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REVIEW

Experimental Approaches to the Study of Somaclonal Variation

THOMAS J. ORTON (Applied Genetics Lab, Agrigenetics Corporation, 3375 Mitchell Lane, Boulder, CO 80301)

Some 200 years ago, Weissman (cited in Sinnott and Dunn, 1936) hypothesized that multicellular organisms were, functionally speaking, temporal, and existed only to cast germplasm forward in time. Through Mendel and Darwin, and an illustrious cast of geneticists until very recent times, the genetic significance of the organism proper has more or less been discounted in favor of sex. In short, we have come to regard mitosis as a mechanism by which single cells give rise to populations of genetically identical cells, while meiosis (or modifications thereof) plays the central role in generating diversity.

A broad and expanding spectrum of observations are not entirely congruent with this view. Collectively, these observations suggest that *de novo* genetic variability in somatic tissues occurs somewhat frequently. For example, over 500 papers have been published describing the existence of genetic variability in cultured plant cells and tissues, and over 4,000 papers have appeared on the subject of genetic instability of mammalian tumors (Orton, 1983). Observations suggest that genetic variability arises spontaneously within differentiated contexts as well, manifested as mosaics in animals and sectors in plants.

Larkin and Scowcroft (1981) advanced the term "somaclonal variation" to denote such variation, and summarized many of its pertinent manifestations as they occur in cultured plant cells and regenerated plants. Other various reviews have also appeared which provide excellent summaries of the phenomenology of somaclonal variation in plants (D'Amato, 1975; Sunderland, 1977; Bayliss, 1980; Chaleff, 1981). The present article will critically examine the experimental approaches that have been utilized to examine somaclonal variation in plants and suggest possible improvements or alternative interpretations.

EXPERIMENTAL DESIGN

There exists a great need to improve and standardize experimental designs such that results are repeatable, and conclusions can be extended to general concepts. The salient findings of virtually all descriptive studies of plant somaclonal variation suffer from narrow applicability or tenuousness due to potentially confounding factors or lack of adequate replication. Moreover, germplasm and precise experimental conditions are often not described in sufficient detail such that results could be repeated in a different time and place. For example, tissue from single plants is often used to represent the general char-

Offprint requests to: Thomas J. Orton

acteristics of that from entire populations, varieties, and species. However, plants of different genotype within a species have been shown to exhibit reproducible differences in the types and degrees of genetic variability they give rise to in culture (McCoy et al., 1982; Browers and Orton, 1982). Hence, extrapolations beyond the individual genotype are tenuous, and the lack of defined genotype, or access to identical genotypes, makes reconstruction of experiments impossible. Further, explant tissue is generally assumed to be in a state of genetic uniformity, permitting the conclusion that spontaneous genetic instability occurs *in vitro*. Polyploidy (D'Amato, 1952) and aneuploidy (Murashige and Nakano, 1966; Heinz et al., 1969) have been noted in differentiated plant tissue, and appear to be a normal characteristic of some tissues (e.g., spinach periblem, tobacco pith, sugarcane meristem).

The major factors that appear to have an impact on somaclonal variation are genetic background, explant source (possibly confounded with genotype), the characteristics of the initiated growth form (e.g., cell association, latent differentiation, intrinsic growth rate), medium composition, rate and method of transfer, and overall culture age (see Orton, 1983, for detailed discussion). Experiments to elucidate the general relative impact or role of these factors on somaclonal variability incorporate the following features: hypotheses and necessary replication to permit statistical treatment, genetic and developmental architecture of explant, the genotype(s) of explant donor(s) with regard to future experiments (genetic studies?) and replication in time, precise standardization and definition of all possible invariate factors, and the fact that genotype × environment and environment × environment interactions may exist. Adoption of fixed standards for these experiments, such as for basal media, light and temperature regimes, and transfer method and frequency would foster repeatability and synergism.

Protoplasts could be used as a means to circumvent problems associated with the lack of definition of the explant, but the necessary assumption of genetic equivalency of different protoplasts still remains. This seems intuitively reasonable in most cases, with the possible exception of older clonally propagated lines such as potatoes. In any event, we presently lack the capability to establish genetic equivalency. Pure or inbred lines or F1 hybrid varieties are, in most cases, reasonably homogeneous with respect to genotype; these should be used in preference to undefined populations such as natural collections, open pollinated outcrossing cultivars, early generation breeding materials, synthetics, and hybrid varieties other than strict F1 types. Where possible genetic studies are planned, inbred lines should be used.

DETECTION AND MEASUREMENT OF VARIATION

Many of the tenuous assumptions and shortcomings in experimental design discussed above were a practical consequence of the lack or difficulty of measuring specific forms of genetic variation. In the ideal case, one would desire the capability to detect mutational events in single cells attributable to base changes, duplications, deletions, transpositions, translocations, inversions, chromosome loss or gain, and polyploidy. Both the physical and functional locus of these classes of mutation is essential. Moreover, the capability of monitoring such changes in chloroplast and mitochondrial genomes independent of nuclear genomic mutations is also necessary.

Cultured cells and tissues may be considered bereft of morphological variation of use in dissecting the phenomenon of somaclonal variation. Hence, even the earliest studies consisted of direct observations of the genetic apparatus. A summary of the techniques of use or proposed for the detection and measurement of genetic variation in somatic tissues is

compiled in Table 1. Perhaps the most crude method is spectrophotometric estimation of cellular DNA content. In this technique, tissues are typically fixed, administered a weak hydrolysis, and stained with Feulgen reagent, which contains a DNA-specific dye moiety. A modified light microscope is then used to measure absorbance in reference to a standard of known DNA content. The main advantages of the technique are the relative ease of sampling, and the fact that interphase, not mitotic, cells are used. Among the disadvantages are high error components and the inability to detect mutations other than nuclear polyploidy. Errors can be reduced by utilizing isolated nuclei, fluorescent DNA-specific dyes, and fluorescence-activated sorting, also permitting extremely large sample sizes (D. Galbraith, personal communication). Under certain circumstances, it may be desirable to study polyploidy alone in isolation from other potentially confounding classes of mutations, but DNA contents alone can only be of descriptive value in the total phenomenon of somaclonal variability.

Mitra and Stewart (1961) were among the first to report striking variability in chromosome number among cells of cultured *Haplopappus gracilis* tissue. Change of chromosome number is a more direct and convincing demonstration of variation than DNA content, and has been by far the most popular descriptive measure in cultured plant tissue. However, such determinations are reasonably tedious and progressively more difficult as chromosome numbers increase. Moreover, only the subpopulation of cells within a culture that is in a state of mitosis can be sampled. The nondividing or quiescent subpopulation may not be genetically equivalent to the proliferating fraction. One would predict that raw chromosome numbers would provide information on both aneuploidy and polyploidy. However, aneuploidy is a condition that is defined by the presence or absence of a specific chromosome or chromosomes, which cannot be extracted from chromosome numbers alone. Finally, the assumption that change in chromosome number directly implies change in cellular information content may in certain instances be erroneous, for example, in the case of Robertsonian chromosome fissions and fusions.

Karyotypic analyses of cultured cells have clearly demonstrated the existence of chromosomal rearrangements, most conspicuously multicentric/constrictional chromosomes probably generated by translocations or fusions, and chromosomes of reduced length resulting from deletion or translocation (Kao et al., 1970; Sunderland, 1977; Orton, 1980). Murata and Orton (1983) conducted extensive karyological studies on cultured celery cells, and estimated that approximately 40% were diploid ($2n = 22$) based on raw chromosome number, the remainder being hypoaneuploid. However, comparisons of presumed diploid cells with those that have been established as normal revealed that they all had distinct chromosome structural changes. These observations suggest that chromosome numbers are also far too crude to permit their use as a tool to study this phenomenon.

Karyotype analysis, even at high resolution, suffers from the uniform morphology of chromatin. Thus, the precise nature and source of striking structural changes is elusive based on cytological observations alone. Differential staining of chromatin has long been an important capability in diagnostic cytogenetics of mammalian organisms, tumors, and culture lines. Unfortunately, plants have proven to be less cooperative in revealing striking patterns in a reproducible way, and do not generally exhibit sufficient interstitial differentiation to permit the fingerprinting of individual chromosomes (Greilhuber, 1977). Nonetheless, Ashmore and Gould (1981) were successful in using the technique of Giemsa-banding to pinpoint specific structural changes in altered karyotypes of cultured crown gall tissues of *Crepis capillaris*. It is evident that the technique of differential chromatin staining will not be of general utility in the visualization of specific karyotypic changes.

TABLE 1. SUMMARY OF THE STRENGTHS AND LIMITATIONS OF TECHNIQUES PROPOSED OR IN USE FOR THE DETECTION AND MEASUREMENT OF GENETIC VARIATION IN CULTURED SOMATIC TISSUES

	TYPE OF VARIATION										
	Quantitative – DNA				*Qualitative*						
Technique	*Total*	*Repetitive/ Unique Fractions*	*AT/GC Content*	*Inverted Repeats*	*Poly-ploidy*	*Aneu-ploidy*	*Deletions/ Inversions/ Translo-cations*	*Somatic Recombina-tion*	*Base Changes*	*Inser-tions*	*Other Considerations*
Microspectrophotometry: flow cytometry	+	–	–	–	+	+, –	–	–	–	–	Relatively easy. High variability component (reduced by flow cytometry)
Differential density gradient separation	–	+, –	+	–	–	–	–	–	–	–	Requires large amounts of tissue and tedious extractions.
Thermal denaturation	–	–	+	–	–	–	–	–	–	–	Requires large amounts of tissue, tedious extraction, and stringent experimental conditions.
Reassociation kinetics	–	+	–	–	–	–	–	–	–	–	Requires large amounts of tissue, tedious extraction, and stringent experimental conditions.
S_1 nuclease resistance	–	–	–	+	–	–	–	–	–	–	Requires large amounts of tissue, tedious extraction, and stringent experimental conditions.

TABLE 1. (CONTINUED)

Technique	TYPE OF VARIATION: Quantitative–DNA: Total	Quantitative–DNA: Repetitive/ Unique Fractions	Quantitative–DNA: AT/GC Content	Quantitative–DNA: Inverted Repeats	Qualitative: Poly-ploidy	Qualitative: Aneu-ploidy	Qualitative: Deletions/ Inversions/ Translo-cations	Qualitative: Somatic Recombina-tion	Qualitative: Base Changes	Qualitative: Inser-tions	Other Considerations
Chromosome number	+,–	–	–	–	+	+	+,–	–	–	–	Relatively tedious; samples only mitotic cells.
Karyotype analysis	+,–	–	–	–	+	+	+,–	–	–	–	Extremely tedious; samples only mitotic cells.
Structural/ informational probes to southern blots	–	+,–	–	+,–	+,–	+,–	+,–	+,–	+	+,–	Requires at least some tissue and the ability to resolve infrequent hybridization events.
Structural/ informational probes to chromosomes *in situ*	–	+,–	–	–	+	+	+	–	–	+,–	Numerous problems (see text).
Genetic/cytogenetic analyses of regenerates	+,–	–	–	–	+,–	+,–	+,–	+,–	+,–	+,–	At least some variation may not be transmitted to regenerates.

\+ Technique yields direct, conclusive information
+,– Technique yields indirect information, or special assumptions or conditions are necessary to permit direct, conclusive information.
– Technique yields little or no pertinent information.

A myriad of physiological/biochemical phenotypes of known genetic control is expressed in cultured plant tissues, and these have been advanced as tools to probe somaclonal variation in a more precise manner than cytologically. Starting with tissue of known (or presumed known) genetic constitution at a given locus or set of loci, changes in the expression of the loci can be used as tools to estimate somaclonal variation at the level of the structural gene. For example, let us assume that we have a diploid organism that is heterozygous at a defined locus, and all three possible phenotypes at the locus can be visualized in cultured tissues. If large quantities of tissue are required to visualize phenotypes, such as electrophoretic protein mobilities, variability at a given point in time in a given culture may be determined indirectly by isolating single cells, raising clonal tissue from each, and determining phenotypes of clones as an inference of the original population of single cells. Qualitative changes in phenotypic expression, such as loss of electromorph activity or altered mobilities, can be seen directly and are probably indicative of underlying mutations. Conceivably, quantitative changes could also be detected given acceptably stringent protocols.

While one is able to improve resolution to the level of single structural loci using this approach, it suffers from lack of conclusiveness with respect to underlying cause or causes. For example, Arnison and Boll (1975) observed changes in isozyme patterns in *Phaeseolus vulgaris* cultures, but were unable to distinguish the relative genetic and developmental (i.e., nongenetic) components of variation. This was a consequence of the lack of understanding of the genetic control of the isozyme phenotypes they used, and the fact that they were known *a priori* to be developmentally unstable. Orton (1980) observed in regenerates from cultures of an interspecific *Hordeum* hybrid that the presence and absence of bands of one constituent parent or the other more or less mirrored the presence of phenotypic characteristics of the same parent. Since both cultures and corresponding regenerated plants were karyologically unstable, variation in isozyme patterns was speculated to have been a manifestation of aneuploidy. Attempts to substantiate this hypothesis were stymied by the inability to distinguish the individual chromosomes of the parents, structural rearrangements that occurred frequently *in vitro*, and pervasive sexual sterility of the regenerates.

Loss of allele expression in cultured heterozygous tissues could conceivably occur as a consequence of a broad range of both genetic and nongenetic causes. However, the involvement of epigenetic changes alone seems highly unlikely, since complete selective allele inactivation at a given locus has not been demonstrated in plants. However, modulation of *Adh* allele expression as a function of tissue has been observed in corn (Freeling, 1975). Moreover, the phenomenon of X-linked allele inactivation is common across a broad range of animal groups, and is associated with permanent heterochromatinization of one of two X chromosomes in somatic cells of females at a prescribed point in development. Epigenetic events would not be expected to be stable across developmental transitions in culture, or especially among sexual progenies of regenerates. Among the possible genetic causes of allele inactivation are point mutation, insertion of a transposable element, somatic recombination, nondisjunction, deletion, and position effects resulting from translocation or inversion.

All of these classes of mutations would be expected to give rise to stable, sexually heritable variation with the possible exception of transposable elements. Further testing should theoretically permit one to distinguish among these possibilities. For example, point mutations should give rise to karyologically and cytogenetically normal cells, and corresponding regenerated plants should transmit the resulting null allele in a Mendelian

fashion. Somatic recombination should be associated with clones of phenotypically homo/hemizygous cells for both alleles, should be normal with respect to chromosome number and structure, and should transmit no null alleles. Loss of allele expression due to nondisjunction should be associated with monosomy, and trisomy should be observed among at least certain of the presumptive heterozygous clones. Although deletions and inversions may not be directly discernible, cytological abnormalities and segregation distortions should reveal their existence in regenerates and corresponding progenies.

Insertion by foreign elements into control or coding regions of a gene could result in its inactivation. For example, selective modulation of allele activity by the transposable element Ds at the *Adh* locus has been reported by Osterman and Swartz (1981). Peacock (1983) has shown that the insertion occurs at the promotor region of *Adh* proximal to the actual coding sequence and that reversion to normal occurs concomitantly with the excision of Ds. Unfortunately, we do not presently have sufficient understanding of the general behavior of transposable elements to permit us routinely to distinguish them phenotypically from point mutations (if stable) or developmental switches (if unstable).

Further, unforeseen complications can arise in attempts to understand the basis of changes in the expression of genes at specific loci. Orton (1983, in press) has observed loss of allele expression in cultured celery (*Apium graveolens*) tissue heterozygous for two linked loci, *Pgm-2* and *Sdh-1*. Specifically, cells progressively exhibited loss of the PGM-2^F band over a period of approximately 30 months in culture, while heterozygosity was retained at *Sdh-1*. The resulting homo/hemizygous phenotype was highly stable and transmitted to regenerates, but abnormal development obviated any inheritance studies. High background variability with respect to chromosome number and structure obscured the ability to discern whether gross karyological changes were responsible. High mutation frequencies and the absence of reciprocal clones were taken as strong indications that point mutation and somatic recombination were not involved. Moreover, since *Pgm-2* and *Sdh-1* are linked but no altered SDH-1 phenotypes were observed concomitantly with loss of PGM-2^F, it was reasoned that simple aneuploidy could not be responsible. Thus, it was concluded that either insertional events or chromosome structural changes were probably the underlying cause of the phenomenon. Unfortunately, high background variability and inviability of regenerated plants stymied attempts to reach stronger, more positive conclusions.

New capabilities in molecular biology have and will continue to provide precise, unequivocal tools for the visualization of genetic variability. This is a direct consequence of the ability to discern phenotypes directly on genomic DNA using sequence-specific restriction enzymes and cloned probes. For example, restriction enzymes have already been used to demonstrate the occurrence of spontaneous mutational events involving mitochondrial (mt) DNA in cultured plant tissues (Pring et al., 1981). Recently, Boeshore et al. (1983) have obtained direct evidence of hybrid composition of the mitochondrial genomes in clonal tissue derived from fused protoplasts from *Petunia* species. The chloroplast genome seems to be relatively more stable than mtDNA in cultured tissues, but further experimentation is necessary to determine whether chloroplast genomes recombine in somatic tissue.

The nuclear genome is, of course, organized and maintained in a much different and more complicated manner than those of the cytoplasmic organelles. Nonetheless, molecular approaches should provide unprecedented insights into the sources and phenomenology of somaclonal variation with respect to genes encoded in the nucleus (Rivin et al., 1983). New rapid molecular cloning techniques that utilize improved vectors have made it

possible to obtain comprehensive stable genomic libraries with a minimum of effort. If taken from standard or reference genotypes, specific constituents of such libraries will be useful as direct genetic or chromosome structural probes in experimental studies. Rivin et al. (1983) have described several cloned probes that reveal restriction site polymorphisms. Molecular polymorphisms can be subjected to standard inheritance and linkage tests. Armed with a set of cloned fragments of known arrangement, one could initiate test cultures and conduct powerful experimental studies on somaclonal variation.

Various modifications of or additions to this scheme can be envisioned that enhance resolution of chromosome structural mutations and aneuploidy. For example, fluorescence activated sorting capability is presently being applied to the purification of metaphase chromosome fractions based on DNA content (Carrano et al., 1979). This permits the direct mapping of library probes to linkage groups, and, potentially, the linear order as well (Kao et al., 1982).

Radiolabeled cloned probes of highly repeated ribosomal RNA genes of rye have been successfully hybridized directly to metaphase chromosomes immobilized on glass slides and visualized directly by autoradiography. This suggests the theoretical possibility that library probes could be used directly as markers for genome architecture via *in situ* hybridization. Problems that must be overcome include (1) developing techniques to permit visual perception of single copy hybridization events, and (2) developing the ability to preserve single metaphase chromosome spreads for recurrent probing. Any reduction of the necessary time and effort associated with autoradiography would be extremely desirable.

Other limited approaches that have been utilized to detect and measure heritable, qualitative changes in nuclear DNA of cloned somatic tissues include CsCl gradient separation, thermal denaturation curves and reassociation kinetics, and resistance to S_1 nuclease digestion (De Paepe et al., 1982). The strengths and limitations of these approaches are summarized in Table 1.

Yet another approach to the study of somaclonal variation is the analysis of variation in regenerates as a direct reflection of that arising spontaneously in corresponding cultured cells or tissues. Since inheritance studies are necessary, the ideal starting materials for such experiments are inbred lines, and presumably homogeneous explants. The main advantage to this approach is that direct morphological phenotypes are restored as markers for the detection and measurement of variation, thus obviating the need for the tedious, expensive laboratory protocols described above. Moreover, direct cytogenetic analyses can be used to detect and quantify certain classes of chromosome structural changes (McCoy et al., 1982).

However, genetic variability present in a given population of cultured plant cells will probably be underestimated by this method, since the process of regeneration is associated with elimination or reduction of chromosomal variability (Bayliss, 1981), and variation is often observed *within* as well as among regenerating units. (Sacristan and Melchers, 1969; Ogura, 1978; Orton, 1980; Ogihara, 1981; Browers and Orton, 1982). Further complications may result from viability or fertility losses encountered as a consequence of somaclonal variation. Hence, using morphological or physiological variation in regenerates as a direct measure of somaclonal variation in cultured tissues is extremely tenuous.

It should be pointed out that regeneration is necessary to carry out inheritance studies to verify the genetic bases of certain of the forms of variation described above. The conspicuous examples of somaclonal variation provided by sugar cane and potato (summarized in Larkin and Scowcroft, 1981) were flawed by the inability to demonstrate conclu-

sively the genetic basis (or base) of variation. Stability over time and recurrent vegetative propagation were invoked instead as weak substitutes.

As a further aside, somaclonal variation may indeed provide a powerful tool to the study of the mechanisms surrounding embryogenesis or organogenesis. By comparing specific manifestations of variation among cultured cells and corresponding regenerating it may be possible to draw inferences regarding genotype and developmental competence.

SUMMARY

As pointed out in the introduction, the process of cell division in higher eukaryotes appears to be naturally error-prone. It has been argued that differences in overt manifestations of somaclonal variation, which emanate from such errors, are a function of intrinsic selective forces, possibly in combination with altered mistake rates (Orton, 1983). For example, a broader range of variant cells would be expected to remain viable in the context of a suspension culture as compared to a highly differentiated plant structure. In addition, there is a strong suggestion that somaclonal variation is mediated or conditioned at least in part by genotype. Other factors that bear on somaclonal variation, such as age, transfer technique, and medium composition, are probably manifestations of selection (or possibly mutagenic activity as in the case of 2,4-D). More research incorporating improved designs and utilizing new techniques to visualize variability will be needed to elucidate the precise roles that selection and mutation play in the generation of genetic variability in somatic tissues. This knowledge should provide a basis for the design of propagation protocols to minimize or directionally maximize its expression.

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