Plant Cell Tissue Organ Culture 4 (1985) 159–169.

Genetic instability during embryogenic cloning of celery

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(Received 8 August 1984; in revised form and accepted 3 December 1984)

Key words: Plant tissue culture, callus, regeneration, somaclonal variation, isozymes, chromosome instability, celery, *Apium graveolens*

Abstract. Genetically marked tissues of celery (*Apium graveolens*) were employed to contrast genetic and chromosomal stability in serially bulk-transferred callus and regenerated plants. After six months in culture, 84% of the callus cells were karologically indistinguishable from normal, while the remainder exhibited chromosome loss and/or fusion. All of 50 clones derived from this tissue expressed the control phenotype with respect to heterozygous isozyme markers. Of 95 plants regenerated from the same tissue, 94 were phenotypically indistinguishable from the original explant donor, and cytogenetic analyses revealed the presence in 4.3% of an accessory chromosome, while the remainder were normal diploids. Analysis of the selfed progeny of these regenerated plants revealed the presence of a new recessive mutation causing abnormal leaf morphology at a frequency of 1.8%. Only one of 40 cells in 12-month-old callus tissue was karyologically indistinguishable from normal, the remainder consisting primarily of hypodiploids. The observation that all 50 clones were phenotypically heterozygous was statistically inconsistent with the hypothesis that hypodiploidy was associated with random complete chromosome loss. The culture had, at this point, lost the ability to regenerate. It is speculated that embryogenic cloning of celery may be suitable under certain circumstances for direct field establishment, but that levels of new genetic variation are sufficiently high to preclude its use for seed production.

Introduction

Among the most promising and immediate applications of plant cell and tissue culture in crop improvement is large scale in vitro cloning. This capability allows the maintainance of sexually unstable heterozygous genotypes indefinitely. Perhaps more significantly, the technique allows maintenance and propagation of genotypes which are difficult or impossible to sexually propagate. Inbreds of dioecious crops such as asparagus [26], highly self-incompatible inbred lines such as in *Brassica* [5], nuclear or cytoplasmic sterile lines [11], and inbred parental lines are some examples. Mass in vitro propagation of vegetation tissues may also hold potential for direct field establishment of crops typically established from transplants [11]. Thus, individual genetically superior plants could be tested for performance in field stands, and scaled up to production proportions.

Two major factors have severly limited the widespread integration of this technology into crop and seed production and breeding: (1) economic

efficiency in terms of net cost for field-ready transplants [11], and (2) genetic variability manifested in regenerated plants [9]. It is generally held that propagation efficiency will improve as a result of practical research on culture and regeneration, mechanical handling, and plant hardening. However, not enough is presently understood of the causes and critical extrinsic or intrinsic factors contributing to genetic instability to permit meaningful speculations as to its control. Reasons for our lack of knowledge, despite the wealth of circumstantial evidence, have been discussed in depth previously [17]. Basic deficiencies include the lack of defined markers and undeveloped experimental systems.

Lassner and Orton [10] have speculated that heterozygous allozymes may be effective markers for the study of de novo genetic variability for the following reasons: (1) A large number of marker alleles can be accumulated in a single plant or tissue with no deleterious effect on viability; (2) markers can be chosen which reside on a broad expanse of the nuclear genome or are linked; (3) they can be assayed from small amounts of living tissue, and many enzymes are expressed constitutively in both differentiated and cultured tissues; and (4) they encode polypeptides of at least partially known function. The present study utilized several known isozyme markers, a disease resistance locus and a flowering behavior locus, to probe the effects of growth in vitro on phenotypic stability at specific defined loci in celery.

Materials and methods

Celery (*Apium graveolens* L.) is a herbaceous species cultivated for succulent, flavorful leaves, petiole, and swollen taproots. The normal karyotype is $2n = 2x = 22$, consisting of 10 pairs of acro/telocentric and one pair of metacentric chromosomes [14, 24]. Original plant materials were obtained from the USDA Plant Introduction Station at Geneva, New York. The hybrid between PI 169001 and PI 257228 was obtained by the method of Honma [8]. The two accessions differ with respect to alleles at seven loci: *Pgm-2*, *Pgm-3*, *Adh-I*, *Pgi-3*, *Mdh-3* [1], *R112* [20], and *F* (Orton; unpublished). The first five loci encode active isozymes or monomers of phosphoglucomutase (*Pgm-2* and *Pgm-3*), alcohol dehydrogenase (*Adh-1*), phosphoglucoisomerase (*Pgi-3*), and malate dehydrogenase (*Mdh-3*): The *R112* locus conditions response to the soul-borne pathgen *Fusarium oxysporum* f. sp. *apii* race 2 (R = dominant, resistance, r = recessive, susceptibility), and *F* controls flowering behavior (F = dominant, annual; f = recessive, biennial). Among these markers it is know the *Pgi-3* and *Mdh-3* are linked and 9.7 map units apart [1] and that *R112* and *Pgm-2* are linked and 15 to 20 map units apart (Orton, unpublished). No linkage studies have yet been conducted with *R112* and the other loci or with *F*.

Callus was initiated from immature petiole segments and maintained and regenerated as described previously [3]. Preparations for cytological studies

were as specified by Browers and Orton [3, 4]. Electrophoresis was performed as described by Shields et al. [25] including the modifications of Arus and Orton [1]. Staining for activity was done as specified by Vallegos [27]. The *R112* phenotype was visualized and scored as described by Orton et al. [20].

The design of the experiment was as follows: Callus was propagated by monthly bulk transfer. Callus tissues were sampled as randomly and completely as possible during transfer. After 6, 12, and 24 months in culture, the karyological constitution of approximately 40 cells of the callus tissue was determined, and 100 representative plants regenerated. Isozyme phenotypes were determined from 50 clones isolated from the bulk callus by dissociating cells and clumps in liquid maintenance medium, passing through a 100 μm mesh screen and culturing the resulting single cells and small clumps on solid maintenance medium. Extracts consisting of approximately 50 mg (fresh weight) of tissue were randomly taken from each clone after approximately one month and subjected to the electrophoresis procedures described above.

When 2,4-D was removed from the maintenance medium, globular proembryos rapidly differentiated into masses of recognizable embryos and plantlets. After these had developed two to three true leaves and a vigorous root system, they were transferred into sterile potting mix, maintained under light in a 25 °C growth room, and covered with clear plastic. The plastic was gradually removed over two to three weeks to permit the plants to harden while establishing in the soil. Surviving plants (95) were transplanted into individual pots and grown to maturity in a glasshouse. The regenerated plants were to be screened for phenotypes at the seven known loci, any striking new variation, and for meiotic behavior. Plants were to have been self-pollinated, and progeny inspected for the appearance of new recessive mutations not present in primary regenerates. For reasons which will become evident in the Results section, it was not possible to follow through with the complete experimental plan.

Results

Six months

Of 44 callus cells on which detailed karyotype analysis was conducted, 37 were indistinguishable from normal (Table 1). Of the remaining seven cells, five appeared to have undergone one or two chromosome fusions. Although technically aneuploid, these cells may have been equivalent to true diploid cells in terms of total information content. However, two apparently true aneuploids were observed whigh showed clear loss of one or two acrotelocentric chromosomes. All 50 of the clones of this callus tested for isozyme phenotypes at the 5 originally heterozygous loci were unchanged.

Table 1. Karyotypes of 44 cells of celery PI 169001 × PI 257228 callus cultures six months after initiation.

Number of cells	Number of chromosomes				
	Acro-telocentric	Metacentric	Altered		Total
			Fusions	Fragments[a]	
1	18	2	0	0	20
2	16	2	2	0	20
1	19	2	0	0	21
1	19	1	2	0	22
2	19	2	0	0	22
37	20	2	0	0	22
normal	20	2	0	0	22

[a]not counted in total

Of the 95 regenerated plants, 94 exhibited phenotypes indistinguishable from the original explant donor at the originally heterozygous loci. More specifically, the heterozygous phenotypes were observed at all 5 isozyme loci and the dominant phenotypes were observed at *R112* (resistant) and *F* (annual). These 94 plants were also phenotypically indistinguishable from the original explant donor in all other respects. The remaining single plant differed from the original plant in all respects. First, the plant exhibited homozygous loci. Secondly, flowering behavior was biennial. Finally, it differed in morphological characteristics too numerous to mention, although the plant did exhibit the dominant R112 phenotype. It is highly unlikely that this plant was a seed contaminant, since this collection of fixed isozyme alleles does not occur in any of the *A. graveolens* germplasm entries in our possession (Arus and Orton, unpublished). Unfortunately, we were unable to obtain progeny from this plant to verify the genetic basis of phenotypic departure, nor were we successful in elucidating its meiotic behavior. Therefore, it is presented only as possible evidence of variation to those seeking to use embryogenesis for high-fidelity genotype cloning of celery.

Definitive data on diakinesis – metaphase I meiotic behavior were successfully obtained for 46 of the 95 regenerated plants. Of these 46 plants, 44 were indistinguishable from normal. The remaining two plants contained an extra unpaired chromatin element, which appeared to be a paired structure (Figure 1a). This element has been termed an accessory chromosome following the terminology established for similar structures in maize. The presence of accessory chromosomes in the somatic tissues of these plants was not determined. No striking phenotypic affects were associated with plants exhibiting these accessory chromosomes.

Sufficiently large populations of selfed progeny to permit meaningful genetic analyses were obtained from 55 of the original 95 regenerated plants. This group included some plants from which cytological data were successfully obtained (see above). Of the remaining infertile plants, those studied cytologically had apparently normal meiotic behavior, and so the reasons for

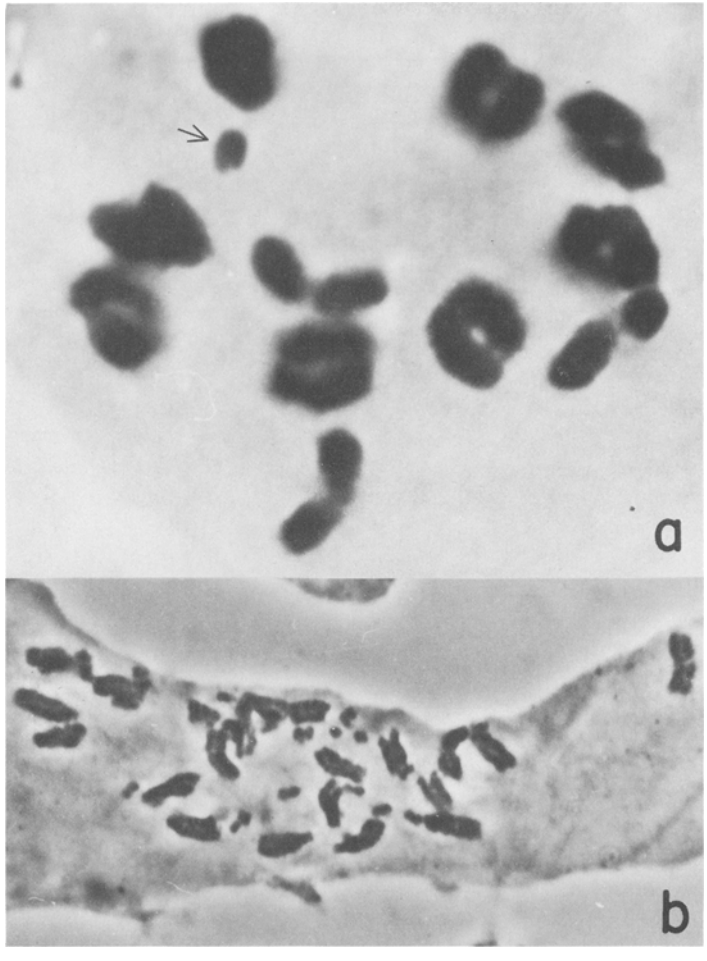


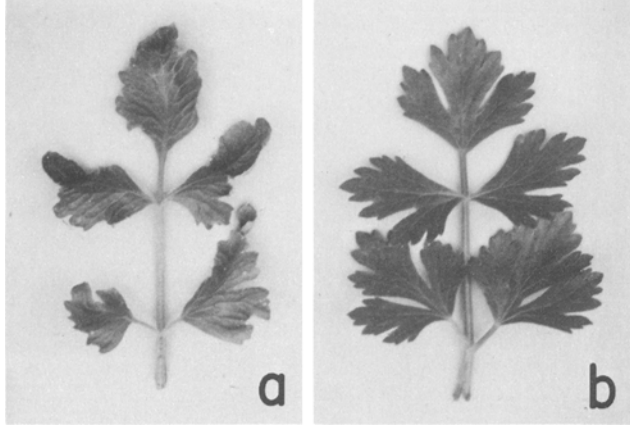
Figure 1. Accessory chromosomes in plants regenerated from six month old PI 169001 X PI 257228 petiole callus cultures (a) Metaphase I cell of regenerated plant containing an accessory chromosome (arrow); (b) Root tip metaphase cell of selfed progeny of this regenerated plant.

infertility remain obscure. Fifteen to twenty progeny of each selfed family were germinated and grown to maturity to look for striking departures from expected phenotypes. Isozyme phenotypes were not tested, but segregations at the *R112* and *F* loci were not different from expected in any of the 55 selfed families. In one of the two selfed families from plants which exhibited accessory chromosomes at meiosis, seedlings exhibited segregation for the presence/absence of the accessory chromosome, and, in one progeny plant, the number of accessory chromosomes ranged from 4 to 14 (Figure 1b, Table 2). The only other character observed among the progeny of a single

Table 2. Transmission of an accessory chromosome from a primary regenerate from the six-month-old celery callus culture to 20 selfed progeny.

Number of selfed progeny	Number of accessory chromosomes per root tip cell[a]
9	0
5	1
2	2
1	1–2
1	2–3
1	6–8
1	4–14

[a]N = 3 root tip cells per progeny



Phenotype conditioned by the recessive abnormal leaf morphology mutation observed among plants regenerated from six month old PI 169001 X PI 257228 petiole callus cultures (a) mutant; (b) normal.

regenerated plant was abnormal leaf morphology (Figure 2). In two separate tests, segregations for the trait were not significantly different from that expected if the original regenerate had been heterozygous for a new mutant recessive nuclear allele (13:3 and 15:5 normal: variant segregation). All of the selfed progeny of plants exhibiting the abnormal leaf morphology were also variant, consistent with this hyothesis.

From the standpoint of attaining genetic fidelity, it is entirely possible that the remaining families did contain new variability, but not striking or qualitative enough to permit unambiguous identification. Moreover, the *r112* (*F. oxysporum* f. sp. *apii* race 2 susceptible) and *f* (annual) phenotypes would have impaired the ability to distinguish variants arising later in development.

Table 3. Karyotypes of 40 cells of celery PI 169001 × PI 257228 callus cultures twelve months after initiation.

Number of cells	Number of chromosomes				
	Acro-telocentric	Metacentric	Altered		Total
			Fusions	Fragments[a]	
1	15	0	2	2	17
1	12	3	3	0	18
1	14	3	1	0	18
1	17	2	0	9	19
1	17	1	1	0	19
1	17	2	1	1	20
1	16	4	0	0	20
1	17	1	2	0	20
1	17	2	1	0	20
1	18	2	0	3	20
2	18	1	1	1	20
3	18	2	0	0	20
2	18	2	0	1	20
4	18	1	1	0	20
1	19	1	1	0	21
6	19	2	0	0	21
1	18	1	2	0	21
1	18	2	1	1	21
1	17	4	0	1	21
1	19	2	1	0	22
1	20	2	0	0	22
1	20	2	0	1	22
1	19	2	2	0	23
1	22	1	1	0	24
1	22	2	1	0	25
1	21	2	3	2	26
1	34	3	0	2	37
1	60	6	7	12	73

[a]not counted in total

Twelve months

In contrast to the 6-month-old culture, divergence from the original karyotype in the 12-month-old culture was almost complete; only one cell was indistinguishable from normal (Table 3). Of the remaining 39 cells most (31) were presumed hypoaneuploids (17 to 21 chromosomes), although many of these exhibited compound chromosomes resulting from fusions, and the conclusion of aneuploidy in an informational sense was not possible. Of the remaining cells, two had 22 distinct chromosomes, but exhibited a fusion or fragment not characteristic of the original karyotype; six cells were hyperdiploid (23 to 26 chromosomes) or presumably polyploid (37 and 73 chromosomes). In contrast to this extensive karyological variation, no departures from the original heterozygous isozyme phenotypes were observed among 50 clones isolated from the 12-month-old callus.

When the 12-month-old callus was placed onto medium which typically fosters the continued development of embryos from globular structures [4, 19], the tissue exhibited some greening, but no recognizable embryos. Repeated attempts all gave the same result. Since the study was conceived to explore the genetic efficacy of embryogenic cloning, and the culture had apparently lost the ability to do so, the experiment was terminated.

Discussion

Williams and Collin [29] examined regenerated plants from nine – to fifteen-month-old cultures of the celery cv. Lathom Blanching for evidence of instability. Chromosome counts conducted on root tips revealed that departures from the normal chromosome number occurred at an appreciable frequency. Specifically, hypodiploidy (27.3% of cells of regenerates), tetraploidy (2.3%), and hypotetraploidy (2.3%) were observed among and within six regenerated plants. Despite this, all of the regenerated plants were morphologically indistinguishable from normal. Thus, the apparent aneuploidy was not associated with striking phenotypic alterations as is generally the case. This hypoeuploidy was probably a consequence of Robertsonian fusions, which lead to a decrease in gross chromosomes number with no corresponding change in informational content and which have been observed in *A. graveolens* [12]. Williams and Collin [29] conclude that celery cell cultures are sufficiently stable that they may be used for propagation in conjunction with conventional breeding. They did not, however, conduct any genetic studies of the cultured tissue itself in support of this possibility. Numerous instances have been described in which regeneration appears to act as a screen against cytologically mutant cells (summarized in ref. [2]).

Despite the presence of striking cytologically mutant cells (16%) in six-month-old cultures of the present study, copious viable somatic embyos were successfully obtained. Among those which survived transplantation to the greenhouse, evidence for cytological change from the original explant donor plant was obtained from only two of 46 tested plants. These both contained a small accessory chromosomal element in addition to an apparently normal complement of 11 bivalents (2N = 22). Despite the fact that such accessory chromosomes were not observed among 44 cells in the culture from which these plants were regenerated, they probably arose in vitro, since they have not been reported in karyotypes of seed-propagated plants [13, 14, 24]. Regenerating embryos were thus derived almost entirely from karyologically normal cultured cells, which were estimated to comprise 84% of the six-month-old population. The dramatic increase of karyologically abnormal cells in the 12-month-old culture was associated with a complete loss of embryogenic potential. While this circumstantial evidence suggests that karyological mutations tend to render cells unable to differentiate normally, it is possible that the loss of embryogenic potential was due to epigenetic changes. Attenu-

ation of karyological variability from cultured into regenerated cells and tissues has been reported previously [2, 15, 16, 23]. Reduction of variability in regenerates from celery cultures has been reported by Browers and Orton [4]. While no homogeneously aneuploid plantlets were observed in this report, two such plantlets were observed in a separate study [18], and both were grossly abnormal.

The consequences of true aneuploidy should be the loss of duplication or information contained in the derivative diploid tissues. Apparent chromosome loss, resulting in functional monosomy and possible nullisomy, was observed both in the six-month-old culture (at least two of 44 cells sampled) and at high frequency in the 12-month-old culture (at least 28 of 40 cells sampled). Any resulting loss of genetic information involving heterozygous loci should occur simultaneously with a change in phenotype to that associated with the retained allele. The explant donor plant was heterozygous at five known isozyme markers, two of which were tightly linked while the other three assorted independently [1]. Hence, it is likely (but not certain) that 4 of the 11 chromosomes were marked with at least one constitutive heterozygous isozyme marker. If (for example with the 12-month-old culture) chromosomes had been lost completely and randomly in the presumed hypoaneuploid cells (i.e. monosomics, 28 of 40 cells or 70% of the culture), then approximately 13 of the 50 clones tested should have shown a change in at least one isozyme marker phenotype from heterozygous to hemizygous. Such was not the case, as the heterozygous phenotype was conserved among all 50 clones. Possible explanations for this incongruency are: (1) chromosomes were not lost randomly, and one of several chromosomes without an isozyme marker were preferentially lost; (2) chromosomes were not actually lost, but were rearranged such that equivalent information was present and expressed on fewer chromosomes; and (3) mono- and nullisomic cells were derived from diploid or hyperdiploid cells at high frequency in vitro, but were relatively inviable as compared to diploid cells. Hence, viable clones were actually a mixture of diploid/hyperdiploid and mono- or nullisomic cells, and would exhibit isozyme phenotypes indistinguishable from homogeneous heterozygous in the assay systems used. No experimental data were obtained in the present study to distinguish among these hypotheses.

The use of in vitro culture as a potentially powerful tool in celery production and breeding has been discussed previously [7, 19, 22, 28]. Phenotypic and genotypic stability are important prerequisites for the successful realization of the potential for mass cloning, as discussed by Lawrence [11]. While the report of Williams and Collin [29] suggests that embryogenic cloning of celery gives rise to a phenotypically uniform population of plants, subsequent reports have demonstrated that phenotypic variation and karyological mutations are possible [4, 18, 21]. In the present report, cytological (with no apparent phenotype) variation and a recessive abnormal leaf morphology mutation were observed among regenerates of the six-month-old

culture at corresponding estimated frequencies of 4.5 and 1.8% (not counting the single grossly abnormal regenerate). If such frequencies could be extrapolated to celery cultures across a broad range of genotypes and culture ages, the use of in vitro culture for direct field establishment would be feasible. However, such frequencies may be too high to permit its use for seed production and in breeding. Also, 42% of the primary regenerants exhibited some loss in fertility, the genetic basis of which is not known. Engler and Grogan [6] have reported that lettuce plants regenerated from leaf mesophyll protoplasts frequently show similar infertility.

There are some suggestions that the degree of variation and embryogenic potential may be altered by cultural manipulations (reviewed in ref. [17]). For example, if uniform tissues can be generated and stored indefinitely, the undesirable effects brought on by prolonged culture may be avoided. All of the reports on celery cited above utilized distinctly different genotypes, and is is not known the extent to which results are generally comparable, or to what extent genetic background influences instability in culture. Browers and Orton [3] have presented evidence which strongly suggests that genotypic background is an important factor in determining the types and frequencies of karyologically abnormal cells in vitro. Thus, there is hope that a combination of basic genetic and empirical cultural research can contribute to the control and understanding of genetic instability in cultured plant cells.

Acknowledgements

This research was supported by grants from the California Celery Research Advisory Board. The valuable assistance of M. E. Durgan and S. D. Hulbert is gratefully acknowledged.

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