somatic variation for maturity, seed size, pod size and stem pigmentation in pigeonpea. The first attempt to create genetic variability through *in-vitro* culture of somatic cells in pigeonpea was made by Chintapalli *et al*. (1997). They regenerated plants from the cotyledonary explants of pigeonpea and reported significant variation for plant height, seed size, seed colour and insect resistance in R2 and R3 generations. The present study reports the results of field evaluation of the selected somaclones for some important agronomic and quality traits.

SOMACLONAL VARIATION

In nature, the genetic diversity and variability within a population are generated via recombination events. Factors such as natural selection, mutation, migration and population size influence genetic variability in different ways. In 1958 a novel, artificially produced, source of genetic variability was reported, the higher plant cells cultured *in vitro* showed a genetic instability that was also characteristic of regenerated cells. The first observation of somaclonal variation was reported. Subsequently, the variability existing in plant tissue and cell cultures received much attention and neologisms were proposed by Larkin and Scowcroft to refer to the results of *in vitro* cultures of plants.

The term 'somaclone' was coined to refer to plants derived from any form of cell culture, and the term 'somaclonal variation' was coined to refer to the genetic variation among such plants. The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process that involves only mitotic division of the cells. In this context, the occurrence of uncontrolled and random spontaneous variation when culturing plant tissue is a major problem. *In vitro,* the conditions of culture can be mutagenic and regenerated plants derived from organ cultures, calli, protoplasts and somatic embryos sometimes can show phenotypic and genotypic variation. Some, or all, of the somaclones may be physically different from the stock donor plants. Usually, variability occurs spontaneously and can be a result of temporary changes or permanent genetic changes in cells or tissue during *in vitro* culture. Temporary changes result from epigenetic or physiological effects and are nonheritable and reversible. In contrast, permanent changes are heritable and often represent expression of pre-existing variation in the source plant or are a result of *de novo* variation. The literature to date indicates that somaclonal variation can range in scope from specific trait to the whole plant genome. Somaclonal variation provides a valuable source of genetic variation for the improvement of crops through the selection of novel variants, which may show resistance to disease, improved quality, or higher yield.

Although somaclonal variation has been studied extensively, the mechanisms by which it occurs remain largely either unknown or at the level of theoretical speculation in perennial fruit crops. A variety of factors may contribute to the phenomenon. The system by which the regeneration is induced, type of tissue, explant source, media components and the duration of the culture cycle are some of the factors that are involved in inducing variation during *in vitro* culture.

Regeneration systems - Regeneration systems can be ranked in order from high to low in terms of genetic stability, as follows: micropropagation by preformed structures, such as shoot tips or nodal explants; adventitiously derived shoots; somatic embryogenesis; and organogenesis from callus, cell and protoplast cultures. Cellular organization is a critical factor for plant growth, whereas *in vitro* the loss of cellular control, which gives rise to disorganised growth, is a characteristic of somaclonal variation.

Although the direct formation of plant structures from meristem cultures, without any intermediate callus phase, minimises the possibility of instability, the stabilising influence of the meristem is sometimes lost *in vitro* cultures.

Somatic embryogenesis and enhanced axillary branching are the methods used most extensively in commercial micropropagation systems. Somatic embryogenesis has the potential to produce the greatest number of plantlets in a short time, and makes possible the use of bioreactors for the large-scale production of somatic embryos and their delivery through encapsulation into artificial seeds. Enhanced axillary branching involves the abolition of apical dominance to achieve the de-repression and multiplication of shoots, and has become a very important method on account of the simplicity of the approach and rapid propagation rate. These methods are considered to produce genetically uniform and true-to-type plants, because the organised meristems generally are believed to be immune to genetic changes. Several reports of experimental studies support this view. However, there is an increasing body of evidence that indicates that in embryogenic cultures, selection in favour of 'normal cells' does not always take place during development and that

growth of mutant cells can occur as well, which can induce variability in the cultures.

Explant source - Genetic fidelity largely depends on explant source. The explant tissue can affect the frequency and nature of somaclonal variation. The use of meristematic tissues, such as the pericycle, procambium and cambium, as starting materials for tissue culture reduces the possibility of variation. In contrast, highly differentiated tissues, such as roots, leaves, and stems, generally produce more variants, probably due to the callus-phase, than explants that have pre-existing meristems. Furthermore, preparation of many explants from only one donor plant increases the possibility of variation in cultures. This illustrates the importance of the donor plant with respect to its inherent genetic composition and genome uniformity in any of its components. Somaclonal variation can arise from somatic mutations already present in the tissues of the donor plant. To test for pre-existing somaclonal variation, the somatic embryos obtained in the first round of regeneration may be subjected to another round of *in vitro* regeneration. Tissues that show pre-existing variation should yield more variability in the first somaclonal generation than in the second generation, and thereafter the variation in the second round can be eliminated or stabilised.

Medium components - The hormonal components of the culture medium are powerful agents of variation. The effect of the type and concentration of plant growth regulators on the incidence of somaclonal variation in different plant species remains a topic of debate. Unbalanced concentrations of auxins and cytokinins may induce polyploidy, whereas under a low concentration or total absence of growth regulators the cells show normal ploidy. In addition, rapid disorganised growth can induce somaclonal variation. Sub- and supraoptimal levels of growth regulators, especially synthetic compounds have been linked with somaclonal variation. Auxins added to cultures of unorganised calli or cell suspensions increase genetic variation by increasing the DNA methylation rate.

Similarly, in callus cultures of strawberries, the presence of the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) is often associated with genetic abnormalities, such as polyploidy and stimulation of DNA synthesis, which may result in endoreduplication. It would seem that growth regulators preferentially increase the rate of division of genetically abnormal cells. High levels of cytokinins do not directly affect the rate of somaclonal variation in the banana cultivars 'Nanjanagudu Rasabale' and 'Cavendish'; in this contest it would seem that the genotype has the greatest effect on somaclonal variation. Conversely, high levels of benzyladenine (BA) cause the number of chromosomes in the banana cultivar 'Williams' to increase. In addition, diphenylurea derivatives are implicated in the incidence of somaclonal variation in bananas.

Duration and number of culture cycles - The frequency of somaclonal variation increases as the number of subcultures and their duration increases, especially in cell suspensions and callus cultures. Moreover, the rapid multiplication of a tissue or long-term cultures may affect genetic stability and thus lead to somaclonal variation. A statistical model has been proposed for predicting the theoretical mutation rate, primarily on the basis of the number of multiplication cycles. However, the model has limited application, due to the complexity of biological systems.

ISOLATION OF SOMACLONAL VARIANTS

Mutants for several traits can be far more easily isolated from cell cultures than from whole plant populations. This is because a large number of cells, say 106-109, can be easily and effectively screened for mutant traits. Screening of as many plants would be very difficult, ordinarily impossible. Mutants can be effectively selected for disease resistance, improvement of nutritional quality, adaptation of plants to stress conditions, e.g., saline soils, low temperature, toxic metals (e.g., aluminium), resistance to herbicides and to increase the biosynthesis of plant products used for medicinal or industrial purposes. The various approaches to the isolation of somaclonal variants can be grouped into two broad categories: (i) screening and (ii) cell selection.

Screening - It involves the observation of a large number of cells or regenerated plants for the detection of variant individuals. This approach is the only feasible technique for the isolation of mutants for yield and yield traits. In general, R1 progeny (progeny of regenerated, Ro, plants) are scored for the identification of variant plants, and their R2 progeny lines are evaluated for confirmation. Screening has been profitably and widely employed for the isolation of cell clones that produce higher quantities of certain biochemicals. Computer based automated cell sorting devices have also been used to screen as many as 1000-2000 cells/second from which desirable variant cells were automatically separated.

Cell selection - In the cell selection approach, a suitable selection pressure is applied which permits the preferential survival/growth of variant cells only. Some examples of cell selection are, selection of cells resistant to various toxins, herbicides, high salt concentration etc. When the selection pressure allows only the mutant cells to survive or divide, it is called positive selection. On the other hand, in the case of negative selection, the wild type cells divide normally and therefore are killed by a counter selection agent, e.g., 5 BUdR or arsenate.

The mutant cells are unable to divide as a result of which they escape the counter selection agent. These cells are subsequently rescued by removal of the counter selection agent. Negative selection approach is utilized for the isolation of auxotrophic mutants. The

positive selection approach may be further subdivided into four categories: (i) direct selection, (ii) rescue method, (iii) stepwise selection and (iv) double selection. In direct selection, the cells resistant to the selection pressure survive and divide to form colonies; the wild type cells are killed by the selection agent. This is the most common selection method. It is used for the isolation of cells resistant to toxins (produced by pathogens), herbicides, elevated salt concentration, antibiotics, amino acid analogues etc.

In the rescue method, the wild type cells are killed by the selection agent, while the variant cells remain alive but, usually, do not divide due to the unfavourable environment. The selection agent is then removed to recover the variant cells. This approach has been used to recover low temperature and aluminium resistant variant cells.

The selection pressure, e.g., salt concentration, may be gradually increased from a relatively low level to the cytotoxic level. The resistant clones isolated at each stage are subjected to the higher selection pressure. Such a selection approach is called stepwise selection. It may often favour gene amplification (which is unstable) or mutations in the organelle DNA.

In some cases, it may be feasible to select for survival and/or growth on one hand and some other feature reflecting resistance to the selection pressure on the other; this is called double selection. An example of double selection is provided by the selection for resistance to the antibiotic streptomycin, which inhibits chlorophyll development in cultured cells. The selection was based on cell survival and colony formation in the presence of streptomycin (one feature) as well as for the development of green colour in these colonies (second feature; only green colonies were selected). This approach has been used for the selection of cells resistant to the herbicide amitrole, 2, 4-D, tobacco mosaic virus (TMV) and aluminium.

PREDISPOSING FACTORS IN TISSUE CULTURE

The biochemical, physiological and developmental environment of tissue culture can predispose microRNA-based effects. Many microRNA genes are involved in hormonal responses, particularly auxin responses. For example, the miR160 family is highly conserved and regulates several AUXIN RESPONSE FACTOR family (ARF) transcripts.

Recent studies have shown that microRNAs and tasiRNAs are involved in complex networks regulating both auxin receptors and ARF transcription factors. In tissue culture with high exogenous levels of plant growth regulators, that might not be the same compounds let alone in the same stoichiometry as in planta, the effects on such complicated networks are likely to be considerable and difficult to predict. Certainly, thidiazuron-induced differentiation of rice callus has generated expression changes in microRNA-targeted genes, although no work has been performed to determine if actual microRNA levels are responsible. Epigenetic changes to microRNA loci and expression levels might help to explain why habituation can develop in vitro yet can also be reversible.

Habituation is where a plant culture in vitro that is normally dependent on exogenous plant growth regulators for continued growth and development loses its dependence on these chemicals after repeated sub-culturing. Similarly, effects on juvenility and phase change in vitro can be related to interactions with small RNA systems.

The developmental effects of growth under such artificial conditions are also likely to contribute to effects generated by microRNAs. In meristems, cells are highly regulated and undergo only a limited number of divisions in an undifferentiated state before differentiation occurs, generating a small, developmentally restricted ‘stem cell’ niche. In callus and immortalized suspension cultures, cells constantly divide in an undifferentiated state without such restrictions – an indefinite number of divisions in this special state, in which epigenetic changes could stabilize.

Furthermore, in callus, plasmodesmatal connections could allow propagation of small RNAs between cells, e.g. Whilst microRNAs tend to behave in a cell-autonomous manner, siRNAs including tasiRNAs (trans-acting siRNAs, produced from specific long noncoding RNAs targeted for cleavage by microRNAs) can move from cell to cell, e.g. In this way, siRNAs generated from microRNA transcripts or their targets could move with little restriction through callosic masses.

Even a small number of cells overexpressing small RNAs could have consequences beyond their physical limitations (Figure 1e). For example, overexpression of microRNAs generating 22-nt isoforms could produce a cascade of secondary siRNAs from target transcripts with transcriptomic and epigenetic consequences.

In tissue culture regimes promoting greater differentiation, disorganized meristematic regions and disordered vasculature might still generate problems. Some micro-RNAs are known to be enriched in meristematic regions. Likewise, complex small RNA populations, including some microRNAs, can be found in phloem sap.

Both short- and long-distance distribution of small RNAs and epigenetic silencing effects in recipient

cells is now an established concept. Even long-distance signaling by microRNAs seems increasingly likely, e.g. Aberrant differentiation and development in vitro can cause these normal systems of restriction and distribution of specific small RNAs to change. Over time, callus can lose its ability to form organized meristems that differentiate into new organs. This loss of competence might also be directly connected to microRNA levels and activity. For instance, in embryogenesis, AGO1 and AGO10 act together to promote apical meristem formation through a vascular-dependent mechanism, whereas ectopic expression of AGO10 promotes formation of ectopic meristems. Interestingly, AGO10 probably binds microRNAs and is involved in translational repression (but not cleavage) of their targets. Disturbing microRNA-based systems, through altering the balance of microRNAs and their effectors such as AGOs, can help to lock cells into an undifferentiated condition. This could be achieved through several mechanisms, such as altered expression of microRNAs that promote the maintenance of an undifferentiated, meristematic state to transition of expression patterns of the small RNA machinery to a fixed state similar to the meristematic region. The transition to such 'stable' or 'fixed' states is also interesting in the context of epigenetic repair mechanisms. For example, an RNA-dependent system operates to repair defects in the methylation levels of TEs in Arabidopsis. Such epigenetic repair mechanisms are likely to account for the instability observed in some cases of somaclonal variation.

However, if these mechanisms themselves become impaired under certain conditions in vitro, it seems plausible that particular epigenetic states can become stabilized resulting in fixation of epigenetic variants.

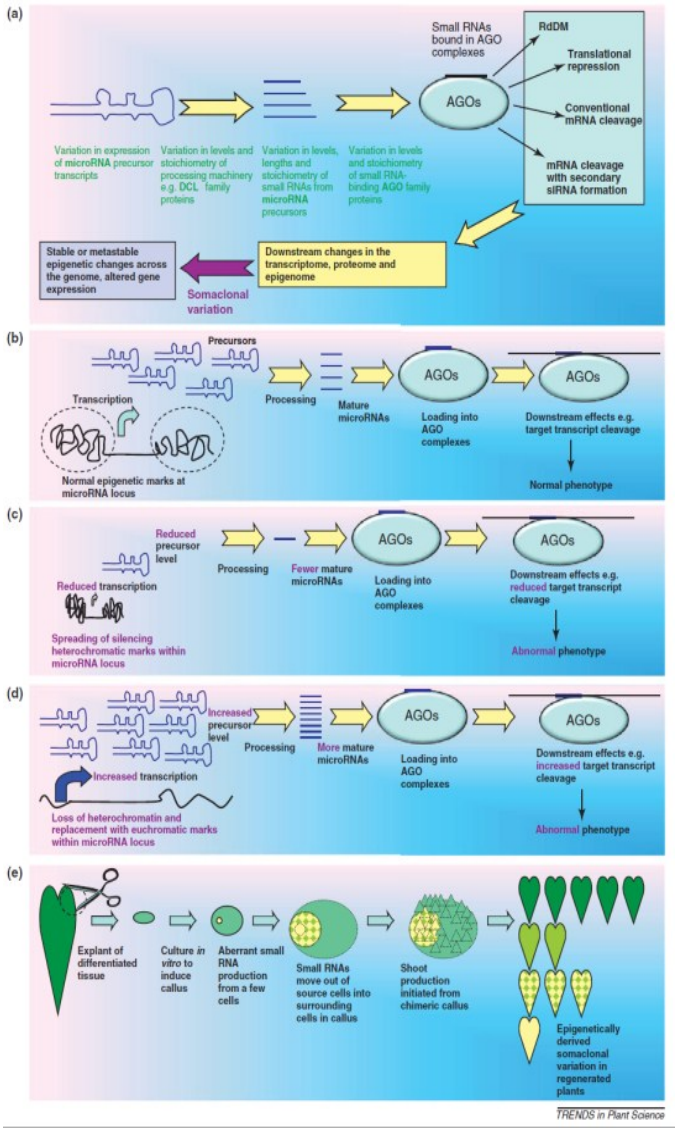

Figure 1. (a) Effects on small RNA epigenetic systems in plant cells in vitro may generate somaclonal variation. Tissue-culture induced changes in small RNA systems that have been experimentally determined (green text) can, via differential biochemical actions of AGO proteins and their complexes, have direct and indirect effects on the transcriptome, proteome and epigenome. These are likely to result in further stable or metastable epigenetic changes in the genome that will result in altered gene expression and phenotypic change (somaclonal variation). Some of these effects can reinforce or cancel out the original inducing changes, for example variation in microRNA expression levels through epiallele formation. (b–d) Epigenetic effects at microRNA loci. Many microRNA loci reside in intergenic regions close to or within silenced repetitive DNA (outlined in dashed lines in b). In cells in vitro, this silent heterochromatin flanking microRNA genes can spread into the promoter and transcribed.

GENETIC CHANGES THAT CONTRIBUTE TO SOMACLONAL VARIATION

During plant growth and development *in vivo*, gross changes in the genome can occur during somatic differentiation, including endopolyploidy, polyteny and amplification or diminution of DNA sequences. The processes of dedifferentiation and redifferentiation of cells may involve both qualitative and quantitative changes in the genome, and different DNA sequences may be amplified or deleted during the cell reprogramming. In addition, this process is related closely to the tissue source and the regeneration system. Gross and cryptic chromosomal changes, or extensive changes in chromosome number, occur early during induction in an *in vitro* culture. Variation in chromosome numbers and structures, and chromosome irregularities (such as breaks, acentric and centric fragments, ring chromosomes, deletions and inversions) are observed during *in vitro* differentiation and among regenerated somaclones. Such rearrangements in chromosomes may result in the loss of genes or their function, the activation of genes previously silent, and the expression of recessive genes, when they become haploid. The irregularities in the chromosomes may be lost during plant regeneration and result in the production of 'normal' plants, or appear in the regenerated somaclones.

Cryptic changes, such as point mutations, are also expected to occur and may affect the chloroplast or mitochondrial genomes. In addition, transpositional events, such as the activation of transposable elements, putative silencing of genes and a high frequency of methylation pattern variation among single-copy sequences, play a role in somaclonal variation.

The tissue culture environment may result in the modification of DNA methylation patterns. Global methylation levels and methylation of specific sites are documented in several crops, e.g. oil palm, grapevine and apple. In addition, epigenetic changes, such as DNA methylation and histone modifications, may be associated with the physiological responses of the plant cells to the conditions *in vitro*. Several epigenetic systems have been studied: variation for morphological traits, such as flower colour and shape, leaf colour and shape, and plant height; resistance to disease; and maturity date.

The rate of these changes varies not only in response to tissue culture conditions, but also among species and even among cultivars of the same species. There are several extensive reports of morphological and genetic variation of several plant species, primarily herbaceous species, but few studies have been conducted on perennial fruit crops, which indicates that knowledge of somaclonal variation in these plants is lacking. Currently, many markers are available to verify the fidelity of perennial fruit crops at the morphological, physiological and molecular levels.

METHODOLOGY

Chintapalli *et al*. (1997) regenerated pigeonpea plants from cotyledonary explants and studied *in-vitro* somaclonal variation under pot culture experiments for some morphological characters in R1 and R2 generations. Field grown R3 population segregated for flower colour, leaf shape, flowering habit, pollen fertility, pod borer damage, seed size, and seed colour. At maturity, over 100 plants were selected and transferred to the pigeonpea breeding unit of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for further utilization in breeding programme. At R4 generation, advanced through pedigree selection, the single plant progenies were sown in Alfisols at the onset of rainy season where a number of promising single plants were visually selected at maturity mainly for more number of pods/plant (as an indicator of more seed yield), seed size, and seed colour. These selections were further advanced for three generations using pedigree method of breeding. In 2000 rainy season, 15 promising progenies were bulk harvested and kept under cold storage.

In 2006, those lines were rejuvenated for their field evaluation for various agronomic characters along with the parent variety 'ICPL 87' in two replications during the rainy seasons of 2007 and 2008. A basal doze of di-ammonium phosphate was applied @ 100 kg/ha to ensure good crop growth and full expression of characters. The seeds were sown in Alfisols on ridges at 60 cm × 10 cm spacing in randomized complete block design (RCBD) at the onset of rains. Each plot consisted of four rows of four metre length. To control the weeds, a pre-emergence herbicide Fluchloralin was applied @ 2 l/ha that was followed by three hand weedings. The experiments were irrigated as and when required. One spray of Monocrotophos 36% EC was applied @ of 1 l/ha to control *Maruca vitrata* damage at early flowering stage. This was followed by two sprays of Methomyl @ 1 l/ha and one spray of Endosulfan 35% EC @ 2 l/ ha for controlling *Helicoverpa armigera* damage during podding stage. Data were recorded for days to maturity, plant height, grain yield, seeds/pod, seed size, and seed colour. Net plots, measuring 9.12 m2 were harvested at maturity.

Two samples from eight inbreds and the control were also assessed for their major cooking quality parameters. The cooking quality analyses were performed using decorticated dry split peas. For protein estimation, random samples of 70 mg *dal* were placed in a digestion tube. One auto-tablet (Kjeltab) and 3 ml of H2SO4 - H3PO4 mixture 95

parts conc. H2SO4, 5 parts of 85% H3PO4 (v/v) were added to the digestion tube and the samples digested at 370°C for 1 h. After cooling, distilled water was added to bring its volume to 75 ml. An aliquot from the digested sample was used for nitrogen estimation in Technicon Auto Analyzer and the nitrogen values were converted into protein by multiplying by a factor of 6.25.

The cooking time was determined by boiling the samples (10.0±0.5 g) in 50 ml of distilled water in a BD-20 heating block digester (Tecator, Sweden). To determine cooking time, the boiled samples were examined at one minute intervals for their softness by pressing them between the forefinger and the thumb. For water absorption study, the samples (5.0 g ± 0.5 g) were boiled for 20 min in 35 ml of distilled water in BD-20 block digester. After boiling, excess water was decanted and the samples weighed. The amount of water absorbed by the samples was calculated and the results were expressed as increase in weight per gram of sample. The percentage of solids dispersed into the cooking water was determined by boiling the samples (5.0 + 0.5 g) for 20 min. The boiled material was passed through a 20 mesh sieve. After thorough washing, the residue was dried at 110°C for 3 h and the loss in sample weight was calculated and expressed as percentage of solids dispersed in the cooking water.

CONCLUSION

In plants, microRNAs have a number of qualities that put them in the frame for causing somaclonal variation. These range from their origins and genomic organization, via their involvement in hormonal responses and meristem identity to their disproportionate effects on the transcriptome and epigenome.

Tissue culture is a potential tool in creating genetic variation through *in-vitro* selection of somaclones for targeted traits. The lines derived from somaclonal variations for distinct morphological traits represent certain genetic changes in near isogenic background, expectedly, arising due to point mutations. Such genetic materials are ideal for studying genetic nature of the characters in detail. Ullrich *et al*. (1991) reported the selection of important somaclonal variants, largely arising due to *in-vitro* genetic mutations.

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