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REVIEW

How to measure somaclonal variation

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CONTENTS

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INTRODUCTION

Plants are generated by a series of cell divisions in meristematic tissues. The apical meristem is formed during the early stages of embryogenesis and consists of two outer cell layers (L_1 and L_2 or tunica) and the inner body (L_3 or corpus). Axillary meristems originate from the apical meristem and have the same histogenic arrangement (Reeve 1948). New apical meristems can also be formed adventitiously from somatic cells in many types of tissues. However, plants generated by the outgrowth of such adventitious meristems are often genetically different from the original plant. The term 'somaclonal variation' was introduced to describe the genetic variation in plants regenerated from any form of cell culture (Larkin & Scowcroft 1981). The term has been adopted widely, but is used in various senses, especially in practical discussions (Soh 1987). In the present article 'somaclonal variation' indicates (i) genetic variation in plants originating from adventitious meristems (usually formed *in vitro* by various cell types, such as somatic cells in organs, cells in callus or single cell cultures, and germ cells), and (ii) genetic variation in cell and callus cultures themselves.

Somaclonal variation constitutes a major problem in present-day micropropagation and is one of the great stumbling blocks for micropropagation via somatic embryos. It also impedes the application of biotechnological breeding techniques. The effects of biological (genotype, explant type), medium (plant growth regulators) and physical (duration

of culture) factors on somaclonal variation have been noted, but so far basic knowledge is fragmentary and only a few general principles have emerged. In the study of somaclonal variation, the absence of a straightforward, rapid assay to measure its extent is a major obstacle (Orton 1983b). Reviews on somaclonal variation focus on its potential for breeding (Larkin & Scowcroft 1981; Evans 1989), on cytological aspects (Bayliss 1980; Lee & Phillips 1988; Pijnacker & Sree Ramulu 1990) or on its origins and causes (Orton 1983a; Karp & Bright 1985; Gould 1986; Sree Ramulu 1987). In the present paper, I will discuss methods to assess the extent of somaclonal variation (page 134). First, brief overviews are given on backgrounds (pages 130 and 131) and on practical aspects (page 133).

SOMACLONAL AND EPIGENETIC VARIATION

In plants regenerated from tissue culture, all kinds of genetic variation have been observed, namely, changes in DNA sequence (point mutations, activation of transposons), in chromosome structure (duplications, translocations) and in chromosome number (leading to polyploidy or aneuploidy). In addition to somaclonal variation, epigenetic (i.e. non-genetic) variation also occurs frequently (Binns 1981; Meins 1983). The main difference between epigenetic and somaclonal variation is that somaclonal variation is transmitted during meiosis whereas epigenetic variation is not. Other differences are that, (i) epigenetic variation is reversible during the life of a plant, whereas somaclonal variation is not; (ii) somaclonal variation only occurs in plants generated from adventitious meristems, whereas epigenetic variation is observed both in these plants and in plants originating from outgrowth of pre-existing apical/axillary meristems; and (iii) epigenetic variation is predictable, whereas somaclonal variation is not (i.e. the same conditions will usually result in the same type of epigenetic variation, but not in the same type of somaclonal variation). Finally, (iv) epigenetic variation is a physiological response and is therefore in one direction (or more accurately, epigenetic variation follows a dose–response curve). Just as with spontaneous mutations, the direction of somaclonal variation is supposed to be entirely random with reference to the functional or adaptive value (cf. contemporary evolutionary theory, e.g. Stebbins 1982). In consequence, a trait affected by somaclonal variation may change in different regenerated plants into opposite directions. It should be noted, however, that because most mutations are deleterious, somaclonal variation will, as a rule, be in the direction of decreased vigour, decreased yield, etc. (Johnson *et al.* 1984; Lee *et al.* 1988; Jackson & Dale 1989), and therefore also in one direction only.

The difference between both types of variation is not an all or nothing phenomenon but a matter of degree; DNA methylation, for example, is transmitted through meiosis but is also reversible (Holliday 1987). Similarly, changes of the ploidy level leaving the chromosome number euploid, and directed DNA rearrangements (as in the case of mating-type switching in yeast, Binns 1981) might not be considered a genuine mutation of the genome. Furthermore, in contrast to (iii) mentioned above, the same mutation may occur independently in many regenerated plants because of the presence of mutation-sensitive regions in the chromosomes.

It should be noted that somaclonal variation occurs on top of spontaneous mutations. The number of point mutations occurring under natural conditions is probably specific for chromosome regions. During tissue culture of maize, the frequency of nucleotide substitution in the *Adh1-1S* allele (1 in 10^2) was found to be 10 000 times higher than the spontaneous mutation rate (Dennis *et al.* 1987). For animal tissues, until recently it was

thought that the mutation rate per locus was 1 in 10^6, but the actual rate is likely to be 1 in 10^5 (Neel 1983).

ORIGINS AND CAUSES

Mericlones, i.e. plants originating from the outgrowth of non-adventitious meristems, are true to type; somaclonal variation is only observed in plants originating from adventitious meristems (Hussey 1983). Thus, genetic stability is maintained in meristems, but is partly lost in non-meristematic tissue. Accordingly, genetic instability occurs from the time a cell leaves the apical/axillary meristem up to the formation of a new adventitious meristem. It is not known what portion of the mutations occurs *in planta* prior to the tissue-culture phase ('pre-existing variation') and what portion during tissue culture.

A number of hypotheses have been put forward to explain the increase of genetic instability in non-meristematic tissue.

Number of cell divisions. Obviously, the number of mutations is proportional to the number of cell divisions between the zygotes of two subsequent generations. In agreement with this, genetic variation increases with prolonged culture (Orton 1985; Armstrong & Phillips 1988; Hartmann *et al.* 1989). It is, however, unlikely that the larger part of this increase is due to prolonged culture: Gould (1986) conservatively estimated the number of cell divisions between two generations of zygotes in plants to be 30; in a suspension culture sub-cultured every week there are three divisions each week (Gould 1986). It is unlikely that the increased frequency of mutations in tissue culture (in the case of *Adh1* in maize 10 000-fold higher, Dennis *et al.* 1987) is due to the 'extra' cell divisions.

Mutagenic substances. It has been assumed that medium components, especially certain plant growth regulators, are mutagenic (e.g. Vajrabhaya 1977; George & Sherrington 1984). The reason for this is that at high concentrations (50 mg l^{-1}) 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) cause chromosomal aberrations (Bayliss 1980). At concentrations used in tissue culture (< 10 mg l^{-1}), though, no such aberrations have been observed (review in Bayliss 1980). Moreover, in an assay for chemical mutagens (colour mutants in stamen hairs of *Tradescantia*) none of the medium components tested (2,4-D, NAA and benzylaminopurine, all at approximately 1 mg l^{-1}, and nutrients) caused an increase in the frequency of mutations (Dolezel & Novak 1984). Bayliss (1980) concludes that 2,4-D does not have a direct mutagenic effect but causes abnormalities indirectly by stimulating disorganized growth.

Response to stress. Conventionally, the genome is considered to be a stable entity. More recently, however, evidence has accumulated to suggest that the genome is actually in a continuous flux with changes occurring in both mitotic and meiotic cycles (Walbot & Cullis 1985). These changes may concern all of the genome, leading for example to polyploidy, or only a portion of the genome, resulting in amplification or reduction of certain parts. Cullis (1981) observed that the genome of flax undergoes changes supposedly aimed at adaptation to the new conditions. McClintock (1984) has advocated the view that when plants are exposed to an environmental stress that lies beyond their capacity to adapt by epigenetic changes, they may enter a state of 'genome shock' that

activates transposons and thereby brings about rapid evolutionary changes. Tissue culture is envisaged as a form of stress and would therefore result in similar genomic changes. In flax, it was found that plants regenerated from callus and plants under environmental stress show variation in the same subsets of highly repeated sequence families (Cullis & Cleary 1986). This suggests that at least part of the genetic variation in tissue culture is caused by stress and forms part of an adaptive strategy. To some extent in contradiction with this view, Heindorff *et al.* (1987) reported that stress pre-treatment of *Vicia faba* root meristems renders these meristems less susceptible to mutagenic treatment.

Directed developmental changes in chromosomes. In plants, development is accompanied by a range of directed changes in nuclear DNA (Altamura *et al.* 1987; Cavallini *et al.* 1988). It might also be that during the induction and/or growth of callus such directed chromosomal changes occur. Plants regenerated from somatic or callus cells with a changed genetic composition are likely to differ genetically from the original plant and resemble the genome of the somatic or callus cells. Recently, Pijnacker *et al.* (1989) have shown that the degree of polyploidization of callus cultures is indeed correlated with the degree of polyploidization in the explant. It has been suggested that because of these chromosomal alterations, mitotic fidelity is difficult to achieve (Sree Ramulu 1987).

Meristematic versus non-meristematic tissues. In animals, the germ line is a group of cells often derived from a specialized region of the zygote. The cells of the germ line are inactive in the somatic body of the animal (Walbot 1985). When plants reach the reproductive stage, the microsporogenic and megasporogenic cells are formed from the apical and axillary meristems. So, in plants these meristems function as the germ lines. From an evolutionary point of view, germ cells must have a balance between copying the parent genome and mutations, allowing an infrequent occurrence of the latter. As genetic stability in the meristems is a prerequisite for such balance, plants must have developed mechanisms in meristems for faithful mitotic division, for repair of DNA, and for removal of cells with a disadvantageous genotype (Klekowski & Kazarinova-Fukshansky 1984). In non-meristematic tissues, such control processes presumably occur to a much lesser extent. The specific nature of cell divisions in meristems is indicated by the finding that the durations of the various phases of the cell cycle are very different in apical meristems and in tissue and cell culture (Gould 1984). Lee & Phillips (1988) proposed that the disturbance of the cell cycle causes a delay in the replication of DNA in heterochromatic regions (which normally replicate late). This would initiate a cascade of events eventually resulting in all types of observed genetic changes. Orton (1979, 1980) observed that the extent of chromosomal variation in callus tissue depends on the type of callus: hard, nodular, slowly growing calli are more stable than friable, growth-uncentralized, rapidly growing calli. Similar observations have been made by Geier (1988). In hairy-root cultures of *Nicotiana rustica* and *Beta vulgaris*, chromosome number and secondary metabolite production are stable, whereas cell suspensions are very variable (Aird *et al.* 1988). This demonstrates that stability is not only maintained in apical and axillary meristems, but also in root meristems.

In the dicotyledons, the sporocytes (both the micro- and megasporocytes) originate from the second cell layer (L_2). In monocotyledons, the megasporocytes originate from L_2 and the microsporocytes from L_2 or L_2 and L_3 (D'Amato 1977). This suggests that a high level of genetic stability is only maintained in L_2 and possibly in L_3. It might be that in

mericlones the genetic stability is not maintained when the structure of the meristem is disturbed. Disorganized meristems are frequently observed in tissue culture (Varga *et al.* 1988).

PRACTICAL ASPECTS OF SOMACLONAL VARIATION

In their influential review, Larkin & Scowcroft (1981) advocate the view that somaclonal variation represents a new source of variability and therefore constitutes a powerful tool for the breeder, especially in combination with a mutagenic treatment and selection *in vitro*. Although the use of somaclonal variation has been successful in this respect, in general the original promises have not been fulfilled and at present it is becoming increasingly clear that somaclonal variation is usually undesirable.

First, in micropropagation of various ornamental crops, namely orchids, anthurium, lily and African violet, the produced plants show aberrations which are, at least in part, due to somaclonal variation. In The Netherlands, the losses exceed US$1 million per year. In most micropropagated crops, somaclonal variation is avoided by micropropagation via axillary buds. However, even then adventitious buds are often formed without notice (Marcotrigiano *et al.* 1987). In addition, micropropagation via somatic embryos in liquid medium, which is a cheap, automatable alternative for present-day methods, is hampered by, among others, the occurrence of somaclonal variation.

Secondly, in most biotechnological breeding techniques plants are regenerated from genetically engineered somatic cells. So, biotechnologically engineered plants are subject to somaclonal variation. The same holds for haploid plants. Undesirable mutations can be removed by strong selection and by incorporation of the regenerated plants in breeding programmes where the desired trait can be separated from the undesirable mutations. Such breeding programmes, however, are in practice not possible for crops with a long generation cycle (trees), for crops which are propagated vegetatively (potato, many ornamentals) and, because of the high costs, for crops from which the number of plants per cultivar is relatively small (many ornamentals). In addition, some undesirable mutations will not be recognized immediately and will therefore not be removed. Such hidden deleterious mutations include recessive mutations, which can only be recognized in the progeny of selfed plants (Malepszy & Nadolska-Orczyk 1989), mutations in genes that are expressed exclusively during certain developmental stages or under specific environmental conditions, and mutations that result in small changes of quantitative traits. Oil palms, for example, show abnormalities only after several years of culture in the field; moreover, in the same clone the plantings of the second and especially the third year show abnormalities but not the plantings of the first year (Corley *et al.* 1986; it is not clear whether the observed aberrations are somaclonal or epigenetic). A similar situation occurs for a spontaneous mutation of *Adh* in maize. *Adh* is not necessary during normal aerobic growth but as *Adh* confers flood resistance, mutations will have a negative effect when the seedling or the roots of the adult plant are flooded (Schwartz 1969). Small changes in quantitative traits due to tissue culture have been noted. They are apparent from an increased coefficient of variation for quantitative traits in the progeny of a selfed regenerated plant (Jackson & Dale 1989). Such small changes will not be recognized readily, but may have large economic effects.

Thirdly, somaclonal variation also affects the production of secondary plant substances by cell cultures, thereby reducing the production by well-established cultures (Deus-Neumann & Zenk 1984).

From the data and hypotheses mentioned earlier, various rules can be derived to cope with somaclonal variation. it is advisable to avoid adventitious meristems. When this is not possible, the adventitious meristems should be induced directly on the explant or the callus interphase should be kept short. In the latter case, 'organized' callus should be selected and friable callus discarded. In genetic engineering, stable genotypes should be used for the introduction of new genes into a crop (it has been shown that various genotypes of one species show large differences in the extent of somaclonal variation, Nagarajan & Walton 1987). The explant used to initiate a culture should consist of young tissue, preferably apical or axillary meristems.

Vasil *et al.* (1983) suggested that plants generated from adventitious embryos are more true to type than plants from adventitious buds. There are, however, various reports describing no or even a negative effect of somatic embryogenesis (Karp & Maddock 1984; Linacero & Vazquez 1986; Armstrong & Phillips 1988). It has been proposed that the optimal conditions during tissue culture enable mutated cells to reproduce and eventually to generate plants. Minimal medium could select against mutated cells and plants regenerated from minimal medium would accordingly be more true to type. However, a minimal medium actually increases the number of aberrant plants as compared with an optimal medium (Orton 1986).

ASSESSMENT OF THE EXTENT OF SOMACLONAL VARIATION

For the development of methods to counteract or avoid somaclonal variation, it is necessary to carry out careful and critical studies as to the effect of various parameters on the extent of somaclonal variation. Examinations have been carried out on the phenotypic and the cytological/molecular levels. In evolutionary studies, it has been observed that the magnitude of a phenotypic change is usually not correlated with the magnitude of the underlying genetic change (for review see Paigen 1986; Patterson 1987). As in horticultural and agricultural practice only the phenotype counts, the correspondence between changes at the phenotypic and at the cytological/molecular level should be considered.

The extent of somaclonal variation is usually determined as the percentage of plants which show one or more qualitative aberrations, for example, the percentage of aneuploid and polyploid plants, or the percentage of plants with distinct morphological alterations such as dwarfism, albinism and aberrant leaf shape. Alternatively, as in somaclonal populations a greater spread of quantitative traits is observed relative to the control (Karp & Bright 1985), the value of the standard deviation (SD) for a trait in a somaclonal population indicates the extent of somaclonal variation. This method has been described in a concomitant communication (De Klerk *et al.* 1990a). A significantly larger value of the SD has been noted in other papers (Wang & Holl 1988; Jackson & Dale 1989; Zheng *et al.* 1989) and can be derived from many articles (Engler & Grogan 1984; Larkin *et al.* 1984; Evans *et al.* 1986; Novak *et al.* 1986; Chen *et al.* 1987).

In the following sections, I will first discuss the assessments of somaclonal variation on the phenotypic level. Subsequently, I will deal with observations on the cytological/molecular levels and their correlation with the phenotypic level.

Measurements on the phenotypic level

The effect of somaclonal variation on the level of the phenotype is usually determined as the percentage of plants that show aberrations for one or more defined characteristics (Lee & Phillips 1987; Lourens & Martin 1987). Two other approaches have been published.

First, studies are carried out with plants bred to be heterozygous for heritable marker traits. Consequently, for these traits mutations are immediately detectable. Armstrong & Phillips (1988) found that in regenerated maize plants various marker traits are not affected by somaclonal variation, whereas there are distinct aberrations in chromosome number and structure. Similarly, Graybosch *et al.* (1987) observed no mutations in six marker genes in regenerated soybean plants. In alfalfa both cytological alterations and shifts in the expression of marker traits occur (Groose & Bingham 1984). In tobacco protoplast culture followed by regeneration, instability was found for two marker genes (Barbier & Dulieu 1983). Groose & Bingham (1986) used an anthocyanin mutation, previously recovered from tissue culture, to study reversion. They found a high frequency of reversion (22%) *in vitro* but a very small frequency (< 1%) *in planta*. The high frequency indicates the involvement of a transposable element.

Secondly, De Klerk *et al.* (1990a) propose to assess the degree of variation in a somaclonal population by determining the value of the SD for a quantitative trait. In the regenerated plants themselves, this should be a trait that is not affected by epigenetic variation. Preferably, the mean value of the trait should be the same for the somaclonal and the control populations. De Klerk *et al.* (1990a) have used the leaf shape (expressed as the natural logarithm of the ratio of the longest rib and its longest perpendicular rib) of regenerated plants of *Begonia* × *hiemalis* as such a parameter. They found that plants regenerated from callus induced at a high concentration of 2,4-D ($1\ mg\ l^{-1}$) have a significantly higher SD than plants regenerated from callus induced at a low 2,4-D concentration ($0{\cdot}1\ mg\ l^{-1}$). There is no effect on the ratio itself. (As noted above, the effect of 2,4-D is likely to be indirect by stimulating disorganized growth.) Wang & Holl (1988) found an increased value of the SD for the leaf shape (length–width ratio) in a somaclonal population of *Trifolium pratense*, but they also observed an increased SD in plants generated from axillary buds.

Because somaclonal variation renders the genome more heterozygous, a sample of plants originating from one selfed regenerated plant should display a greater spread than a control sample. Accordingly, Jackson & Dale (1989) reported an increased SD for various quantitative traits in families originating from selfed regenerated plants of *Lolium temulentum* (Fig. 1). They did not observe alterations for chromosome number, chromosome structure, and patterns of four isozyme systems, indicating that the 'SD-assay' is very sensitive. An increased value of the SD for various traits was also noted by Zheng *et al.* (1989) in regenerated rice plants.

The advantages of the quantitative SD-assay over the qualitative assays are (i) for each determination the number of plants needed is smaller (20–30 instead of 100), (ii) the observation of these parameters does not depend upon the eye of the observer, (iii) in a short period of time many individuals can be evaluated, and (iv) the SD-assay seems to be much more sensitive.

Numerical chromosome variation

Chromosome numbers are determined by chromosome counts in mitotic cells (in regenerated plants usually in the root tips), by measurement of the DNA content per cell (either by a cytophotometric determination after Feulgen staining or by flow cytometry), by chloroplast counts in stomatal guard cells, or by measuring stomatal size. The latter two methods are the easiest, albeit not always accurate (see Sree Ramulu *et al.* 1983); the other methods are laborious. With regard to chromosome counts in root tips, it is important to note that the roots are formed mostly adventitiously on shoots and that this may affect the

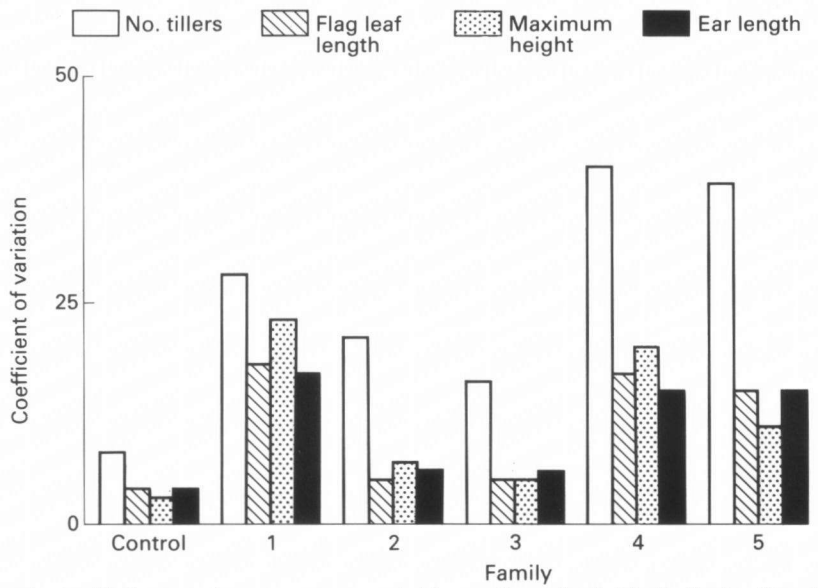

Fig. 1. Five plants of *Lolium temulentum* were regenerated from callus and selfed. In the five families of R_2-plants (R_2 is the progeny of the selfed regenerated plants) and in a control group, various quantitative traits were determined and the coefficients of variations calculated. (Based on data from Jackson & Dale 1989.)

determination of the chromosome number. As the regeneration of shoots acts as a screen against cytological mutant cells (Browers & Orton 1982b; Natali & Cavallini 1987), so the subsequent regeneration of roots will act as a second and different screen (because both the medium conditions and the types of tissue from which the primordia originate, are different for shoots and roots). In agreement with this, large differences exist in rooting capacity between euploid and aneuploid shoots (Fish & Karp 1986). Consequently, changing the rooting conditions will affect the percentage of observed aberrations.

Changes of the chromosome number are the genetic changes observed earliest in tissue culture (Mitra *et al.* 1960) and in plants regenerated from tissue culture (Sacristán & Melchers 1969). These changes have been reviewed at length (Bayliss 1980; Lee & Phillips 1988). By determining the chromosome number, the effects of the genotype (Browers & Orton 1982a; Nagarajan & Walton 1987), of medium composition (Fish & Karp 1986; Creissen & Karp 1985; Sree Ramulu *et al.* 1983), of the type of tissue (Browers & Orton 1982a; O'Connell *et al.* 1986; Sree Ramulu *et al.* 1986; Wersuhn & Sell 1988) and of the length of culture (Orton 1985; Natali & Cavallini 1987; Franklin *et al.* 1989) on the degree of somaclonal variation in callus tissues and regenerated plants have been studied.

It has been observed that plants with an aberrant chromosome number have an altered phenotype. However, such plants may also have a normal phenotype and plants with the correct chromosome number may have an aberrant phenotype (Fig. 2) (Liu & Chen 1976; Creissen & Karp 1985; Fish & Karp 1986; Gill *et al.* 1986; Maddock & Semple 1986; Sree Ramulu *et al.* 1986; Chen *et al.* 1987; Jackson & Dale 1989; Osifo *et al.* 1989). Sree Ramulu (1987; Table 3) found for three somaclonal populations that the percentage of plants with an aberrant chromosome number is not correlated with the percentage of plants with phenotypic aberrations.

Discrepancies between chromosomal and phenotypic data are also observed in evolutionary studies. In angiosperms, the genome size varies between less than 1 pg to well

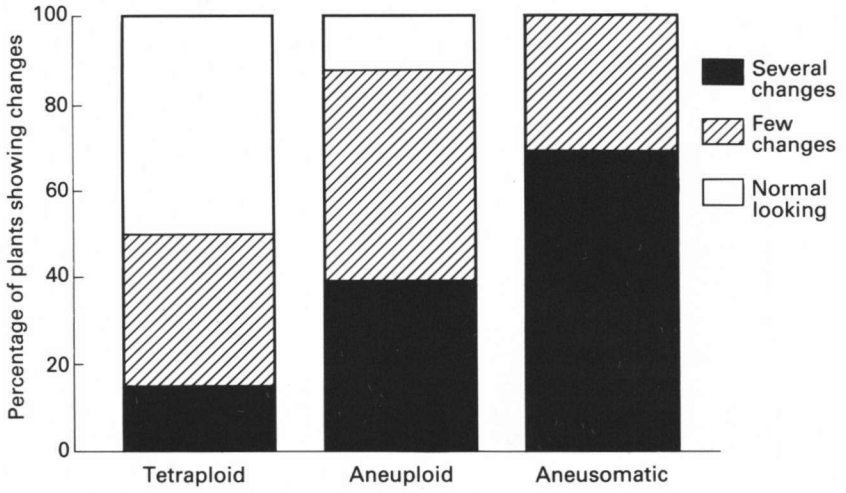

Fig. 2. Potato plants were regenerated from protoplasts. For plants with an aberrant chromsome number (aneuploid and aneusomatic) and for plants with the correct chromosome number (tetraploid), the percentages of plants with an aberrant phenotype are shown. In the regenerated plants several morphological characters were scored (vigour, leaf shape, branching, etc.) and three classes of plants were recognized: without changes (normal looking; □), with variation in 1–3 characters (few changes; ▨) and with variations in >3 characters (several changes; ■). (Based on data from Sree Ramulu *et al.* 1986.)

over 100 pg DNA per diploid nucleus, but there is no correlation between DNA content and organismic complexity (Walbot & Cullis 1985; Vedel & Delseny 1987; Price 1988). This phenomenon is widely observed in evolution and is referred to as the 'C-paradox'. The differences in DNA content are caused by a change of the ploidy, by increase of repeated sequences and by duplications of parts of the genome (Walbot & Cullis 1985). The number of genes is probably the same among the angiosperms. In *Nicotiana* (4 pg DNA per diploid cell; Vedel & Delseny 1987), this number has been estimated at approximately 15 000 (Goldberg *et al.* 1978), i.e. about 0·6% of the genome. The larger part of the variation in the size of the genome has apparently no major effect on these 15 000 genes. Two hypotheses have been put forward to explain why there is such a surplus of DNA and why this surplus varies. As the amount of DNA is correlated with ecological factors, especially with the duration of the growing season, DNA quantity itself may be of adaptive significance (Price 1988). Alternatively, it has been suggested that a very large portion of DNA is not functional but parasitic (Orgel & Crick 1980). The intra- and interspecific variation of DNA content is highly relevant for the understanding of somaclonal variation. It should be noted here that mutations in the genome of a diploid plant species (e.g. maize) differ from mutations in a polyploid species because of the buffering capacities of the extra chromosomes in the latter.

In conclusion, large chromosomal changes occur in plants and have only a small effect on the phenotype. In mammals, the situation is very different. All mammals have a genome size of 4–5 pg DNA per diploid cell. This is not caused by different evolutionary ages, as the evolution of the angiosperms started some 120 million years ago and that of the mammals about 80 million years ago (Stebbins 1982). Moreover, only few chromosomal abnormalities are tolerated in animals. These differences indicate that data on mutations obtained from mammals cannot be transferred to the angiosperms.

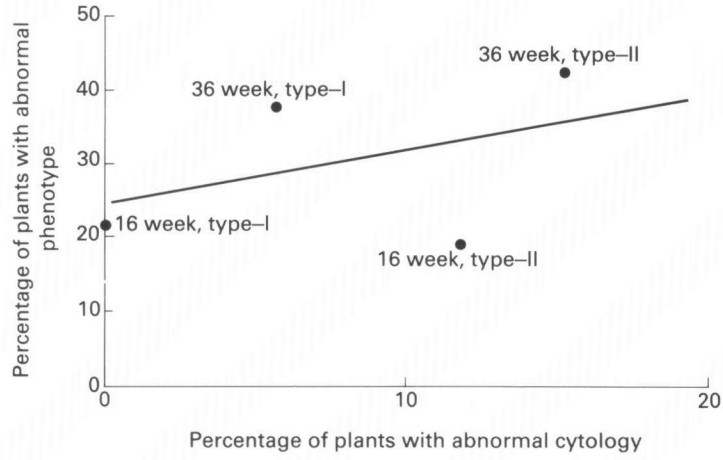

Fig. 3. Maize plants were regenerated from two types of callus after 16 or 36 weeks of culture. Type I callus was maintained in medium without proline and selected for organogenic growth. Type II callus was maintained in medium with 25 mM proline and selected for friable, embryogenic growth. The four groups of plants were examined for aberrant chromosome number and structure, and for phenotypic aberrations. For the four groups, the percentages of phenotypically aberrant plants were plotted against the corresponding percentages of plants with aberrant chromosomes. The regression line is shown; the correlation is not significant. (Based on data from Armstrong & Phillips 1988.)

Structural chromosome variation

It has been observed that in regenerated plants with the correct chromosome number, many plants show structural chromosomal changes (Lee & Phillips 1988). The detection of alterations of the chromosomal structure is very laborious and consequently not suitable for a routine assay. In addition, as in the case of chromosomal numbers, phenotypically aberrant plants may have the same chromosome structure and vice versa (Armstrong & Phillips 1988). From the data presented by Armstrong & Phillips (1988) a slight, but statistically non-significant correlation between changes in chromosome structure and number, and phenotypic changes can be deduced (Fig. 3).

DNA restriction fragments

Many authors have examined changes in restriction fragments of mitochondrial DNA (Breiman *et al.* 1987; Hartmann *et al.* 1989; Shirzadegan *et al.* 1989), chloroplast DNA (Day & Ellis 1985; Shirzadegan *et al.* 1989), introduced T-DNA (Peerbolte *et al.* 1987), nuclear DNA sequences coding for specific proteins (Brettell *et al.* 1986a; Breimann *et al.* 1987; Dennis *et al.* 1987), or nuclear ribosomal DNA and other repeated DNA sequences (Cullis & Cleary 1986; Brettell *et al.* 1986b; Breiman *et al.* 1987; Karp *et al.* 1987). As the nuclear genome is far more complex than organelle DNA (Vedel & Delseny 1987) and as the chloroplast DNA is relatively stable both in evolution and in tissue culture (Palmer & Stein 1986; Shirzadegan *et al.* 1989), most studies have been carried out on mitochondrial DNA. These studies have only rarely been aimed at establishing the effect of tissue culture conditions on somaclonal variation. For mitochondrial DNA, Hartmann *et al.* (1989) found that the extent of change depends on the length of the tissue culture period.

Only a few times has the relationship with phenotypic changes been noted. Albino plants were found to have mutated chloroplast DNA (Day & Ellis 1985). Regenerated potato plants deficient in nuclear ribosomal DNA genes (75% reduction of 25S ribosomal DNA) have the same phenotype as control plants (Landsmann & Uhrig 1985). Incidentally, Cullis & Charlton (1981) reported a deamplification of ribosomal DNA under stress in flax.

In evolutionary studies, descent trees have been constructed based on differences in DNA sequences and differences in morphology. In an excellent study on inbred strains of laboratory mice, Fitch & Atchley (1987) observed that these trees do not correspond at all (the tree based on molecular data reflects the correct phylogeny). This shows again that the magnitude of mutations at the molecular level does not correspond well with the magnitude of morphological change and that assays on the molecular level should be examined very critically for their correlation with morphological changes.

Electrophoresis patterns of proteins and isozymes

The genetic stability of cultured tissues or regenerated plants is often examined through isozyme patterns (Orton 1980; O'Connell *et al.* 1986; Karp *et al.* 1987; Kobayashi 1987; Maheswaran & Williams 1987). Furthermore, electrophoretic patterns of seed storage proteins have been examined in regenerated plants with the aim of improving the nutritive value of seeds (Larkin *et al.* 1984; Maddock *et al.* 1985; Cooper *et al.* 1986; but also see Metakosky *et al.* 1987). Data from these studies should be interpreted with care.

First, when the regenerated plants themselves are examined, it may be that epigenetic factors affect the patterns. This has probably been the case in a study by Allicchio *et al.* (1987), who claim somaclonal variation for esterase isozymes in regenerated potato plants. They reported a new esterase isozyme at the same position on the gel in three out of eight regenerated plants. It is very unlikely, however, that the same mutational event occurs three times. Rather, either the plants had originated from one cell in which the mutation had taken place, or the extra band was due to an epigenetic change. For an assay based on isozymes, systems should be chosen that are expressed very stably during the development of a plant and that are not affected much by environmental factors. Peroxidase isozymes are frequently examined in these studies (Heinz & Mee 1971; Maheswaran & Williams 1987). However, the amount of various peroxidases is strongly dependent on the physiological conditions and on the developmental status. For example, in micropropagated *Malus* shoots, which outwardly all look the same, the activity of basic peroxidases varies 12-fold (De Klerk *et al.* 1990b).

Secondly, in an excellent study, Orton (1985) examined celery plants that were heterozygous for five isozymes. After 12 months of culture, he did not observe a single change in the isozymes in 50 callus clones originating from these plants. For chromosome structure and number, though, there were gross shifts in all but one of 40 callus cells examined.

Thirdly, there is no relationship between the change of isozyme patterns and morphological changes (Heinz & Mee 1971, see Fig. 4; the same can be derived from data of Sanford *et al.* 1984; Wang & Holl 1988; Jackson & Dale 1989). In agreement with this, a study in maize revealed that the extent of morphological differences between five lines is not correlated with qualitative differences between two-dimensional protein patterns, but with quantitative differences (Damerval *et al.* 1987). From this, it can be derived that in an assay of somaclonal variation based on isozyme or protein electrophoresis, the patterns should be compared not for the absence or presence of specific bands, but for the amount of protein per band. To date, such a study has not been made in regenerated plants.

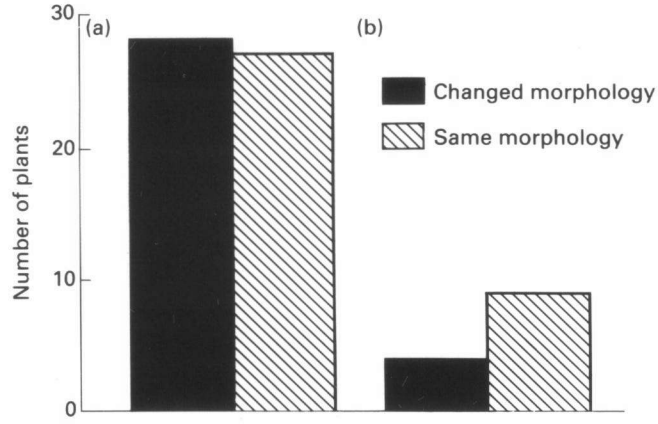

Fig. 4. Sugarcane plants were regenerated from callus and examined for four isozyme systems (amylase, peroxidase, glutamic oxalacetic transaminase, and esterase), and for phenotypic aberrations. For plants with changed isozyme patterns (a) and for plants with unaltered isozyme patterns (b), the numbers of plants with an aberrant (■) or normal phenotype (▨) are shown. (Based on data from Heinz & Mee 1971.)

CONCLUSIONS

Plants generated from adventitious buds often show genetic variation ranging from point mutations to numerical chromosome changes. A large portion of this so-called 'somaclonal variation' is deleterious to the performance of the plants. Because the regeneration of adventitious meristems is one of the key steps in biotechnological breeding and propagation methods, somaclonal variation may turn out to be one of the main stumbling blocks in the practical application of these methods. To develop protocols for avoiding and/or counteracting somaclonal variation and to understand its backgrounds, a simple assay of somaclonal variation is needed. Such an assay may be on the phenotypic or on the molecular/cytological level.

On the phenotypic level, the extent of somaclonal variation is usually assessed by determining the percentage of aberrant plants. This assay has at least three disadvantages. First, it is necessary to evaluate a large number of plants for each determination (at least 100). If, for example, the effect of five auxin concentrations on somaclonal variation is examined, it will be necessary to culture and judge 5×100 regenerated plants. Secondly, the measurements should preferably be made in the adult progeny of selfed, regenerated plants. So, in most cases the actual measurements will be far more than 1 year after the start of tissue culture. Thirdly, for many traits the evaluation of qualitatively aberrant plants depends upon the eye of the observer.

Somaclonal variation has also been determined on the molecular and cytological levels. Most of these determinations are laborious. As they are only qualitative, it is also necessary to determine the percentage of aberrant plants and, consequently, to examine large numbers of plants. Just as in evolutionary studies (Paigen 1986; Patterson 1987) and in studies on cultivars (Damerval *et al.* 1987), no good correlation exists between the extent of mutations at these levels and phenotypic changes. An advantage of these methods (with the exception of isozyme patterns), however, is that the determination can be made shortly after the start of the tissue culture period in the regenerated plants themselves. A second advantage is that the measurements are objective.

It is suggested in this article and in a concomitant paper (De Klerk *et al.* 1990a), that the value of the SD of quantitative phenotypic traits in a somaclonal population may be an accurate measurement of the extent of somaclonal variation. Such an assay has several advantages, namely, its sensitivity, the small number of plants needed in each somaclonal population (20–30) and the objectivity of the quantitative parameters. However, in the regenerated plants themselves epigenetic factors might disturb the measurements. An assay based on the value of the SD is corroborated by many data in the literature (e.g. Jackson & Dale 1989; Zheng *et al.* 1989), but has not yet been examined thoroughly.

An assay for somaclonal variation can be used, and is actually indispensable for several purposes. First, as somaclonal variation has been found to depend on the genotype, stable genotypes may be selected for genetic engineering and propagation via somatic embryos. Secondly, such an assay will allow the development of protocols that minimize variability. Thirdly, by identifying the factors that have an effect on variability, such an assay will enable the study of the mechanisms underlying somaclonal variation.

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