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MORPHOLOGICAL AND MOLECULAR ANALYSIS OF GENETIC STABILITY IN MICROPROPAGATED *FRAGARIA* × *ANANASSA* CV. POCAHONTAS

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SUMMARY

Micropropagated strawberry plants (*Fragaria* × *ananassa* L.) grown on 5 μ*M* and 15 μ*M* BA medium or cold-stored were grown in the field to examine morphological variation. Except for plant height, morphological characteristics did not differ for field-grown plants micropropagated on 5 μ*M* and 15 μ*M* BA medium. Cold-stored plants were less vigorous, both vegetatively and reproductively, than BA-treated plants. Random amplified polymorphic DNA (RAPD) markers were used to determine if cold storage or supraoptimal levels of N^6-benzyladenine (BA) in the culture medium caused genetic changes leading to somaclonal variation. No mutations were observed in 246 loci amplified by the 29 primers tested. Possible changes in methylation patterns of ribosomal DNA genes of strawberries were also examined. Changes in methylation patterns were observed in only one DNA sample from plants grown on 15 μ*M* BA medium and in one of the cold-stored plants. Length polymorphism was observed in two samples from plantlets derived from one explant. The low levels of RAPD variation and methylation observed, and the apparently epigenetic changes in morphological characteristics in plants used in this study, indicated that mutations had not occurred.

Key words: benzyladenine; cold storage; morphology; somaclonal variation; strawberry; tissue culture.

INTRODUCTION

Commercial micropropagation of strawberries (*Fragaria* × *ananassa* L.), in which multiplication of plants is maximized through high concentrations of plant growth regulators, can induce a relatively high proportion of somaclonal variation (Sansavini et al., 1990). Changes include leaf color variants and dwarf plants, among others (Swartz et al., 1981; Cameron and Hancock, 1986; Sansavini et al., 1990; Irkaeva and Matveena, 1997). These variants pose a problem for production of uniform, true-to-type plants. Nehra et al. (1994) found that two cultivars of strawberry responded differently to various forms of in vitro propagation, and in both cases variants were found in callus-derived plantlets, but not those derived from meristems or via direct leaf regeneration. Some studies have shown that modified characteristics are epigenetic and disappear over time (Koruza and Jeleska, 1993). Numerous authors have reported that genetic changes including insertions, deletions, point mutations, and rearrangements occur during tissue culture (Anderson et al., 1991; Kane et al., 1992), but few of the phenotypic symptoms found are heritable (Sansavini et al., 1990; Karp, 1995).

Most somaclonal variations occur in plants regenerated from cultures that have undergone a dedifferentiation phase. Temporary variations may be due to methylation changes in the DNA. Methylation pattern changes have been detected in suspension cultures of soybean [*Glycine max* (L.) Merr.] and maize (*Zea mays* L.) callus culture regenerants (Quemada et al., 1987; Kaeppler and Phillips, 1993). Callus-derived 'Redcoat' strawberry plants differ significantly from standard runner plants for several vegetative characteristics (Nehra et al., 1992).

The role of growth regulators as agents of mutation is under study by several investigators. Arnholdt-Schmitt (1993) observed that indole-3-acetic acid (IAA) and inositol in the growth medium induced DNA rearrangements and methylation changes in carrot (*Daucus carota* L.) callus cultures. However, tissue culture medium cytokinin concentration did not influence cropping or vegetative performance of runner progeny of micropropagated strawberry plants (Beech et al., 1988).

The objective of this study was to determine whether cold storage or benzyladenine (BA) concentration in the culture medium during multiplication influenced genetic stability of 'Pocahontas' plantlets as detected by morphological evaluation of field-grown plants and RAPD molecular markers in plantlets in vitro or in the field.

MATERIALS AND METHODS

Culture initiation. Five runners of *Fragaria* × *ananassa* cv. Pocahontas were collected from clonally propagated screenhouse mother plants, washed in running tap water for 10 min, and disinfested for 10 min with 5.25% sodium hypochlorite [10% (vol/vol)] commercial bleach containing a drop of

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Tween 20 (Sigma Chemical Co., St. Louis, MO). Explant bases were recut and each runner tip was placed in a 16-mm glass culture tube with 10 mL of NCGR-FRA medium (Reed, 1991) consisting of MS (Murashige and Skoog, 1962) salts and vitamins with the addition of 1.42 m*M* NaH_2PO_4, 0.434 m*M* adenine sulfate, 5.71 μ*M* IAA, 0.26 μ*M* gibberellic acid (GA_3), 4.4 μ*M* BA, and 0.3% agar (Bitek, Difco, Detroit, MI) plus 0.125% Gelrite (Kelco, San Diego, CA). The medium was adjusted to pH 5.7, heated, dispensed into tubes, and autoclaved for 20 min at standard temperature and pressure. Cultures were maintained under a 16-h photoperiod (25 μmol $m^{-2}s^{-1}$). After 3 wk, plantlets were transferred to Magenta GA7 boxes (Magenta Corp., Chicago, IL) with 40 mL NCGR-FRA medium. Plantlets produced from each of the five explants were maintained separately. The same basal medium was used in both experiments with changes in plant growth regulators as noted below.

Cold stored plantlets. Five 'Pocahontas' plantlets which originated from the same mother plants and cold-stored in vitro at 4° C for 4 yr were tested. Plantlets were stored in gas-permeable plastic bags ("Star*pacs", Agristar Inc, Garner Enterprises, Willis, TX) with 10 mL of NCGR-FRA medium free of adenine sulfate, BA, IAA, and GA, but with more agar (0.35%) and Gelrite (0.145%) than used for standard culture (Reed, 1991). During the cold-storage period, plantlets were removed yearly and repropagated on NCGR-FRA medium with 4.4 μ*M* BA for three to six subcultures of 3 wk each, and returned to Star*pacs containing NCGR-FRA medium without growth regulators for further storage. After 4 yr of storage, 10 plantlets were transferred to individual Magenta boxes with 40 mL of NCGR-FRA medium and grown under controlled environmental conditions as described above for four subcultures before DNA sampling.

BA-treated plantlets. Six wk after initiation, 10 plantlets were randomly selected from stock cultures of each explant and placed on medium with 5 or 15 μ*M* BA (10 plantlets from each original explant on each medium). All plantlets produced were subcultured four times onto the same medium at 3-wk intervals; all propagules were used for further propagation. The fifth transfer produced enough plantlets so that only one offshoot from each plantlet was randomly chosen for transfer. Explants were subcultured for 8 mo. before sample collection for DNA extraction.

Magenta boxes were placed in the growth room in a completely randomized design. Boxes were labeled with the BA treatment, explant number, and transfer date. Plantlets from each explant were distinctly maintained with four boxes (20 plants each) per original explant.

Morphological analysis of field-grown plants. One-hundred plantlets from each BA treatment and 50 cold-stored plantlets were acclimatized for field planting. Plants were potted in seedling mixture (peat moss, perlite, and vermiculite 1:1:1 by volume) in trays, placed in a mist bed for 1 wk, then moved to greenhouse benches. After 7 to 10 d, rooted plants were moved to 600-cc pots containing a 2:1:1 potting mixture (fir bark, peat, and vermiculite) and acclimatized in the greenhouse for 3 to 4 wk. In April, plants were transplanted to the field (Salem gravely silt loam) in matted rows 76.2 cm apart with 30.5 cm between plants in a completely randomized design. Four d after being transplanted, plants were fertilized with 15:16:17 fertilizer at the rate of 4.8 N–5.12 P–5.44 K. Irrigation was provided as needed by overhead sprinklers.

Morphological characteristics were measured for each transplant in early (flowering data) or mid-June (fruit and vegetative characters) including leaf length, leaf angle (the angle between the lines connecting the first serration and the base of the leaf), leaf width, petiole length, number of runners per transplant, plant height, spread, vigor (rated 1–5 with 5 being most vigorous), number of trusses, average number of fruits per truss, primary fruit weight, color (rated 1–5 with 1 being white and 5 being dark red) and shape (1-globose, 2-conical, 3-malformed), and total fruit weight. Leaf characteristics were measured on three randomly selected central leaflets per plant.

Data were subjected to analysis of variance and multifactorial analysis with the Statgraphics Statistical Graphics System (Statgraphics Graphics Corp., Rockville, MD) and SAS Analytical System (SAS Institute Inc., Cary, NC). Means of morphological characteristics were separated by the least significant difference test at $P \leq 0.05$.

DNA isolation. Eighty samples, one from each explant for each BA treatment, were analyzed by random amplified polymorphic DNA (RAPD) analysis. DNA was isolated from fresh or frozen samples of whole plantlets stored at −70° C. Young leaf samples were collected from the screenhouse mother plants to provide control DNA. Total cellular DNA from samples of each explant was extracted by a modified minipreparation protocol as described by Torres et al. (1993) with 2% cetyltrimethylammonium bromide (CTAB) extraction buffer (1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl, pH 8.0). RNA was removed by a second precipitation. The DNA was quantified with Hoechst 33258 dye with a TKO-100 Minifluorometer (Hoefer Scientific Instruments, San Francisco, CA) and diluted to 5 ng μL^{-1}. These samples were analyzed with RAPD-polymerase chain reaction (RAPD-PCR). Samples were collected on four separate dates from 1 wk to 3 mo. apart. Freshly emerged leaves were also collected from 2-mo.-old pot grown cold-stored plants, and DNA was extracted for PCR amplification.

For methylation experiments, total cellular DNA was isolated by a modified Doyle and Doyle (1990) DNA preparation with 4% CTAB (0.5% polyvinylpyrrolidone, 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl, pH 8.0). Samples were treated with RNAase (100 μg mL^{-1}) again when absorbance values at 365 n*M* were higher than 1.8. DNA of young whole daughter plants from field-produced runners from the BA-treatments, cold-stored, and control plants was extracted, and DNA profiles were compared to those from in vitro samples.

DNA (RAPD) analysis. PCR amplification was performed with arbitrary 10-mer Operon oligonucleotides (Operon Technologies, Inc., Alameda, CA) with a cocktail adapted from Huff et al. (1993). For initial studies, we optimized the PCR reaction for strawberry DNA by varying the range of DNA templates, DNA polymerase, and $MgCl_2$ concentrations. The PCR amplifications were performed in 12-μL volumes. Comparison reaction conditions included 0.5 ng strawberry DNA per μL, Stoffel buffer (10 m*M* KCl and 10 m*M* Tris-HCl), 2.5 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 0.5 μ*M* Operon decamer primer, and 0.042 u of Amplitaq DNA polymerase Stoffel fragment (Perkin-Elmer Corp., Norwalk, CT) per μL.

DNA amplifications were performed in a PTC100 MJ thermocycler (MJ Research, Inc., Watertown, MA) in 96-well flexible assay plates (Falcon, Oxnard, CA) with reaction mixtures overlaid with three drops of mineral oil. The thermocycle profile was adapted from Huff et al. (1993). Each thermocycle consisted of DNA melting at 94° C for 30 sec, primer annealing at 36° C for 1 min, and a 1° C sec^{-1} ramp leading to 72° C for 2 min for primer extension. This cycle was repeated 43 times followed by final extension at 72° C for 5 min. The thermocycle profile was preceded by an initial denaturation for 7 min at 94° C and 1 min annealing at 36° C.

Amplification products were separated on 7.5% acrylamide/bis gel (37:1) in 0.375 *M* Tris, pH 8.8 buffer, and visualized with silver staining according to Caetano-Anolles and Gresshoff (1992). After electrophoresis, gels were fixed in a 7.5% acetic acid solution for 10 min followed by three washes of 4 to 5 min in double-distilled water. Silver impregnation was performed with neutral silver solution (0.15% silver nitrate and 0.056% formaldehyde) for 10 min followed by a brief double-distilled water rinse. Images were developed with a solution of 3% sodium carbonate, 0.056% formaldehyde, and 2 mg sodium thiosulfate per L, and gels were dried between membranes. Gels were visually scored for presence (1) or absence (0) of selected loci. All PCR reactions were replicated with a second subsample from the DNA extraction.

Primer screening. Approximately 100 decamer arbitrary primers were screened for suitability of producing dark, low background bands. The PCR conditions used for this purpose included (per μL) 0.5 ng DNA and 1.0 u Amplitaq Polymerase (Stoffel fragment) of reaction volume and other conditions indicated earlier. Primers were screened with a bulk sample of cold-stored plantlets (leaf samples from each original explant). Three replicates were tested and DNA was extracted from young leaves of screenhouse mother plants for controls.

Methylation analysis of rDNA genes. Methylation changes in cold-stored and BA-treated samples as compared to control screenhouse-mother plants were analyzed with Southern blots. About 15 μg of DNA from each sample was digested overnight with *Hpa*II and *Msp*I restriction enzymes (New England Biolabs, Beverly, MA) with a 3.11-kb *Bam*HI fragment containing 18S, 5.8S and a part of the 26S rRNA genes. Samples were further digested with a fresh aliquot of enzymes for 4 h and run on 0.8% agarose (GIBCO BRL, Gaithersburg, MD) gels. The DNA was Southern-transferred overnight onto nylon membranes. Protocols were performed according to procedures outlined in Current Protocols in Molecular Biology (Ausubel et al., 1995).

Bacteria were cultured overnight in Luria Broth medium (Ausubel et al., 1995) with 1 g ampicillin per mL, and plasmid DNA was prepared with a Wizard miniprep kit (Promega, Madison, WI). The DNA was digested with *Bam*HI restriction enzyme and insert DNA was then purified, labeled with random priming, and hybridized overnight to a nylon membrane at 37° C. Membranes were placed in film cassettes with Kodak film (Eastman Kodak Co., Rochester, NY) and autoradiographed for 1 wk at −70° C.

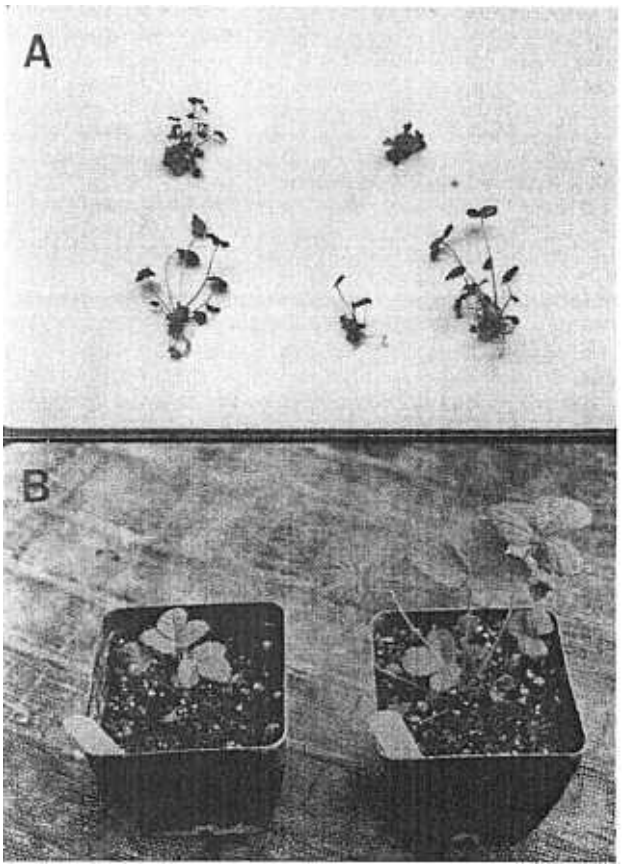

FIG. 1. 'Pocahontas' plants grown in vitro for 8 mo. *A*, In vitro-grown plantlets from BA medium containing 5 μ*M* (*bottom*) or 15 μ*M* (*top*) BA. *B*, Appearance 4 wk after being transplanted to pots from medium containing 5 μ*M* (*right*) or 15 μ*M* (*left*) BA. Plants from 5 μ*M* BA remained taller during the entire greenhouse phase of growth.

TABLE 1

MORPHOLOGICAL ANALYSIS OF FIELD-GROWN 'POCAHONTAS' STRAWBERRY PLANTS FROM IN VITRO PLANTLETS MULTIPLIED ON NCGR-FRA MEDIUM WITH 5 μ*M* OR 15 μ*M* BA FOR 8 MONTHS OR STORED AT 4 °C FOR 4 YR ON MEDIUM WITHOUT BA[a]

	In-vitro growth conditions		
Character	5 μ*M* BA	15 μ*M* BA	Cold-stored
Leaf angle (mm)	107.8 z	109.3 z	104.8 y
Leaf length (mm)	74.1 z	76.1 z	70.6 y
Leaf width (mm)	66.1 z	67.1 z	62.7 y
Petiole length (mm)	123.0 yz	126.4 z	119.0 y
Plant height (cm)	6.8 y	7.1 z	6.4 x
Plant spread (cm)	6.8 z	7.2 z	6.2 y
Plant vigor[b]	4.4 z	4.5 z	3.8 y
Runnering	11.4 y	11.6 y	13.6 z
Number of trusses	1.6 z	1.6 z	0.5 y
Total fruit number	13.2 z	14.3 z	5.0 y
Primary fruit weight (g)	12.2 z	11.4 z	3.5 y
Primary fruit color[c]	4.3 z	3.9 z	1.3 y
Total fruit weight (g)	33.5 z	30.2 z	7.0 y

[a]Means in a row followed by the same letter are not significantly different by the least significant difference test ($P \leq 0.05$).
[b]Plant vigor rated 1 (little growth) to 5 (very vigorous).
[c]Fruit color rated 1 (white) to 5 (dark red).

RESULTS AND DISCUSSION

Tissue culture morphology. 'Pocahontas' in vitro-grown plantlets on 15 μ*M* BA medium were compact and pale green, while those on 5 μ*M* BA were longer and dark green (Fig. 1). Cold-stored plants were not morphologically different from 5 μ*M* BA plants.

Field plant morphology. All morphological characteristics except petiole length varied greatly between the BA-treated and cold-stored plants (Table 1). BA-treated plants varied only in height, and field-grown plants from both BA treatments were taller than cold-stored plants. Cold-stored plants were less vigorous in general and produced more runners and fewer flower trusses and fruit than the BA-treated plants. Cold-stored plants showed delayed ripening of fruits (as indicated by fruit color). Lack of vegetative vigor might have contributed to reduced reproductive vigor of cold-stored plants. Chilling could inhibit flower induction in strawberry plants and freezing injury of initiated floral buds, but there was no reason to believe that cold-stored and non-cold-stored plants would vary in this regard. Vernalization decreases days to flower initiation, but long-term cold storage is not the same as vernalization (Higgins and Stimart, 1990). The increased number of runners on the cold-stored plants might be due to decreased reproductive vigor which would channel photoassimilates into runners.

One field-grown plant from the 15 μ*M* BA treatment had distorted, crinkled, and rough leaves, with poor vegetative vigor and no flower production. The runner-produced daughter plants also had distorted leaves. Cyclamine mites (*Stenotarsonemus pallidus* Banks) were not detected on the distorted plants, and these aberrant features could not be attributed to cyclamine mite infestation. At the end of the season this plant began to bear normal leaves, suggesting that these variations were epigenetic. No other gross variants were observed. These results were similar to those of Nehra et al. (1994) who found no variants for plants derived from meristem culture or direct leaf regeneration for strawberry cultivars Veestar and Redcoat. In contrast with our results, Irkaeva and Matveeva (1997) observed many morphological changes in *F. vesca* L. seedlings following culture on 0.2 to 1.2 μ*M* BA. Multiple shoot-like structures, simple leaves, reduced anthocyanin, and shortened leafstalks were noted. They found that quantitative characteristics of these changes varied with the BA concentration, length of culture, and genotype. These differences in response might be due to the genetic variability commonly observed in seedlings in contrast with the relative stability of clonally propagated cultivars used in this study.

RAPD analysis of BA treatments. BA treatments did not yield polymorphism in any DNA samples when amplified with 29 primers (Table 2). Likewise, the same 29 primers used on samples of cold-stored plants yielded monomorphic profiles, indicating genetic stability after micropropagation and 4 yr of cold storage. The number of bands in a profile varied between 3 to 18 depending on the primer with 246 clearly distinct bands (loci) analyzed.

Methylation studies of BA treatments. One RAPD primer produced repeatable bands among retests but was not consistent over treatments nor between duplicate plants. This suggested that methylation might be occurring at specific loci. Differences in methylation patterns were observed between field plants and in vitro samples for one treatment (15 μ*M* BA treatment, explant 5) when probed with a 3.11-

TABLE 2

PRIMERS USED FOR DNA AMPLIFICATION OF IN VITRO-STORED STRAWBERRY PLANTLETS AND SCREENHOUSE-GROWN MOTHER PLANTS, THEIR SEQUENCES, AND THE NUMBER OF FRAGMENTS AMPLIFIED

	Sequence	Number of amplified fragments
OPA1	CAGGCCCTTC	8
OPA2	TGCCGAGCTG	1
OPA7	GAAACGGGTG	8
OPA11	CAATCGCCGT	8
OPA20	GTTGCGATCC	
OPB10	CTGCTGGGAC	12
OPC9	CTCACCGTCC	6
OPC17	TTCCCCCCAG	6
OPD1	ACCGCGAAGG	8
OPD2	GGACCCAACC	4
OPD11	AGCGCCATTG	19
OPD18	GAGAGCCAAC	4
OPF2	GAGGATCCCT	
OPF3	GGGATATCGG	8
OPF7	CCGATATCCC	14
OPF9	CCAAGCTTCC	2
OPF10	GGAAGCTTGG	10
OPF17	AACCCGGGAA	8
OPH4	GGAAGTCGCC	
OPH5	AGTCGTCCCC	
OPH8	GAAACACCCC	8
OPH11	CTTCCGCAGT	9
OPH12	ACGCGCATGT	10
OPH14	ACCAGGTTGG	12
OPH15	AATGGCGCAG	10
OPM17	TCAGTCCGGG	7
OPM20	AGGTCTTGGG	8
OPZ2	CCTACGGGGA	13
OPZ6	GTGCCGTTCA	12

kb *Bam*HI fragment. The remaining samples showed no indications of methylation changes. In all other samples *Hpa*II, as expected, was unable to digest DNA when methylated. Differences in hybridization signals were also seen and may indicate differences in copy number of rRNA genes. Changes in methylation patterns were also observed in one sample from the cold-stored plants. Methylation studies were conducted on only a limited number of samples with no clear implications, and these results need to be reconfirmed (data not shown).

The single variant plant observed in BA treatments could be due to changes in gene expression, but by the end of the season that plant and its runners began to appear normal, suggesting the change was epigenetic. Sansavini et al. (1990) reported that some variations were masked by other characteristics and tended to appear in later seasons. We did not continue the study beyond one season. Absence of polymorphism in RAPDs and lack of major changes in DNA methylation indicated that genetic changes were not apparent in BA-treated or cold-stored plants. The absence of polymorphisms in the specifically amplified DNA we used does not exclude the possibility that mutations occurred in other repetitive DNA segments or coding regions (genes) of the genome.

Several steps are important in avoiding somaclonal variation in micropropagation. Micropropagated plants must be produced from preexisting meristems (not adventitious buds), and regenerated from young tissues and stable genotypes, and callus and protoplasts must be avoided (Jain and De Klerk, 1998). Numerous reports have pointed to the importance of proper explant choice (Adelberg et al., 1994). In this study, BA concentration in the culture medium during multiplication had no major effects on genetic stability of 'Pocahontas' plantlets across 246 RAPD loci and a broad range of morphological characters. Cold storage has a significant effect on the reproductive vigor of field-grown plants, presumably due to reduction in general plant vigor.

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