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EXAMINATION OF A POPULATION OF SOMACLONES FOR VARIATION IN DNA CONTENT, ISOZYME BANDING PATTERNS, AND TISSUE CULTURE RESPONSE IN SUGARBEET (Beta vulgaris L.)

By

Yong Rhee

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ABSTRACT

EXAMINATION OF A POPULATION OF SOMACLONES FOR VARIATION IN DNA CONTENT, ISOZYME BANDING PATTERNS, AND TISSUE CULTURE RESPONSE IN SUGARBEET (Beta vulgaris L.)

By

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Somaclonal variation is genetic change occurring in plants regenerated from callus or other in vitro culture. Nineteen sugarbeet somaclones (regenerated plants) were evaluated for somaclonal variation for three parameters Firstly, for tissue culture response, intensity of leaf disc callus induction and of shoot regeneration on media with and without cytokinin were not significantly different from the source genotype; shoot regeneration occurred only on the medium with exogenous cytokinin. Secondly, isozyme banding patterns with starch gel electrophoresis of 6-phosphogluconic acid, malate dehydrogenase, shikimate dehydrogenase, and glutamate dehydrogenase from leaves, as well as alcohol dehydrogenase from callus showed no somaclonal variation. Thirdly, no significant difference in DNA content in nuclei of leaf cells as determined by flow cytometry was found. Additionally, a narrow leaf variant with altered lateral stalk organization did not differ from the source genotype in isozyme banding patterns and DNA content. The narrow leaf variation was not recovered in F_1 progeny. In an initial direction of research, haploids were evaluated for their callus induction and shoot regeneration prior to intended use in recovery of recessive somaclonal variation. But, an unexpectedly low response was found in haploids in comparison with the source diploids.

To my parents

ACKNOWLEDGMENT

I dedicate this thesis to my beloved parents. They always encouraged and gave endless support to me. They also showed me the importance of considering others before taking care of myself. I believe that this philosophy will be the very basis of my whole career in my life.

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EXAMINATION OF A POPULATION OF SOMACLONES FOR VARIATION IN DNA CONTENT, ISOZYME PATTERNS, AND TISSUE CULTURE RESPONSE IN SUGARBEET (Beta vulgaris L.)

LITERATURE REVIEW

Plant cell and tissue culture procedures has been considered as a potentially important tool to produce clonal plants in vitro, to study metabolism and its inheritance, and to serve in plant transformation protocols. Genetic fidelity among cells or plants is usually desired and assumed. The cell and tissue culture procedure, however, does not always produce clonal cells or plants, and frequently results in numerous genetic changes. Larkin and Scowcroft (1981) have argued that these genetic changes are a general phenomenon of cell and tissue culture to which they gave the name 'somaclonal variation'. Controlling this variation is desirable and will provide a powerful source of useful mutants, or for plant improvement (Orton, 1984; Karp, 1991; Phillips et al. 1994). But, in contrast to its potentials for plant biotechnology, the underlying genetic bases of somaclonal variation have been not yet elucidated completely.

From these viewpoints, this review is aimed at providing general information on somaclonal variation, hoping that the information presented will show a good guideline for further research for somaclonal variation with the recognition of the importance of this variation. For this purpose, the emphasis will be on both the various causes of somaclonal variation and the general approaches for measuring somaclonal variation.

It has been known that chromosomal abnormalities with changes in chromosome number and structure occur at a high frequency in some types of regenerated plants. Polyploidy and aneuploidy are found in callus and regenerated plants (Bayliss, 1980; D'Amato, 1985). Two main factors have been considered to play an important role in the chromosome number changes in callus and regenerated plants: firstly, the composition of the medium, especially kinds and /or concentrations of the plant growth regulators was considered. Some studies suggested that auxins and /or cytokinins induced chromosomal abnormalities, probably leading to chromosome number variation in callus or regnerated plants (Bayliss, 1973; Ogura, 1982). Secondly, the duration of the culture of callus may cause chromosome aberrations with the function of time after callus initiation. It has been reported that chromosomal variation increased in proportion to the duration of the callus culture over time after callus initiation (Ogura, 1982). Swedlund and Vasil (1985) also reported that the proportion of diploid cells decreased, while that of tetraploid and aneuploid cells increased after 6 months of callus culture in pearl millet embryogenic callus.

Chromosome structural changes or karyotypic changes of different types (deficiencies, translocations, duplications, inversions) are common in callus and regenerated plants. Murata and Orton (1983,1984) observed chromosome fusions in celery suspension culture. They reported that chromosome fusion seems to be a primary source of chromosomal aberrations such as translocation, duplication, and deficiency, which are probably involved in chromosome number variation within celery suspension cultures. Callus and regenerated plants can display cytologically observable structural

changes in their chromosomes (D'Amato, 1985). The consequence can be production of chimeric genes with promoter substitutions which alter expression conditions and genetic transmission of specific genes perhaps by deletion or duplication of one copy of a gene. In addition, recombination or chromosome breakage can occur in hot spots of particular chromosomes, thereby affecting some regions of the genome. For example, Karp and Maddock (1984) reported that 29 % of the 192 regenerants from four different wheat cultivars showed chromosome interchanges. And Lee and Phillips (1988) have cited evidence for chromosome translocations, deletions, inversions, and other minor reciprocal and nonreciprocal rearrangements in other many plant species such as ryegrass, potato, maize and oats, and argued that late-replicating heterochromatin may occasionally replicate so late in mitosis of cultured cells that bridge formation and subsequent chromosome breakage occurs at anaphase. This would be lead to a breakage-fusion-bridge cycle with consequent genetic effects, such as deletions and chromosome interchanges.

Transposable elements can be activated and alter gene expression in callus and regenerated plants (Peschke et al. 1987). Cell and tissue culture procedures are highly mutagenic to plant cells (Karp and Bright, 1985). And so, they can be a cause for genome alterations or rearrangements (McClintock, 1984), stimulating transposable element activities. Evidence has become more numerous for transposable elements' activation in callus and regenerated plants, although this evidence is generally limited to maize (Evola et al. 1985; James and Stadler, 1989; Peschke and Phillips, 1991). In alfalfa, the involvement of transposable element activities was suggested in the unstable

mutation to white flower with high frequencies of reversion in color in vitro (Groose and Bingham, 1986). Activation of uncharacterized silent elements has also been suggested based on the recovery of unstable variants consisting of an extremely high frequency of green and yellow sectors in tobacco in vitro (Lorz and Scowcroft, 1983).

An increasing number of plant genes are proving to be normally encoded and expressed in multigene families usually in clusters in one or more positions in the genome. Somaclones have been found with altered patterns of products from such families. The gliadin storage protein of wheat (Cooper et al. 1986; Larkin et al. 1984; Maddock et al. 1985), the hordeins of wild barley (Breiman et al. 1987), the beta-amylases of wheat (Ryan and Scowcroft, 1987), and the esterases of potato (Allichio et al. 1987) are the examples for this case. Some of these mutants express proteins not present in the donor plants. There may be heritably repressed genes in these gene families that are exposed following callus initiation.

It has been reported that specific genes can amplify or deamplify themselves during differentiation or in response to environmental pressures. Depending how gene expression is regulated, this could mean that the production of mRNA and protein from that gene is up or down regulated. If such amplification or deamplification of DNA sequence copies also can occur in callus or regenerated plants, it may account at least in part for somaclonal variation. Cullis and Cleary (1986) showed that the copy number of repeated DNA in flax varied when exposed to various environmental stimuli. Amplification and deamplification of DNA have been reported in both callus and regenerated plants of flax. Another example is the amplification seen in alfalfa following

cell selection. In a cell line selected for resistance to a competitive inhibitor of glutamine synthase, the tolerant cell line contained 4-11 times the number of glutamine synthase gene sequences (Donn et al. 1984). Examples of dramatic deamplification have been found in potato (Landsmann and Uhrig, 1985) and barley (Breiman et al. 1987).

Plant DNA has been known to be partially methylated and the role of methylation in gene expression of plants has been reviewed (Hepburn et al. 1987). DNA methylation can affect plant gene regulation and expression at the level of transcription (Palmgren et al. 1991). It was proposed that every steps in callus cutlure and regeneration could alter DNA methylation patterns in regenerated plants (Phillips et al. 1990; Kaeppler and Phillips, 1993). For instance, in carrot, genomic DNA methylation was shown to be tissue specific and influenced by plant growth, culture age, genotype, and primary cultures during the transition from linear to stationary growth in vitro (Arnholdt-Schmitt et al. 1995). The maize plants regenerated in vitro showed both increases and decreases in methylation (Brown 1989; Muller et al. 1990). It has been also suggested that DNA methylation could be correlated with transposable element activities (Chandler and Walbot, 1986; Banks et al. 1988) and chromatin structure (Klaas and Amasino, 1989) in vitro.

The measurement of the extent of somaclonal variation is important for several reasons. Firstly, as somaclonal variation has been found to depend on the genotype (Puolimatka and Karp, 1993), stable genotypes may be selected for genetic engineering and propagation via somatic embryos. Secondly, such a measurement will allow the development of protocols that minimize variations. Lastly, by identifying the factors that

have effect on generation of somaclonal variation, such a measurement will enable the study of the mechanisms underlying somaclonal variation. Actually, such measurement may be on the morphological, on the cytological or on the biochemical / molecular level.

On the gross morphological level, the extent of somaclonal variation is usually determined as the percentage of plants which shows one or more qualitative variations, for example, the percentage of plants with distinct morphological alterations such as dwarfism, albinism and aberrant leaf shape (Lee and Phillips, 1987). Alternatively, De Klerk et al. (1990) proposed to measure the extent of variation in a somaclonal population by determining the value of the Standard Deviation (SD) for a quantitative trait and have used the leaf shape (expressed as the natural logarithm of the ratio of the longest rib and its longest perpendicular rib) of regenerants of *Begonia* × *hiemalis* as such a parameter. Wang and Holl (1988) found an increased value of the SD for the ratio of length-width of the leaf shape in a somaclonal population of *Trifolium pratense*. An increased value of the SD for various traits was also reported in regenerated rice plants (Zheng et al. 1989).

On the cytological level, chromosome number or ploidy of somaclones is determined by chromosome counts in mitotic cells from the regenerant's root tips, by measurement of the DNA content per cell either by a microspectrofluorophotometric determination after Feulgen staining, or by flow cytometry. But chromosome counts can only be made on dividing cells, and thus the sample is limited and potentially biased. Furthermore, chromosome counts do not measure changes in chromosome volume and DNA content, and therefore there may be significant changes that such chromosome counts do not reveal (Karp and Bright, 1985). Microspectrofluoro-photometric

techniques avoid these problems, as they measure cells in all phases of the cell cycle, and flow cytometry is probably the most rapid method of these for measuring DNA content. It has been found that flow cytometric analysis of DNA content can successfully be accomplished measuring the relative fluorescence of stained nuclei (Galbraith et al. 1983). For example, the ploidy stability of somaclones was evaluated by nuclear DNA contents with flow cytometry and it was found that ploidy of somaclones from short term callus culture was significantly different from somaclones' ploidy from long term callus culture (Jacq et al. 1992). But most of these methods do not permit individual cell selection and are limited to fluorescence dyes. The detection of small alterations of the chromosomal structure is very laborious. But karyotypic analyses of cultured cells have clearly demonstrated the existence of translocations, fusions, and deletions (Orton, 1980). With differential staining of chromatin using Giemsa banding, Ashimore and Gould (1981) showed specific structural changes in altered karyotypes of cultured crown gall tissues of *Crepis capillaris*. Interestingly, it has been observed that in regenerated plants with the correct chromosome number, many plants show structural chromosomal changes (Lee and Phillips, 1988). However, phenotypically aberrant plants may have the same chromosome structure as the phenotypically normal plants and vice versa (Armstrong and Phillips, 1988).

On the biochemical level, the genetic fidelity of callus or regenerated plants is often examined at the isozyme level. This approach has been conducted with many plant species such as barley (Orton, 1980), tomato (O'Connell et al. 1986), orange (Kobayashi, 1987), and beet (Sabir et al. 1992). Qualitative changes such as loss of

electromorph activity or altered mobilities can be seen directly and are probably indicative of underlying mutation, and can also be tracked by transmission genetics. For a measurement based on isozymes, enzyme systems should be chosen that are expressed very stably during plant development and that are not affected much by environmental factors. For this reason, peroxidase isozymes were frequently examined in these studies (Maheswaran and Williams, 1987).

New capabilities in molecular biology have and will continue to provide precise, unequivocal measurement of somaclonal variation. This is a consequence of the ability to discern variations of DNA directly using sequence-specific restriction enzymes and cloned probes. For example, band polymorphisms were observed from sugarbeet somaclones by restriction enzyme digestions based RFLP (Sabir et al. 1992) and RAPD (Munthali et al. 1996). As the nuclear genome is far more complex than cytoplasmic organelle DNA (Vedel and Delseny, 1987) and as the chloroplast DNA is relatively stable in callus or regenerated plants (Shirzadegan et al. 1989), most molecular studies for somaclonal variation have been carried out on mitochondrial DNA. Hartmann et al. (1989) found that the extent of change of mitochondrial DNA in wheat depends on the length of the tissue culture period. Furthermore, genome rearrangements in sugarbeet mitochondrial DNA were detected in callus and suspension-cultured cells (Dikalova et al. 1993) as well as somaclones from callus culture (Brears et al. 1989; Dikalova et al. 1993). Rivin et al. (1983) argued that new rapid molecular cloning that utilizes improved vectors has made it possible to obtain comprehensive stable nuclear genomic libraries. If taken from standard or reference genotypes, specific constituents of such libraries will be

useful as direct genetic or chromosome structural probes for detection of variation.

In vitro micropropagation is being used increasingly as a production process to rapidly propagate elite genotypes and genetically engineered plants. But, somaclonal variation could be a serious disadvantage to such operations if adventitious shoot production is involved, because clonal uniformity may not be sustained. Furthermore, somaclonal variation is a random process which produces both desirable and deleterious variants (Evans and Sharp, 1986). It can be very important therefore that reliable procedures are developed to control this unwanted source of variation.

Since its intial recognition, somaclonal variation has been also regarded as a new source of genetic variability for plant improvement in spite of its limitations (Larkin and Scowcroft, 1981; Bingham and McCoy, 1986; Ryan et al. 1987). One of the major benefits of somaclonal variation is the creation of additional genetic variability in coadapted, agronomically useful cultivars, without the need to resort to hybridization. This takes on added appeal if efficient in vitro selection is possible or if rapid plant screening procedures are available. Characteristics for which the recovery frequency of somaclonal mutants can be enriched in vitro include resistance to herbicides (Saunders et al. 1992; Donn et al. 1984), and tolerance of unfavorable environmental or chemical conditions (Levall and Bornman, 1993). Somaclonal variation for other characters for which there is no adequately defined in vitro response, such as seed protein quality (Zeng et al. 1989), photosynthetic efficiency or yield, can be of benefit, provided efficient whole plant screening protocols are available (Evans and Sharp, 1986).

INTRODUCTION

There has been continual success in producing superior crop varieties by conventional plant improvement. However, it is likely that there are still a lot of valuable gene combinations or additions that can not be achieved by conventional methods. At the present time, considerable advances have been made in the genetic engineering of crop plants through the coupling of recombinant DNA techniques with shoot or embryo regeneration from plant tissue cultures. Amongst the current problems facing the advance of this technology is the occurrence of somaclonal variation, that is, genetic infidelity arising due to cell proliferation in callus or suspension culture. The term was originally coined by Larkin and Scowcroft (1981) to describe "the variations displayed among the plants derived from any form of cell and tissue culture". Its origins and causes (Gould, 1986; Sree Ramulu, 1987) have been discussed speculatively, but so far basic knowledge is fragmentary and only a few general principles have emerged.

Somaclonal variation is of particular interest because it may provide hints as to the basic mechanisms underlying genotypic and phenotypic stability in normal plant development. It is also of considerable practical importance in the application of plant tissue culture in systems for plant improvement. When tissue culture is used for plant propagation or as a system for genetic manipulation, unwanted variation must be minimized. On the other hand, spontaneous and induced genetic changes arising in

culture may provide novel forms of variation (Meins, 1983), particularly if efficiently screened.

From this viewpoint, if somaclones were obtained from adventitious shoots, for the further evaluation of somaclonal variation, it would be advisable to carry out a careful and critical assessment of various parameters on the extent of somaclonal variation. Assessments can be carried out with evaluations at the phenotypic level of plant morphology and electrophoresis banding patterns of isozymes, as well as at the genotypic level of chromosome number, structure, DNA content, and DNA restriction fragments (De Klerk, 1990; Orton, 1983).

Sugarbeet (Beta vulgaris L.), a root crop plant grown mainly in temperate regions, is an economically important agricultural plant and supplies nearly 40% of the world supply of sugar (Smith, 1987). The leading sugarbeet producing countries are France, Germany, Russia, Poland, Ukraine, and the United States (in alphabetical order).

A member of the Chenopodiaceae, sugarbeet is an allogamous species with a biennial life cycle. In the first season, the plant grows a foliar rosette and sucrose is stored in a large fleshy taproot. Following an overwintering experience and exposure to long-day conditions, a large bushy flower stalk emerges on which as many as 5000 seeds ripen under favorable conditions. The fruit is a nutlet, and, depending on the number of potential seeds per fruit, there are two kinds of fruits - monogerm and multigerm. Sugarbeet sometimes produces a precocious flower stalk during the first season in its life cycle. This phenomenon is called bolting and is highly undesirable in sugarbeet production.

In sugarbeet, with reliable shoot regeneration methods available fairly recently, comprehensive reports on the scope of somaclonal variation are lacking. Nevertheless, in several studies, variation derived from in vitro culture has been detected in sugarbeet plants regenerated from callus, cell suspensions, and protoplasts. This variation includes apparent halving of chromosome number (Steen et al. 1986), changes in isozyme patterns (Steen et al. 1986), including an overproducer of glutamate dehydrogenase (Sabir et al. 1992), in band polymorphism based on RFLP (Sabir et al. 1992) and RAPD (Munthali et al. 1996), genome rearrangements in mitochondrial DNA (Brears et al. 1989; Dikalova et al. 1993), altered in vitro response (Saunders and Doley, 1986; Detrez et al. 1989), recurrence of a novel intumescence character in regenerants from different donor plants (Yu et al. 1991), three monogenic dominant herbicide resistances (Saunders et al. 1992; Wright and Penner, unpublished), UV-tolerant somaclonal variants (Levall and Bornman, 1993), as well as a leaf necrosis trait and a heritable pigment variant of possibly unstable nature (Saunders et al. 1990). Some of these variants have not yet been well characterized. Furthermore, there appear to have been no reports of somaclonal variation in sugarbeet for quantitative agronomic traits (Saunders et al. 1990). So, more extensive and detailed studies of somaclonal variation in sugarbeet remain to be conducted.

At least nineteen sugarbeet somaclones were available that had been regenerated from leaf disc callus of diploid cytoplasmic male sterile genotype 8266-10 on a medium which contained 30 mM glutamine as the only nitrogen source. Glutamine was used as selective regime in a selection experiment to seek sugar beet somaclones with the better

nitrogen assimilation, perhaps by recovering a glutamate dehydrogenase overproducer similar to that found by Sabir et al. (1992). It was proposed that these somaclones should be evaluated for possible phenotypic identification of somaclonal variants, some of which would be available for incorporation into sugarbeet breeding programs in the future.

This research was aimed at detecting any possible somaclonal variation in these nineteen somaclones for several parameters such as tissue culture response, DNA content examination, and isozyme banding patterns. Additionally, a somaclonal variant with a distinct morphological aberration - a narrow leaf (Fig 1.1) and abnormal lateral stalk organization (Fig. 1.2 and Fig 1.3) - was obtained separately from the source clone 8266-10 and named as SC-NL. The narrow leaf variant was examined at the biochemical and cytogenetical level, namely, for isozyme banding patterns and DNA content. Molecular approaches were not performed. Information on transmission genetics might be available from seed harvested on the somaclones at a later date, but was not reported at this time.

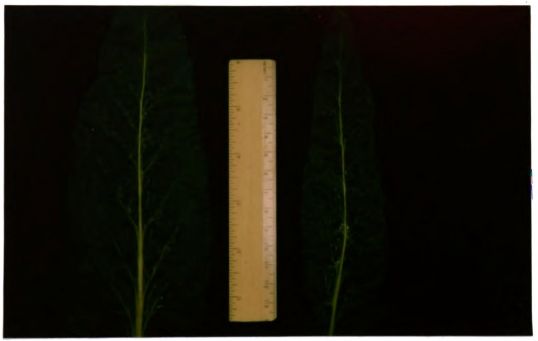

Figure 1. A morphologically aberrant somaclonal variantion. One variation is narrow leaf size in Fig 1.1 (right: source genotype 8266-10, left: SC-NL). Another variation is abnormal lateral stalk organization in Fig 1.2 (right: source genotype 8266-10, left: SC-NL) and Fig 1.3 (right: source genotype 8266-10, left: SC-NL). This variant was named the narrow leaf somaclonal variant (SC-NL).

(Fig 1.2) The young abnormal lateral stalk from SC-NL (right: source genotype 8266-10, left: SC-NL).

(Fig 1.3) The fully grown abnormal lateral stalk from SC-NL (right: source genotype 8266-10, left: SC-NL).

MATERIALS AND METHODS

EXPERIMENT 1. TISSUE CULTURE RESPONSE OF LEAF DISCS

Plant maintenance

Sugarbeet somaclones (regenerants) and source plants were used as donors of leaf explants. Source plant genotype 8266-10 was a cytoplasmically male sterile BC_3 (third backcross generation) individual from the recurrent parent clone 436-3 (with acknowledged shoot regeneration ability, Saunders and Daub, 1984), cytoplasmic male sterility and biennial nature. The nineteen somaclones had been produced independently by callus induction and shoot regeneration from control plant 8266-10. The somaclones and control plants were grown in pots with Baccto high porosity professional planting mix (55-65 % of horticultural sphagnum peat perlite, Michigan Peat Co.) in the greenhouse without supplementary lighting and fertilized weekly with Peters 20-20-20 water soluble commercial nutrient mix (Peters Professional Co, Marysville OH).

Leaf sample preparation

Leaf discs were taken from one smaller and one larger leaf at 20-40 % of fully expanded leaf size from source plants as well as each somaclone. Detached leaves were surface sterilized with two twenty minute soakings in 15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by three rinses with sterile distilled water. Leaf discs were excised from each leaf blade, excluding leaf veins, using a #3 cork borer (0.7 mm in diameter).

Media preparation

The medium designated B_1 was prepared from MS medium (Murashige and Skoog, 1962) which consists of inorganic and organic mineral salts, 3% sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1.0 mg/L thiamine·HCl (Table 1). 0.9% (w/v) Difco Bacto agar was included. 1.0 mg/L 6-benzyladenine (6-BA) was added prior to autoclaving as the only growth regulator used for B_1 medium. The final pH was adjusted to 5.95 with KOH prior to autoclaving for 30 minutes at 121 °C (15 lb/in^2). Basal medium MO was identical but lacked 6-BA. Medium was dispensed into 20 × 100 mm disposable plastic Petri dishes after autoclaving, with 35 ml per dish.

Culture establishment

One leaf disc was used per dish. Dishes were sealed with two strips of parafilm (American National Can Co, Neenah WI). Thirty six leaf discs were used for source plant and each somaclone, respectively. The experimental system was a completely randomized design. All cultures were maintained at 31 °C in the dark. Six weeks after initiating the leaf disc cultures, observations of the responses began and were terminated after 10 weeks with B_1 media and 14 weeks with MO media (because callus initiation took several weeks longer on MO media as reported by Doley and Saunders 1989). The proportion of leaf discs for each entry producing callus, or shoots from that callus. Statistical comparisons of each somaclone with source genotype 8266-10 were made by the chi-square test with the Yates correction factor for one degree of freedom.

Table 1. Inorganic and organic constituents of MS* media (Murashige and Skoog, 1962)

Constituents	mg/L
INORGANIC	
KNO_3	1,900
NH_4NO_3	1,650
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.60
H_3BO_3	6.20
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
ORGANIC	
Sucrose	30,000
Myo-inositol	100
Thiamine·HCl	1.0
Nicotinic acid	0.5
Pyridoxine·HCl	0.5

* For B_1 media, 1.0 mg/L of BA was added to MS media.

For MO media, no BA was added to MS media.

EXPERIMENT 2. ISOZYME BANDING PATTERNS WITH STARCH GEL ELECTROPHORESIS

Sample preparation

Young healthy leaves, approximately 2-4 cm long, were collected in the greenhouse from control 8266-10, each somaclone from SC 1 to SC 19, and SC-NL immediately before they were used. The genotype 80-111 (Aicher and Saunders, 1990) was used as a reference of banding mobility for 8266-10. Three 1 cm diameter leaf discs (= 0.03 g) were excised from each leaf blade, excluding leaf vein, using a #3 cork borer (0.7 mm in diameter). The discs were homogenized using a glass pestle in porcelain spot plates containing 200 μL of extraction buffer. Fresh calli (= 0.6 g) of each somaclone from SC 1 to SC 19 was used for Adh. Callus was not available for SC-NL. The calli were also homogenized in 250 μL of extraction buffer. The extraction buffer consisted of 0.1 M Tris-HCl, pH 7.0 buffer, containing 18% glycerol, 5% (w/v) soluble polyvinylpyrrolidone (PVP-40T), 0.5% Triton X-100 and 1.0% 2-mercaptoethanol which was added just before use. The spot plates were placed on blocks of ice in trays to maintain temperatures near 0 °C throughout sample preparation.

Systems and gel electrophoresis

The apparatus was a slight modification of that described by O'Malley, Wheeler and Guries (1980). Three buffer systems were used to resolve all of the enzyme systems examined. System I consisted of a electrode buffer with 0.065 M L-histidine, 0.02 M citric acid monohydrate, pH 5.7 and a gel buffer that was a 1:6 dilution of the electrode buffer (Cardy, Stuber and Goodman, 1980). System II consisted of a 0.125 M Tris-citric

acid, pH 7.0 electrode buffer modified from Cheliak and Pitel (1984). The gel buffer was a 0.02 M DL- histidine·HCl, 0.56 mM Na_2EDTA buffer adjusted to pH 7.0 with Tris (Trizma base). System III consisted of the same electrode buffer as System II. The gel buffer was a 0.05 M DL-histidine·HCl, 1.4mM Na_2EDTA buffer adjusted to pH 7.0 with Tris (Trizma base). Gels were prepared with 600 ml gel buffer, 75 g potato starch (Sigma Chemical Company) and for system I gels only, 21 g sucrose. The heated and aspirated gel solution was poured into a plexiglass frame with the dimensions: 21.7 cm long × 14.0 cm wide × 1.5 cm deep. The gels were allowed to cool for 2 hours, and were then covered with 2 layers of plastic wrap and kept in the room temperature overnight. Before loading the samples, the gel was kept in a refrigerator at 4 °C for 30 minutes. Twenty one paper wicks (Whatman Electrophoresis Paper No. 3MM), 4 × 15 mm in size, were soaked in leaf extract, blotted on paper toweling, and inserted into a slice in the starch gel 6 cm from the cathodal side of the gel, for system I, II, and III, respectively. After loading the samples, the gel was placed in a refrigerator at 4 °C, and the gels were run at 20 watts constant power. After 30 minutes the wicks were removed and ice bags were placed on top of the gels, and then electrophoresis was resumed for a total of 5 hours for system I, 6 hours for system II, and 6 hours for system III. The enzyme which was examined using system I was 6-phosphogluconic acid dehydrogenase (6-Pgdh). The enzymes which were examined using system II were malate dehydrogenase (Mdh) and shikimate dehydrogenase (Skdh). The enzymes which were examined using system III were glutamate dehydrogenase (Gdh) and alcohol dehydrogenase (Adh).

Gel staining

After electrophoresis, gels were sliced into 1/16″ thick slices using plastic spacer and a fine steel guitar string. Although 5 or 6 thin slices were obtained from a single gel, the top and bottom slices were discarded because they gave poor band resolution. The third and fourth slices were harvested and immersed in stain solutions for 1 hour at 37 °C. Adh was stained for 2 hours in room temperature. The 6-Pgdh, Skdh, and Mdh enzyme stains were adapted from Cheliak and Pitel (1984). The 6-Pgdh was stained with 50 mg $MgCl_2$, 20 mg 6-phosphogluconic acid, 10 mg NADP, 10 mg nitro blue tetrazolium (NBT) and 1 mg phenazine methasulphate (PMS) in 50 ml 0.1 M Tris-HCl pH 8.0 buffer. Skdh was stained with 40 mg shikimic acid, 10 mg NADP (β-nicotinamide adenine dinucleotide phosphate), 10 mg NBT and 1 mg PMS in 50 ml 0.1 M Tris-HCl pH 8.0 buffer. Mdh was stained using 25 ml 0.5 M DL-malate pH 7.0 buffer and 12.5 mg β - nicotinamide adenine dinucleotide (NAD), 10 mg NBT, and 1 mg PMS in 25 ml 0.1 M Tris-HCl pH 8.0 buffer. Adh was stained with 10 mg NAD, 10 mg NBT and 2 mg PMS in 49 ml 0.1 M Tris-HCl pH 8.5 buffer. 1 ml of 95% ethyl alcohol was added just before staining. Gdh was stained with 4 g L-glutamic acid, 20 mg NAD, 20 mg NBT and 1 mg PMS in 50 ml 0.1 M Tris-HCl pH 8.0 buffer. Gels were kept in the dark during staining to prevent excess background staining and to protect light sensitive reagents. After staining, gels were rinsed with distilled water two or three times and fixed by 6 (distilled water) : 4 (95% ethyl alcohol) solution. Zymograms of source genotype 8266-10 and nineteen somaclones were scored and examined for banding patterns. All methods for system I, II and III are summarized in Table 2.

Table 2. Summary of electrophoretic materials and methods

	System I	System II	System III
pH	5.7	7.0	7.0
Electrode buffer (EB)	0.065 M L-histidine 0.020 M citric acid adjust pH with citrate	0.125 M Tris adjusted to pH 7.0 with Tris acid anhydrous	0.125 M Tris adjusted to pH 7.0 with Tris acid anhydrous
Gel Buffer (GB)	1:6 dilution of EB	0.02 M DL-histidine·HCl 0.56 mM EDTA to pH 7.0 with Tris	0.05 M DL-histidine·HCl 1.4 mM EDTA to pH 7.0 with Tris
Gel	75.0 g potato starch and 21.0 g sucrose in 600 mL GB	75.0 g potato starch in 600 mL GB	75.0 g potato starch and 21.0 g sucrose in 600 mL GB
Supplied Power	20 watts (50 mAmps, 400 V)	20 watts (75 mAmps, 270 V)	18 watts (70 mAmps, 250 V)
Duration	5.5 hours	6 hours	6.5 hours
Resolved Enzyme	6-Pgdh	Skdh Mdh	Gdh Adh*

* The source of Adh in this study is only from callus.

EXPERIMENT 3. NUCLEAR DNA CONTENTS AS DETERMINED BY FLOW CYTOMETRY

Solutions required

The following stock solutions were prepared before sample preparation. $MgSO_4$ buffer (pH = 8.0; 10 mM $MgSO_4$, 50 mM KCl and 5 mM Hepes) (van den Engh et al. 1984), 10 % (w/v) Triton X-100, propidium iodide (PI) stock solution (5 mg/mL, covered with aluminum foil and stored at 2-4 °C), and Alsever's solution (pH = 6.1; 0.55 mg/mL citric acid, 20.5 mg/mL glucose, 4.2 mg/mL sodium chloride, 8 mg/mL sodium citrate). Chicken red blood cells (CRBC) was used as an internal standard. 1:100 dilution of the delivered fresh CRBC (Colorado Serum Co, catalog no. cs 1151) in Alsever's solution gives approximately 10^7 CRBC/mL. This concentration was recommended for a good DNA peak for chicken red blood cells as the internal standard (Arumuganathan and Earle, 1991). The diluted solution was stored at 2-4 °C. RNAase (DNAase free) was purchased from Boehringer Mannheim Biochemicals (catalog no. 1119915) and stored below 0 °C.

The following solutions were freshly prepared using the stock solutions, protected from light with aluminum foil, and kept on ice. Solution A (15 mL enough for up to 12 samples) consisted of 14.3 mL $MgSO_4$ buffer (ice-cold), 15 mg DL-dithiothreitol, 300 μL propidium iodide (PI) stock, 375 μL Triton X-100 stock. Solution B (3 mL enough for up to 12 samples) was prepared by 3 mL solution A, 7.5 μL RNAase (DNAase free), 3.0 μL chicken red blood cell suspension (CRBC).

Preparation of nuclei by chopping plant tissues

Young leaves, approximately 2-4 cm in length (Akinerdem, 1991) from healthy source genotype 8266-10, nineteen somaclones, SC-NL in the green house were used. The excised leaves were washed by distilled water to remove debris and chemical residues. The weighed tissues (approximately 100 mg) were placed in plastic Petri dishes (35 mm × 10 mm) on ice. 2 mL of the prepared solution A was added and the tissues were finely chopped using a sharp scalpel. After chopping, the tissue homogenate was filtered through a nylon filter (41 μm pore size) into a microcentrifuge tube, and the resultant solution was centrifuged at high speed (× 14,000 rpm) for 1 minute followed by discarding of the supernatant. The pellet was resuspended in 400 μL solution B and incubated for at least 15 minutes at 37 °C. After this incubation, the sample was ready for the flow cytometer. Every procedure was performed on ice and in the dark.

Estimation of nuclear DNA content

Nuclear DNA content was analyzed on an Ortho Diagnostics Cyto-fluorograph 50-H / Intel 80386 computer system using Acqcyte data acquisition software (Phoenix Flow Systems, San Diego, Calif., USA) with an argon-ion laser to 488 nm. Each measurement was based on propidium iodide fluorescence from 400 - 4000 plant nuclei. The amount of DNA was estimated using MultiCycle version 2.5 (Phoenix Flow Systems) as the fluorescence of the nuclei stained with propidium iodide relative to that of chicken red blood cells (CRBC). The results were obtained in the form of a DNA histogram (Figure 6). The DNA amount of CRBC (2C = 2.33 pg, Galbraith et al. 1983) was used to convert the DNA content estimates for the source plant, the nineteen

somaclones, and SC-NL into absolute amounts (pg), using the following equation (Arumuganathan and Earle, 1991a):

$$\text{Plant nuclear DNA amount (pg / 2C)} = \frac{[\text{mean position of plant nuclear peak} \times 2.33]}{[\text{mean position of CRBC nuclear peak}]}$$

Data of DNA contents were taken based on three observations and analysis of variance (ANOVA) was made from the source genotype 8266-10, the nineteen somaclones, and SC-NL.

RESULTS

EXPERIMENT 1. TISSUE CULTURE RESPONSE OF LEAF DISCS

The goal of this experiment was to determine if the in vitro responses of any of the nineteen somaclones differed significantly from those of the source genotype 8266-10.

The parameters examined for in vitro response were leaf disc callusing proportion and subsequent shoot regeneration proportion of the source genotype 8266-10 and the nineteen somaclones on both B_1 and MO media. Two kinds of media (B_1 and MO) were used because leaf discs respond to each somewhat differently for callus induction: B_1 initiates callus and shoots after one month with some genotypes, while MO initiates callus more slowly and produces somatic embryos with some genotypes (Doley and Saunders, 1989). The leaf discs were taken from smaller and larger leaves from each somaclone because optimal leaf size for somaclone leaf disc callus induction had not been established, even though standard practice otherwise is to use leaves of 20-40 % of final size.

Overall, no somaclone differed significantly from the source clone as to in vitro leaf disc response for any of the four parameters. For callus induction, on B_1 medium, 72-89 % of leaf discs of somaclones from both smaller and larger leaves produced calli compared to 92 % of callus induction in leaf discs of the source genotype 8266-10 (Table 3). The trends were the similar on MO medium, although the range of callus induction

(61-83 %) from both smaller and larger leaf of somaclones was lower on MO medium than on B_1 medium. All somaclones showed statistically insignificant callusing response on MO medium in comparison with the source genotype 8266-10 (83 %) (Table 4). For shoot formation, 61-83 % of leaf discs from both smaller and larger leaves of somaclones produced shoots on B_1 medium with no significant deviation of shooting proportions from the source genotype 8266-10 (80 %) as in Table 5. On MO medium, no shoot or somatic embryos (0 %) were regenerated from either the source genotype 8266-10 or any of the nineteen somaclones (Table 6).

EXPERIMENT 2. ISOZYME BANDING PATTERNS WITH STARCH GEL ELECTROPHORESIS

The isozyme evaluation was performed to detect any variation in banding pattern for the somaclones in comparison with the source genotype 8266-10 for five enzymes 6-phosphogluconic acid dehydrogenase (6-Pgdh), malate dehydrogenase (Mdh), shikimate dehydrogenase (Skdh), glutamate dehydrogenase (Gdh), and alcohol dehydrogenase (Adh). At best, despite considerable effort and troubleshooting, the banding patterns were not clear and were often overstained. So, it was hard to clearly translate the banding patterns.

The banding patterns of the nineteen somaclones seemed to display no somaclonal variation for the enzymes 6-Pgdh (Fig 2), Skdh (Fig 3.2), Gdh (Fig 4.1), and Adh (Fig 4.2). With Mdh (Fig 3.1 and Fig 5), all somaclones exhibited the absence of a fast band in the Mdh-3 region sometimes seen in the source genotype. This is the position of coordinate bands reported by Aicher and Saunders (1990) that have inconsistent occurrence and appear to be physiologically influenced. No other differences were seen

for Mdh (Fig 3.1). In zymograms of Mdh and Gdh, bands of the source genotype 8266-10 were first compared with those of standard genotype 80-111 as the reference to identify 8266-10's band mobilities. The banding patterns were identical between these two genotypes. Thus the zymotype of 8266-10 was Mdh-1, FF; Mdh-3, SS; Gdh, SS as in Aicher and Saunders (1990). The bands of the nineteen somaclones and SC-NL (Fig 5) were similar to 8266-10's bands for Mdh and Gdh. For 6-Pgdh, Skdh, and Adh (this enzyme was not examined in SC-NL because callus was not available in SC-NL), even though 80-111's bands were not available from Aicher and Saunders, (1990), no banding patterns from the nineteen somaclones and SC-NL were seen as different from 8266-10 and 80-111.

EXPERIMENT 3. NUCLEAR DNA CONTENTS AS DETERMINED BY FLOW CYTOMETRY

The purpose of the experiment was to examine DNA content variation of source genotype 8266-10, nineteen somaclones, and SC-NL and all DNA histograms obtained were similar to that described in Figure 6. The DNA content of source diploid 8266-10 was 1.73 pg / 2C. The mean DNA contents (pg / 2C) of the nineteen somaclones ranged from 1.49 to 1.78 and SC-NL was 1.59 (Table 7). Analysis of variance showed no significant variation of DNA content among source genotype 8266-10, the nineteen somaclones, and SC-NL (Table 8).

Table 3. Callus formation from leaf discs of smaller and larger leaves from source genotype 8266-10 and derivative somaclones on B_1 medium 10 weeks after culture initiation.

	Callusing proportion (%)		χ^2 value♠	
Leaf disc origin	Smaller leaf	Larger leaf	Smaller leaf	Larger leaf
8266-10	16/18 (88.9)	17/18 (94.4)	---	---
SC 1	14/18 (77.8)	16/18 (88.9)	1.516	2.632
SC 2	15/18 (83.3)	16/18 (88.9)	1.391	2.632
SC 3	15/18 (83.3)	15/18 (83.3)	1.391	2.618
SC 4	15/18 (83.3)	16/18 (88.9)	1.391	2.632
SC 5	15/18 (83.3)	15/18 (83.3)	1.391	2.618
SC 6	14/18 (77.8)	16/18 (88.9)	1.516	2.632
SC 7	13/18 (72.2)	15/18 (83.3)	3.891	2.618
SC 8	15/18 (83.3)	15/18 (83.3)	1.391	2.618
SC 9	15/18 (83.3)	16/18 (88.9)	1.391	2.632
SC 10	14/18 (77.8)	15/18 (83.3)	1.516	2.618
SC 11	15/18 (83.3)	16/18 (88.9)	1.391	2.632
SC 12	15/18 (83.3)	15/18 (83.3)	1.391	2.618
SC 13	14/18 (77.8)	16/18 (88.9)	1.516	2.632
SC 14	14/18 (77.8)	16/18 (88.9)	1.516	2.632
SC 15	13/18 (72.2)	16/18 (88.9)	3.891	2.632
SC 16	14/18 (77.8)	15/18 (83.3)	1.516	2.618
SC 17	14/18 (77.8)	15/18 (83.3)	1.516	2.618
SC 18	15/18 (83.3)	15/18 (83.3)	1.391	2.618
SC 19	13/18 (72.2)	15/18 (83.3)	3.891	2.618

♠ Comparisons of each somaclone were made with the source genotype 8266-10 using the chi-square test; values of χ^2 were calculated with the Yates correction factor with one degree of freedom; somaclones showed no significant deviation from 8266-10 at 99 % confidence level.

Table 4. Callus formation from discs of smaller and larger leaves from source genotype 8266-10 and derivative somaclones on MO medium 14 weeks after culture initiation.

	Callusing proportion (%)		χ^2 value♠	
Leaf disc origin	Smaller leaf	Larger leaf	Smaller leaf	Larger leaf
8266-10	14/18 (77.8)	16/18 (88.9)	---	---
SC 1	13/18 (72.2)	14/18 (77.8)	1.071	1.516
SC 2	13/18 (72.2)	15/18 (83.3)	1.071	1.391
SC 3	12/18 (66.7)	15/18 (83.3)	1.009	1.391
SC 4	12/18 (66.7)	14/18 (77.8)	1.009	1.516
SC 5	13/18 (72.2)	15/18 (83.3)	1.071	1.391
SC 6	12/18 (66.7)	15/18 (83.3)	1.009	1.391
SC 7	13/18 (72.2)	14/18 (77.8)	1.071	1.516
SC 8	11/18 (61.1)	13/18 (72.2)	2.438	3.891
SC 9	11/18 (61.1)	14/18 (77.8)	2.438	1.516
SC 10	11/18 (61.1)	13/18 (72.2)	2.438	3.891
SC 11	13/18 (72.2)	14/18 (77.8)	1.071	1.516
SC 12	11/18 (61.1)	15/18 (83.3)	2.438	1.391
SC 13	13/18 (72.2)	13/18 (72.2)	1.071	3.891
SC 14	12/18 (66.7)	13/18 (72.2)	1.009	3.891
SC 15	13/18 (72.2)	15/18 (83.3)	1.071	1.391
SC 16	11/18 (61.1)	15/18 (83.3)	2.438	1.391
SC 17	11/18 (61.1)	14/18 (77.8)	2.438	1.516
SC 18	13/18 (72.2)	15/18 (83.3)	1.071	1.391
SC 19	13/18 (72.2)	15/18 (83.3)	1.071	1.391

♠ Comparisons of each somaclone were made with the source genotype 8266-10 using the chi-square test; values of χ^2 were calculated with the Yates correction factor with one degree of freedom; somaclones showed no significantly deviant proportions of callusing from 8266-10 at 99 % confidence level.

Table 5. Shoot formation from discs of smaller and larger leaves from source genotype 8266-10 and derivative somaclones B_1 medium 10 weeks after culture initiation.

	Shoot proportion (%)		χ^2 value♦	
Leaf disc origin	Smaller leaf	Larger leaf	Smaller leaf	Larger leaf
8266-10	16/18 (88.9)	15/18 (72.2)	---	---
SC 1	15/18 (83.3)	14/18 (77.8)	1.391	0.983
SC 2	14/18 (77.8)	14/18 (77.8)	1.516	0.983
SC 3	14/18 (77.8)	11/18 (61.1)	1.516	5.433
SC 4	15/18 (83.3)	13/18 (72.2)	1.391	1.167
SC 5	13/18 (72.2)	11/18 (61.1)	3.891	5.433
SC 6	14/18 (77.8)	11/18 (61.1)	1.516	5.433
SC 7	15/18 (83.3)	13/18 (72.2)	1.391	1.167
SC 8	15/18 (83.3)	12/18 (66.7)	1.391	2.900
SC 9	13/18 (72.2)	14/18 (77.8)	3.891	0.983
SC 10	14/18 (77.8)	13/18 (72.2)	1.516	1.167
SC 11	13/18 (72.2)	12/18 (66.7)	3.891	2.900
SC 12	13/18 (72.2)	14/18 (77.8)	3.891	0.983
SC 13	13/18 (72.2)	11/18 (61.1)	3.891	5.433
SC 14	15/18 (83.3)	14/18 (77.8)	1.391	0.983
SC 15	15/18 (83.3)	13/18 (72.2)	1.391	1.167
SC 16	14/18 (77.8)	14/18 (77.8)	1.516	0.983
SC 17	13/18 (72.2)	12/18 (66.7)	3.891	2.900
SC 18	14/18 (77.8)	13/18 (72.2)	1.516	1.167
SC 19	14/18 (77.8)	13/18 (72.2)	1.516	1.167

♦ Comparisons of each somaclone with the source genotype 8266-10 using the chi-square test; values of χ^2 were calculated with the Yates correction factor with one degree of freedom; somaclones showed no significant deviation from 8266-10 at 99 % confidence level.

Table 6. Shoot fromation from discs of smaller and larger leaves from source genotype 8266-10 and derivative somaclones on MO medium 14 weeks after culture initiation.

	Shoot proportion (%)♠	
Leaf disc origin	Smaller leaf	Larger leaf
8266-10	0/18 (0.0)	0/18 (0.0)
SC 1	0/18 (0.0)	0/18 (0.0)
SC 2	0/18 (0.0)	0/18 (0.0)
SC 3	0/18 (0.0)	0/18 (0.0)
SC 4	0/18 (0.0)	0/18 (0.0)
SC 5	0/18 (0.0)	0/18 (0.0)
SC 6	0/18 (0.0)	0/18 (0.0)
SC 7	0/18 (0.0)	0/18 (0.0)
SC 8	0/18 (0.0)	0/18 (0.0)
SC 9	0/18 (0.0)	0/18 (0.0)
SC 10	0/18 (0.0)	0/18 (0.0)
SC 11	0/18 (0.0)	0/18 (0.0)
SC 12	0/18 (0.0)	0/18 (0.0)
SC 13	0/18 (0.0)	0/18 (0.0)
SC 14	0/18 (0.0)	0/18 (0.0)
SC 15	0/18 (0.0)	0/18 (0.0)
SC 16	0/18 (0.0)	0/18 (0.0)
SC 17	0/18 (0.0)	0/18 (0.0)
SC 18	0/18 (0.0)	0/18 (0.0)
SC 19	0/18 (0.0)	0/18 (0.0)

♠ No shoots or somatic embryos were not formed from leaf discs of smaller and larger leaves on MO medium.

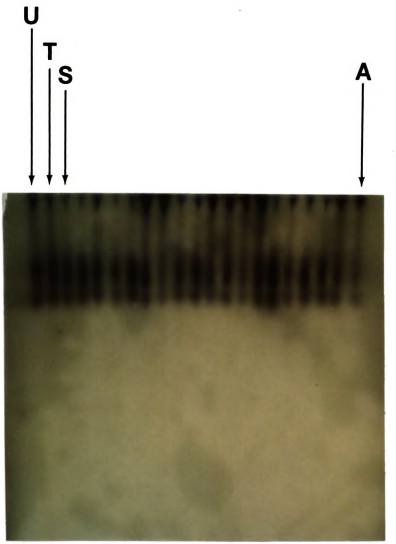

Figure 2. The banding patterns of 6-Pgdh from control (source) plant 8266-10 and nineteen derivative somaclones. Lane A through S were not labeled but they correspond from SC 1 to SC 19, T: 8266-10, U: 80-111.

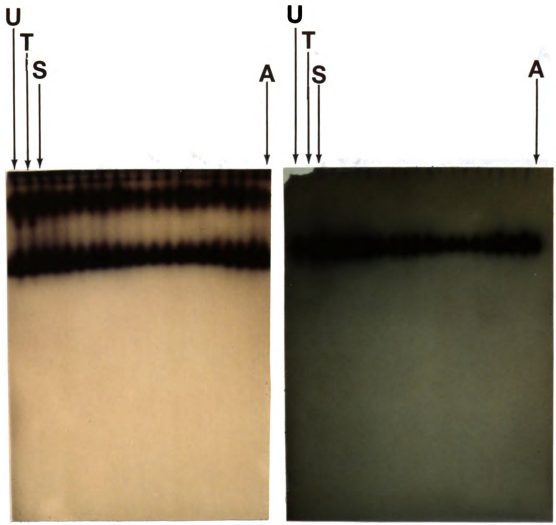

Fig3.1 Fig3.2

Figure 3. The banding patterns of Mdh (Fig 3.1) and Skdh (Fig 3.2) from control (source) plant 8266-10 and nineteen derivative somaclones. Lane A through S were not labeled but they correspond from SC 1 to SC 19, T: 8266-10, U: 80-111.

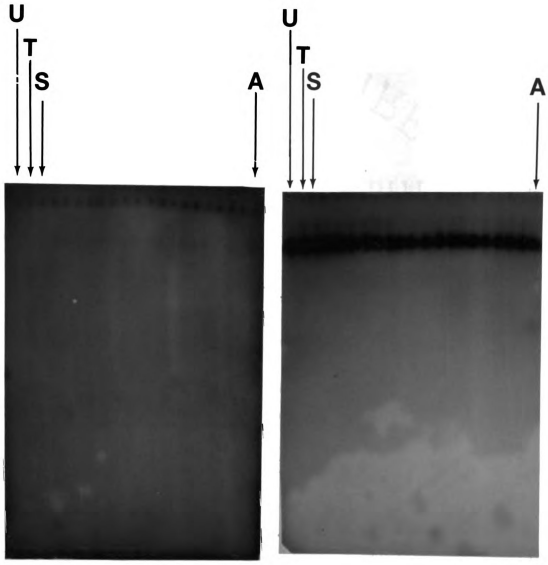

Fig 4.1 **Fig 4.2**

Figure 4. The banding patterns of Gdh (Fig 4.1) and Adh (Fig 4.2) from control (source) plant 8266-10 and nineteen derivative somaclones. Lane A through S were not labeled but they correspond from SC 1 to SC 19, T: 8266-10, U: 80-111.

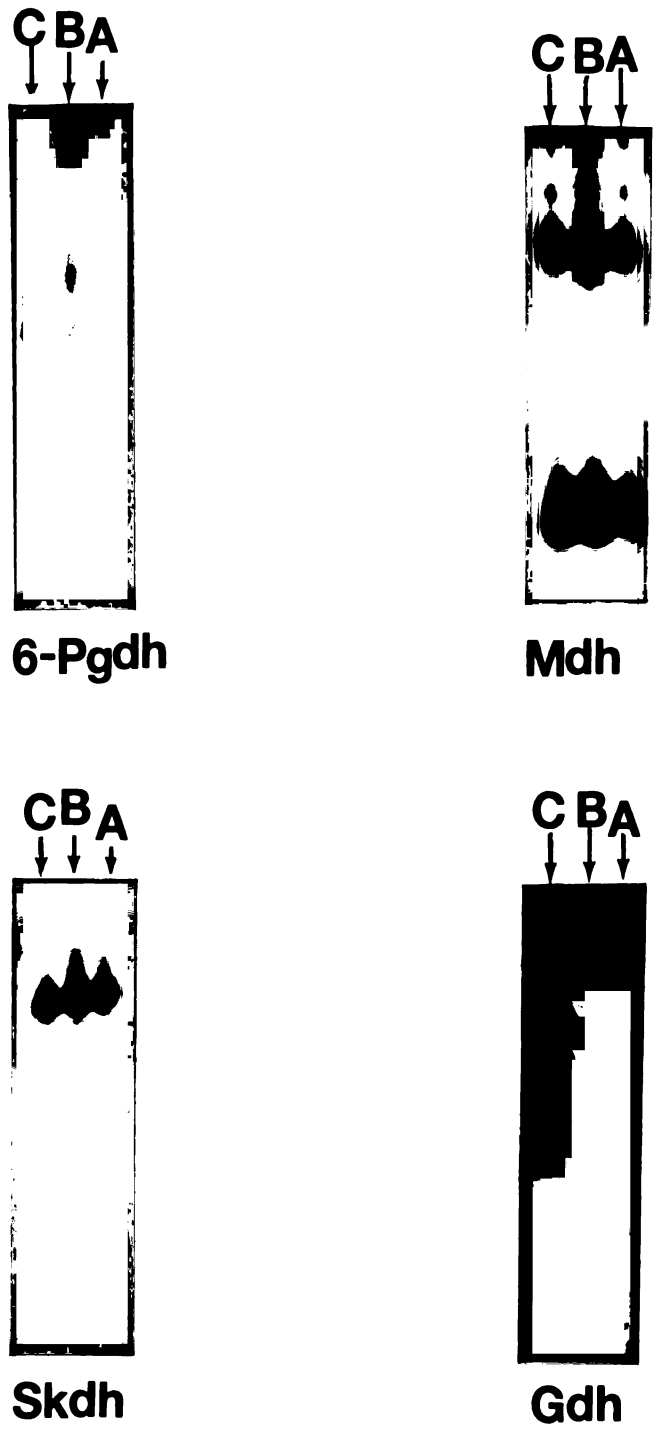

Figure 5. The banding patterns of 6-Pgdh, Mdh, Skdh, and Gdh of control (source) plant 8266-10 and narrow leaf somaclonal variant (SC-NL). A: SC -NL, B: 8266-10, C: 80-111.

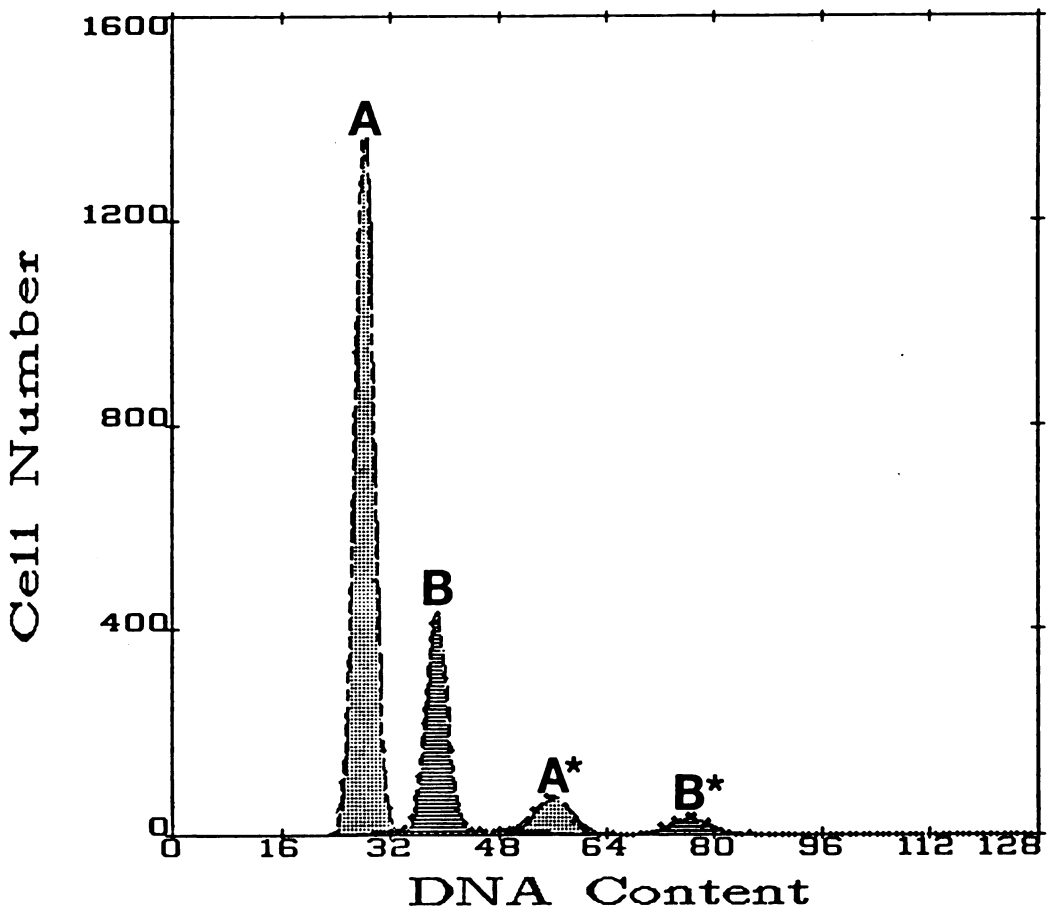

Figure 6. Flow cytometric analysis of DNA content in the leaf cell nuclei from source clone 8266-10, the nineteen somaclones, and SC-NL . Histogram shows the number of nuclei as a function of relative DNA content. Nuclei were isolated from freshly excised young leaves. The two peaks A and A* represent $G_1$and G_2 phase nuclei with 2C and 4C complements of sugarbeet plant DNA, respectively. B and B* are for G_1 and G_2 of chicken red blood cells (C.R.B.C) as the internal standard.

Table 7. DNA contents of leaf cells from control (source) plant 8266-10 and nineteen derivative somaclones by flow cytometry.

Genotype	Replication	DNA content (pg)	Mean[a]	t-value
8266-10	1	1.63	1.73	---
	2	1.89		
	3	1.68		
SC 1	1	1.62	1.65 (ns[b])	0.980
	2	1.71		
	3	1.62		
SC 2	1	1.65	1.69 (ns)	0.209
	2	1.73		
	3	1.68		
SC 3	1	1.59	1.64 (ns)	1.006
	2	1.62		
	3	1.71		
SC 4	1	1.52	1.60 (ns)	1.453
	2	1.62		
	3	1.66		
SC 5	1	1.56	1.49 (ns)	2.028
	2	1.31		
	3	1.61		
SC 6	1	1.52	1.72 (ns)	0.054
	2	1.61		
	3	2.05		
SC 7	1	1.59	1.63 (ns)	1.414
	2	1.62		
	3	1.69		
SC 8	1	1.66	1.81 (ns)	0.428
	2	2.15		
	3	1.62		
SC 9	1	1.56	1.74 (ns)	0.077
	2	1.74		
	3	1.91		
SC 10	1	1.63	1.65 (ns)	0.843
	2	1.58		
	3	1.73		
SC 11	1	1.59	1.63 (ns)	1.195
	2	1.67		
	3	1.62		

Table 7. (Cont'd)

Genotype	Replication	DNA content (pg)	Mean [a]	t-value
SC 12	1	1.56	1.78 (ns[b])	0.250
	2	2.14		
	3	1.63		
SC 13	1	1.59	1.59 (ns)	1.673
	2	1.62		
	3	1.57		
SC 14	1	1.57	1.63 (ns)	1.195
	2	1.67		
	3	1.66		
SC 15	1	1.65	1.74 (ns)	0.067
	2	1.87		
	3	1.68		
SC 16	1	1.07	1.49 (ns)	1.084
	2	1.72		
	3	1.67		
SC 17	1	1.48	1.67 (ns)	0.474
	2	1.73		
	3	1.81		
SC 18	1	1.69	1.64 (ns)	1.006
	2	1.54		
	3	1.69		
SC 19	1	1.67	1.64 (ns)	1.006
	2	1.68		
	3	1.58		
SC NL	1	1.61	1.59(ns)	1.673
	2	1.57		
	3	1.59		

[a] The comparisons of means were made between each somaclone and control 8266-10 by t-test.

[b] ns = non significance at 99 % at confidence level ; from each calculated t-value, each mean is not significantly different from the mean of control 8266-10.

Table 8. Analysis of variance of source genotype 8266-10, the nineteen somaclones, and the narrow leaf variant (SC-NL).

Source of variation	Degree of freedom	Sum of squares	Mean square	F value (F_s)♠
Genotype	20	0.2523	0.0189	0.738
Error	42	1.0752	0.0256	

♠ $F_s < F_{0.01\,(20,\,42)} = 2.370$; $F_s < F_{0.05\,(20,42)} = 1.840$

DISCUSSION

In all the experiments, the nineteen somaclones and narrow leaf somaclonal variant (SC-NL) showed no significant variation from source genotype 8266-10.

Delbreil and Jullien (1994) have reported somaclonal variation for in vitro response, i.e. improved somatic embryogenesis in *Asparagus officinalis*, and indicated that in vitro response could be a good parameter for examining somaclonal variation. Additionally, Saunders and Doley (1986) reported a more intensely shoot regenerating somaclone in sugarbeet, one which could have been selected by the regeneration medium. So, in vitro response for callus induction and shoot regeneration was chosen as one set of parameters to be examined in this study. But, originally, when the experiments started, the available population size of somaclones including narrow leaf somaclonal variant (SC-NL) was small and thus it was realized that the probability for detecting any variation from somaclones was relatively low for in vitro tissue culture response.

Sabir et al. (1992) identified somaclonal variations for eight isozyme banding patterns, using 764 sugarbeet regenerants. Steen et al. (1986) also found isozyme variants in somaclones of sugarbeet. Thus, precedents were on record for isozyme variants, including the most interesting over-expression of Gdh (Sabir et al. 1992). With the isozyme parameter, it is possible that some cryptic variation in somaclones and the narrow leaf variant could be underestimated or not detected. This may be quite possible

due to several reasons. Firstly, the genetic variations in the non-coding portions of the DNA (intron) will not be detected by electrophoresis because only transcription and translation products of structural genes by coding portion (exon) are accessible to electrophoretic analysis. Secondly, some mutations in coding sequence for an enzyme may result in a slight size alteration for tertiary structure of an enzyme or in a substitution of amino acids that does not alter the charge or activity of the gene product (enzyme). So, these cryptic mutations will not be detected as a change in migration mobility of an isozyme band. Thirdly, the redundancy of genetic triple code will make some changes in base sequence of code not expressed as amino acid sequence of the encoded gene product (isozyme). Lastly, from the zymograms with simple, single bands in the source genotype 8266-10, nineteen somaclones, and SC-NL, it was concluded that the alleles for the isozymes examined may be at homozygous loci. So, if the mutations in null alleles for the enzymes examined occur, they would not be detected at homozygous loci. For these reasons, even though no genetic variation was found among the nineteen somaclones and SC-NL for the banding patterns of isozymes, the possibility for undetected genetic variation should not be excluded. More reliable molecular approaches such as DNA sequencing might be necessary to detect any mutations.

Especially, there was no relationship between the isozyme patterns examined and the morphological variations in the narrow leaf variant (SC-NL). A similar study in maize also showed that the extent of morphological differences between five lines was not correlated with qualitative differences between two-dimensional enzyme banding patterns, but a variation was found when isozyme dosages were examined (Damerval et

al. 1987). It may be suggested that the patterns showed may be compared not only for the absence or presence of specific bands, but for the amount of enzyme per band (band intensity). However, no difference in either band number, position or intensity was noted between SC-NL and source genotype 8266-10.

For examination of DNA content in somaclones and SC-NL, the precise measurement of DNA content should be considered for detecting the variation of DNA content with several possible explanations. Firstly, the condition of the leaf samples, especially, health of source plants may be considered. In fact, it was difficult to maintain uniform plant health over fifteen months in which leaves were sampled. Physiology of donor plants may affect the synthesis of nuclear DNA or cell cycle. Therefore, nonuniformity of conditions of donor plants may contribute to variability for the measurement of DNA in leaf cells of the somaclones. Secondly, variability in execution of the procedure for nuclei preparation from plant materials may occurs. Therefore, if possible, procedure variations were to be more carefully controlled by the experimenter, more precise data for DNA content might be obtained and significant differences found in a population of somaclones and SC-NL. So, considerable effort should be made to more tightly standardize conditions of sampling in terms of physiological conditions of leaves and conditions of preparation of nuclei. Lastly, the debris of cell organelles may interrupt the precise measurement of DNA content in somaclones and SC-NL. Especially, chlorophyll from the incompletely destroyed chloroplast may emit the exogenous fluorescence with the fluorescence of the stained DNA of isolated nuclei in flow cytometry. This is also a possible cause for somewhat higher DNA content in normal

diploid 8266-10 (1.73 pg / 2C). The established DNA content in diploid sugarbeet is 1.57 pg / 2C (Arumuganathan and Earle, 1991b).

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CHAPTER II

IN VITRO RESPONSE OF SUGARBEET (Beta vulgaris L.) HAPLOIDS

INTRODUCTION

Haploidy refers to the condition of any organism, tissue or cell having the chromosome constitution of the normal gametes of the species involved (Chase, 1952). Since the first in vitro induction of haploids by androgenesis in *Datura* (Guha and Maheshwari, 1964), haploids from various plant species have been produced by in vitro culture techniques such as anther and microspore culture, ovary and ovule culture .

Haploids have been of value in plant biotechnology for two main reasons. Firstly, doubled haploids (DH) can be obtained by diploidization of haploid shoots or cells by chromosome doubling. The resultant doubled haploid is a homozygous diploid, and by selfing gives rise to a pure line, otherwise obtained only after generations of selfing. Usually, diploidization of haploid cells is possible by the use of colchicine. For example, haploid cells of *Atropa* were diploidized by adding colchicine to exponentially growing suspension cultures (Rashid and Street, 1974).

Secondly, haploid cells of diploid species are useful in finding somatic cell mutants (Bajaj, 1983). By plating suspension cultures from haploid explants, they can be handled like microbes, and, with the availability of large populations, it is possible to select haploid recessive mutants arising from somaclonal variation or chemical

mutagenesis. If diploid cells are used, an arising dominant mutation creates a heterozygote at the locus, and is expressed. Recessive mutations also create heterozygotes but the mutant phenotype is not expressed, and the mutation is unavailable for the selection process in that cell population. In contrast to that, the advantage of a haploid cell containing only one copy of each gene is that all mutations arising in that cell are expressed, since any recessive alleles are not masked by the presence of dominant alleles. So, only a system using haploid cells can effectively recover useful recessive mutants. Mutants have been obtained from haploid cell cultures from various plant species. For example, somatic cell mutants selected for herbicide resistance (Chaleff and Parsons, 1978) and for antibiotic resistance (Maliga et al., 1973) have been reported for haploid tobacco, and brought to the whole plant level.

However, for optimal haploid cell use in somatic cell genetics, it is essential that the cultures remain at least predominantly haploid during the stages when mutants are being selected. Haploidy in cell cultures is known to be unstable and within a matter of weeks the ploidy of many cells may revert to the diploid state, or higher levels (Sacristan, 1971; D'Amato, 1977, 1985; Novak and Vsykot, 1975). So, spontaneous increase in ploidy level can be a major obstacle to the use of haploid cell lines in mutant selection. The advantage of haploid cells for mutant selection is lost when spontaneous increase in ploidy occurs in cultures, especially when diploidization, for example, occurs relatively early. Bajaj (1972) regenerated both haploid and diploid plants from haploid mesophyll protoplasts of *Nicotiana tabacum* and put forward various possible explanations for this phenomenon: (1) some of the leaf vein cells that gave raise to the

protoplasts were diploid to begin with, (2) spontaneous fusion during protoplast isolation, and (3) repeated endomitosis (endoreduplication) or nuclear fusion in callus cultures. Observations favored the third mechanism for the differences seen in ploidy levels. Likewise, mixtures of haploids and diploid plants were regenerated from mesophyll protoplasts of haploid *Petunia hybrida* (Binding, 1974). Also, Bajaj et al. (1978) observed a wide range of ploidy differences in the plants regenerated from mesophyll protoplasts of haploid *Atropa belladonna.* They obtained haploid, diploid, triploid, and hexaploid plants , along with various types of aneuploids.

Since the first in vitro induction of androgenetic haploid plants by anther culture (Guha and Maheshwari, 1964), induction of haploids by tissue and cell culture has attracted considerable attention. It is evident that in vitro induction of haploidy can speed up the production of haploids for plant breeding programs (Reinert and Bajaj, 1977; Vasil, 1980; Maheshwari et al., 1982; Bajaj, 1983). Especially, ovule or ovary culture has enabled haploid production in certain species for which anther culture is not so effective, thus extending the number of species for which haploids can easily be available.

Given the potential advantages of haploidy, sugarbeet breeding would also be facilitated by the availability of haploids. Sugarbeet haploids have successfully been obtained as a result of pollen irradiation before pollination (Bosemark, 1971; Seman, 1983), crosses between diploid and tetraploid plants of *B. vulgaris* (De Jong and De Bock, 1978), between wild species and *B. vulgaris* (Bosemark, 1971), or between red beet and *B. vulgaris* (Seman, 1983). But, these conventional productions of haploids are

time-consuming, laborious, and not very efficient.

Procedures for in vitro induction of haploidy have also been developed for sugar beet. Although anther or microspore culture has not been successful for haploid production in sugarbeet (Banba and Tanabe, 1972; Atanasov, 1973; Rogozinska and Goska, 1982; Van Geyt et al., 1985), gynogenesis, i.e., in vitro culture of unfertilized ovules or ovaries, has proven to be more promising for the production of haploids than the other techniques used in sugarbeet (Hosemans and Bossoutrot, 1983). Since the first successful report of ovule culture of sugarbeet, the haploid production frequency has increased dramatically (Bornman, 1985; D'Halluin and Keimer, 1986; Van Geyt et al., 1987; Doctrinal et al., 1989, 1990).

In the experiment described below, on the basis of callusing and shoot regeneration, a comparison was made on the in vitro response of leaf discs between the source diploid REL-1 (with good callusing as well as regeneration ability as in Saunders et al., 1992) and two sugarbeet haploids OV-9, OV-16, respectively, which had been produced from REL-1 by in vitro gynogenesis. A second comparison was made between the source diploid 6822-08 and three haploids OV-68-1, OV-68-2, and OV-68-3 which were also produced from the source diploid 6822-08 by in vitro gynogenesis. But their ploidy had not been confirmed cytologically. However, all fit the previous description of beet haploids as conspicuously reduced in vigor and size, and OV-9 and OV-16 had been grown in replicate long enough to display complete sterility. The three presumptive haploids from 6822-08 had been developed directly from their ovules, minimizing the chance of somaclonal deviation from haploidy. Literatures of sugarbeet gynogenesis

report a preponderance of haploidy in the products of ovule culture (Bossoutrot and Hosemans, 1985; Van Geyt et al., 1987). So, there is a high probability of haploidy for these five products of ovule culture. Furthermore, from observation point of view, all of these five products of ovule culture showed high sensitivity to environment, delayed development, slower growth rates. They had also a smaller mass and a greater number of small leaves. All of these observed characteristics were similar to common characteristics of sugarbeet haploid plants as in Seman's report (1983).

In vitro response data of these five sugarbeet haploid plants provided basic information about the suitability of these haploid plants for the experiment of spontaneous diploidization in callus and regenerated plants and suspension cultures. The haploids could ultimately be the source material for developing a somatic cell selection system in sugarbeet.

MATERIALS AND METHODS

Plant materials

Sugarbeet haploid plants OV-9 and OV-16 had been produced by in vitro gynogenesis from the source diploid clone REL-1 which has good callus induction and shoot regeneration ability (Saunders et al., 1992) and was available for leaf discing in the greenhouse. Other haploids OV-68s (OV-68-1, OV-68-2, and OV-68-3) were produced from the diploid 6822-08 by ovule culture in this experiment. The haploid plants and diploid plants were grown in 6″ plastic pots with Baccto high porosity professional planting mix (55-65 % of horticultural sphagnum peat perlite, Michigan Peat Co.) in the greenhouse without supplementary lighting in 1995 and fertilized with Peters 20-20-20 water soluble commercial nutrient mix (Peters Professional Co, Marysville OH) at the intervals of two weeks.

Ovule culture of 6822-08

Donor plant material. The induction of another haploid with a different genetic background from diploid REL-1 derived haploids OV-9 and OV-16 was attempted. As the ovule donor genotype, a diploid 8266-10 was used. Flower buds of 8266-10 were collected from the incompletely expanded secondary or tertiary branches of the inflorescences in the greenhouse without supplementary lighting as in Doctrinal et al. (1989).

The isolation of ovules and culture establishment. After washing the excised flower buds with distilled water, the flower buds were sterilized twice in a 15% commercial hypochlorite bleach solution with 0.01% sodium lauryl-sulfate for 20 minutes. The explants were rinsed three times in sterile distilled water and were dried between sterile papers. Dissection of floral buds was done with a sharp dissecting needle under a dissecting microscope under aseptic conditions. The isolated ovules were put on the induction medium M_{20} which is MS medium (Murashige and Skoog, 1962) with 0.25 mg/L BA (6-benzyladenine) as the only growth regulator. Five ovules were put on each plate. A total of 700 ovules were cultured. The plates with cultured ovules were kept in a growth chamber at 24 °C ± 1 °C under cool-white fluorescent light (about 25 $\mu mole \cdot m^{-2} \cdot s^{1}$).

The response of cultured ovules. During the next 60 days, regular observations were made once a week to recover any prospective haploid embryos from the ovules (Figure 1). Three embryos were recovered independently from three ovules and labeled as OV-68-1, OV-68-2, and OV-68-3. The recovered haploid embryos were transferred to new M_{20} medium for further development and shoot growth. The shoots were propagated in M_{20} medium and kept under the same culture conditions as ovule culture.

The recovery of whole plants. Haploid shoots were transferred to N_3 medium for rooting. N_3 medium is MS medium with 3.0 mg/L NAA (α -naphthaleneacetic acid) as the only growth regulator and kept under the same culture conditions as ovule culture. The plantlets with roots were then potted into soil and grown in the greenhouse without supplementary lighting.

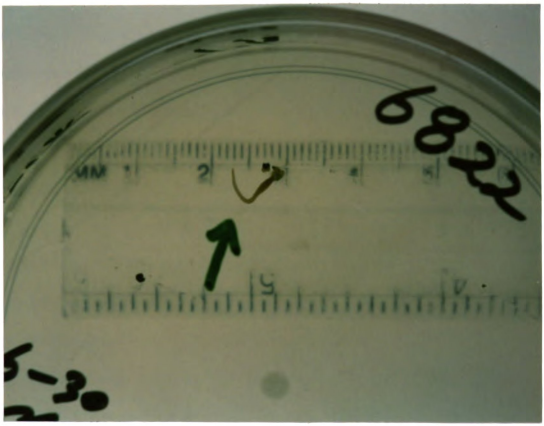

Figure 1. The emerging haploid embryo from the cultured ovule of diploid source clone 6822-08 after about 80 days since culture initiation.

Leaf sample preparation

Leaf discs were taken from incompletely expanded (20-40 % of final leaf size) young leaves of diploid plants REL-1 and 6822-08 as well as haploid plants OV-9, OV-16, and OV-68s. Detached leaves were surface sterilized with two twenty minutes soakings in 15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by three rinses with sterile distilled water. Leaf discs were excised from each leaf blade, excluding leaf veins, using a #3 cork borer (0.7 mm in diameter).

Media preparation

B_1 medium was prepared from MS medium (Murashige and Skoog, 1962) which consists of inorganic and organic mineral salts, 3% sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1.0 mg/L thiamine·HCl. Final concentration of agar was 0.9% (w/v) Difco Bacto agar. The final pH was adjusted to 5.95 with KOH prior to autoclaving for 30 mins at 121 °C (15 lb/in^2). 1.0 mg/L BA (6-benzyladenine) was added prior to autoclaving as the only growth regulator used for B_1 medium. After autoclaving, media were dispensed into 20 × 100 mm disposable plastic Petri dishes with 35 ml per dish.

Culture establishment

One leaf disc was put in each plate. Dishes were sealed with two straps of parafilm (American National Can Co, Neenah WI). All cultures were maintained at 31 °C in the dark. Eight weeks after initiating the cultures, the data for callusing and shoot regeneration were collected from the dishes of haploids OV-9, OV-16, OV-68s (OV-68-

1, OV-68-2, and OV-68-3) and the source diploids REL-1, 6822-08, on the basis of the proportion of discs callusing and regenerating shoots. The data were analyzed by chi-square test with the Yates correction factor for one degree of freedom.

RESULTS AND DISCUSSION

The goal of this experiment was to obtain information about callus induction and shoot regeneration for five haploids (OV-9, OV-16, OV-68-1, OV-68-2, and OV-68-3). OV-9 and OV-16 showed extremely low proportions of leaf disc callusing and shoot regeneration in comparison with source genotype REL-1; REL-1 had high percentage of callus induction (95 %) and shoot regeneration (70 %). However, OV-9 produced callus only from 7 % of leaf discs and shoots from 5 %. OV-16 showed about 6 % of callusing proportion and 4 % of shooting proportion (Table 1).

On the other hand, in order to obtain other haploids with a different genetic background from REL-1, two diploid genotype 6822-08 and FC 701/5-4 were identified as the candidates for ovule donor genotypes from the populations of SP 6822 and FC 701/5 (these two populations had earlier shown good callus induction and shoot regeneration ability as in Saunders and Shin, 1986), respectively, and were tested for callus induction and shoot regeneration.

These two diploid genotypes 6822-08 and FC 701/5-4 showed high proportions of leaf disc callus induction and shoot regeneration. Callus and shoots were produced from about 90 % and 80 % of leaf discs of 6822-08, respectively (Table 2). FC 701/5-4 showed 80 % of callusing proportion and 70 % of shoot regeneration (Table 2). However, in this experiment, 6822-08 was chosen as the ovule donor genotype because it

Table 1. Callus induction and shoot formation from leaf discs of the diploid source plant REL-1 and its haploid plants OV-9 and OV-16

Genotype	Callusing proportion (%) χ^2 value♠	Shooting proportion (%) χ^2 value♠
REL-1	133/140 (95.0) ---	101/140 (72.1) ---
OV-9	10/140 (7.1) 2258.43**	7/140 (5.0) 312.58**
OV-16	8/140 (5.7) 2332.74**	5/140 (3.6) 326.05**

♠ Values of χ^2 were calculated with Yates correction factor with one degree of freedom;

** = significantly different from the diploid source clone REL-1 at 99 % confidence level.

was available first as a flowering plant in the greenhouse.

Table 2. Preliminary test of callus induction and shoot regeneration in two diploid genotypes 6822-08 and FC 701/5-4.

Genotype	Callus induction (%)♠	Shoot regeneration (%)
6822-08	52/60 (87.0)	49/60 (81.7)
FC 701/5-4	47/60 (78.3)	39/60 (65.0)

♠ one leaf disc (total 60 leaf discs) was put on each plate for 6822-08 and FC 701/5-4, respectively.

From 6822-08, three ovules each responded and directly produced (without callus) a presumptive haploid shoot, each of which was multiplied and rooted as OV-68-1, OV-68-2, and OV-68-3. When the callus induction and shoot regeneration abilities of three haploid genotypes were tested, they showed extremely low proportions of leaf disc callusing and shooting in comparison with their source diploid 6822-08 (Table 3). In fact, none of the three haploid genotypes regenerated shoots. From these results, it was concluded that none of the five haploid genotypes were adequate as materials for somatic cell selection or studies of spontaneous diploidization.

Several possible explanations for this low in vitro response of five haploid individuals in two different genetic backgrounds may be given. Firstly, unconscious selection of eggs/ovules that produced haploid plants with low intensity of callus and

Table 3. Callus induction and shoot regeneration of the diploid source 6822-08 and its derivative haploids OV-68-1, OV-68-2, and OV-68-3

Genotype	Callusing proportion (%) χ^2 value♠	Shooting proportion (%) χ^2 value♠
6822-08	28/30 (93.3) ---	24/30 (80.0) ---
OV-68-1	1/30 (3.3) 378.14**	0/30 (0.0) 117.05**
OV-68-2	1/30 (3.3) 378.14**	0/30 (0.0) 117.05**
OV-68-3	3/30 (10.0) 323.35**	0/30 (0.0) 117.05**

♠ χ^2 values were obtained with Yates correction factor with one degree of freedom;

** = significantly different from the diploid source clone 6822-08 at 99 % confidence level.

shoot production may be made when ovules were taken for culture. Secondly, haploid plants may be intrinsically less responsive than diploids. Thirdly, haploid leaf samples may be sensitive to surface sterilization reagents. Before making leaf discs, leaf samples were surface-sterilized by 15 % commercial hypochlorite bleach solution with 0.01 % sodium laurylsulfate. The leaves of haploid plants are thinner than leaves of diploid plants. So, during surface sterilization, the sterilization reagents may have damaged the thin leaves of haploid plants and injured leaf cells. Lastly, the choice of appropriate leaf size from the haploid plants for leaf disc culture may be not made. Actually, leaves of haploid plants are smaller than the leaf of a diploid. So, in a haploid plant, it is more difficult to measure the degree of expansion of a leaf. Standard practice is to use 20-40 % of final leaf size. So, in this experiment, there is a possibility that fully expanded small leaves of haploid plants were taken as samples. If fully expanded leaves were used for explants, the in vitro responses of the explants would be quite different from young incompletely expanded leaves. Therefore, the choice of leaf sample size may be critical for normal in vitro responses of haploid genotypes.

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