Genetic Signatures from Amplification Profiles Characterize DNA Mutation in Somatic and Radiation-induced Sports of Chrysanthemum

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ABSTRACT. The chrysanthemum (*Dendranthema grandiflora* Tzelev.) cultivars 'Dark Charm', 'Salmon Charm', 'Coral Charm' and 'Dark Bronze Charm' are either radiation-induced mutants or spontaneous sports of 'Charm' and constitute a family or series of plants that primarily differ in flower color. These cultivars, which were difficult to differentiate genetically by DNA amplification fingerprinting (DAF), were easily identified by using arbitrary signatures from amplification profiles (ASAP). Genomic DNA was first amplified with three standard octamer arbitrary primers, all of which produced monomorphic profiles. Products from each of these DNA fingerprints were subsequently reamplified using four minihairpin decamer primers. The 12 primer combinations produced signatures containing $\approx 37\%$ polymorphic character loci, which were used to estimate genetic relationships between cultivars. Forty-six (32%) unique amplification products were associated with individual cultivars. The number of ASAP polymorphisms detected provided an estimate of the mutation rate in the mutant cultivars, ranging from 0.03% to 1.6% of nucleotide changes within an average of 18 kb of arbitrary amplified DAF sequence. The ASAP technique permits the clear genetic identification of somatic mutants and radiation-induced sports that are genetically highly homogeneous and should facilitate marker assisted breeding and protection of plant breeders rights of varieties or cultivars.

Chrysanthemum [*Dendranthema grandiflora* Tzelev. or *Chrysanthemum morifolium* Ramat. (synonym) (Anderson, 1987)], mentioned in the writings of Confucius, was probably cultivated before 500 BC and is endemic to Asia. It was introduced to Europe in 1789 and to the United States after the nineteenth century. These earlier plants bear little resemblance to the multitude of cultivars available today (Smith, 1975). Many of the new cultivars of chrysanthemum are developed from a single progenitor either spontaneously or by radiation-induced mutation (sports) (Dowrick and El-Bayoumi, 1966). Because of their close genetic relatedness, there is a need in the industry to clearly differentiate vegetatively derived accessions. Until recently, reliable techniques capable of discriminating between genetically homogeneous plants were not available (Hubbard et al., 1992). However such limitations have been largely alleviated with the advent of a number of DNA profiling methodologies.

DNA can be typified by a wide assortment of fingerprinting techniques that are usually based on either the hybridization of nucleic acids or their selective enzymatic amplification. Among the myriad of amplification-based nucleic acid scanning fingerprinting strategies (Caetano-Anollés and Trigiano, 1997) are randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1991), which do not require previous characterization of the genome, employ short oligodeoxynucleotides as arbitrary primers, and are versatile, universal and relatively simple to perform. Characterization of cultivars or a family of closely related chrysanthemum cultivars has been attempted using RAPD (Wolff, 1996; Wolff et al.,

1995; Wolff and Peters-Van Rijn, 1993) and DAF analysis (Scott et al., 1996). The RAPD technique distinguished nonrelated cultivars of chrysanthemum (Wolff and Peters-Van Rijn 1993), but could not identify cultivars belonging to a family and differing only in flower color (Wolff et al., 1995). However, genetic differences between chimeral cell layers in leaf and floret tissue has been demonstrated recently using the RAPD technique (Wolff, 1996). Because of the relatively high information content of the fingerprints, DAF was used successfully in the identification of members of several chrysanthemum families (Scott et al., 1996). However, unique amplification products were not found for all individual cultivars in the Charm family, although phenetic analyses could assign positions of the individual cultivars relative to one another. Therefore, a method sensitive enough to absolutely differentiate these very closely related cultivars with exclusive markers was needed.

The generation of arbitrary signatures from amplification profiles (ASAP) is a two-step molecular amplifier, whereby DAF fingerprints are reamplified with a different primer (Caetano-Anollés and Gresshoff, 1996.) The ASAP process is a cascade amplification that produces allelic fingerprints of fingerprints and increases detection of polymorphic DNA (Caetano-Anollés, 1996). ASAP has been used to distinguish closely related individuals of bermudagrass [*Cynodon dactylon* (L.) Pers.] Caetano-Anollés, unpublished data), geraniums (*Pelargonium* spp.) (Starman and Abbitt, 1997a), poinsettia (*Euphorbia pulcherrima* Willd.) (Starman and Abbitt, 1997b) and a fungus, *Discula destructiva* Redlin (Caetano-Anollés et al., 1996). In the present study, we used ASAP to assess the genetic diversity within members of the Charm family of chrysanthemum and to uncover markers that unequivocally identify individual cultivars.

Materials and Methods

Plant material was donated by Yoder Brothers, Inc. (Barberton, Ohio), and consisted of rooted cuttings of five cultivars of the

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Fig. 1. Pedigree of the chrysanthemum series Charm. 'Charm' was a selection from the cross of two seedling derived plants (Sp1 and Sp2). All other cultivars were selected following gamma irradiation (2000 to 2200 rad) except for 'Dark Bronze Charm', which was a spontaneous sport.

Charm series, nonhardy pot-type chrysanthemums with decorative flowers. 'Dark Charm', 'Salmon Charm', 'Coral Charm', and 'Dark Bronze Charm' were 1983–86 selections from 'Charm' that resulted from either a spontaneous sport or a mutation induced by ionizing (gamma) radiation at 2000 to 2200 rad levels (Fig. 1). These cultivars differ primarily in flower color ranging from markedly distinctive to subtle changes in hue. 'Pomona' and 'Davis' were designated as a genetic outgroup in the study. Plants were grown and maintained in the laboratory and individuals of the five cultivars were genetically indistinguishable based on an earlier DAF study (Scott et al., 1996).

DNA was extracted from 25 to 50 mg of frozen (-70°C) young, not yet fully expanded, light-green leaves either as described by Scott et al. (1996) or using a commercial extraction kit (Puregene; Gentra Systems, Inc., Minneapolis, Minn.). DAF analysis of genomic DNA was according to Caetano-Anollés et al. (1991). Reactions were assembled in a total volume of either 10 or 20 μL , contained 3 μM primer, 2.5 mM MgSO_4 , and 0.1 $\text{ng}\cdot\mu\text{L}^{-1}$ template DNA in TTNK10 buffer (Caetano-Anollés and Gresshoff, 1994), and were amplified in a thermocycler (Easy Cycler Twin Block Systems; Ericomp, Inc., San Diego, Calif., for 35 cycles consisting of 10-s steps at 96 and 30 $^{\circ}\text{C}$. Prior experimentation demonstrated that an extension step at 72 $^{\circ}\text{C}$ did not alter the DAF profiles (Scott et al. 1996). Three standard (nonstructured) octamer primers were used and had the following (5' to 3') sequences: GAGCCTGT, GTAACGCC and AATGCAGC. DAF reactions were diluted either 1:24 or 1:49 with sterile, distilled water and used as template in ASAP reactions (Caetano-Anollés and Gresshoff, 1996). ASAP cocktails were assembled as above except that primer and MgSO_4 concentrations were 9 μM and 4 mM, respectively. Each of the four minihairpin decamers contained at their 5' termini the constant GCGAAGC sequence (H) and had one of the following arbitrary 3' nucleotide sequences: GCA, GGT, CAT, and CTA. Thirty-five cycles of 30 s at 96 $^{\circ}\text{C}$, 30 s at 30 $^{\circ}\text{C}$ and 30 s at 72 $^{\circ}\text{C}$ were used for amplification. Twelve ASAP profiles were

generated using the products of three DAF reactions as templates for each of the four minihairpin primers. All reactions were repeated at least twice.

DAF and ASAP products were diluted 1:1 or 1:5 with sterile water and separated electrophoretically on 4.5% polyacrylamide gels backed on polyester film (Gelbond; FMC, Corp., Rockland, Maine) (Trigiano et al., 1995). DNA was visualized using a fast and sensitive silver staining method capable of detecting minute quantities of DNA (Bassam et al., 1991).

Silver-stained gels were examined on a light box and bands of 50 to 750 base pairs in length representing unordered genetic characters were scored as either present (1) or absent (0). Because of the complexity of the banding pattern, products of higher molecular weights were not entered in the data set. At least three gels for each ASAP reaction were compared to assess and ensure that profiles were consistent. In a few instances, some faintly stained products were inconsistently present from gel to gel and were not entered in the analysis. Phenetic analyses were performed using the numerical taxonomy and multivariate analysis system (NTSYS-pc; version 1.8, Exeter Software, Setauket, N.Y. For principal coordinate analysis (PCO), Jaccard similarity coefficients were calculated and squared genetic distances aligned using the double center option. Eigenvectors were then calculated from the transformed matrix using the square root (lambda) scaling option. The results were graphically displayed without normalization of scales. Data were also analyzed using unweighted pair group cluster analysis using arithmetic means (UPGMA). A genetic similarity matrix was computed and cluster analysis completed with options set from a maximum of 25 tied trees.

Theoretical calculation of the number of the arbitrarily amplified target sites was done using the methods of Chapco (1995) and Caetano-Anollés (1994).

Fig. 2. DNA amplification fingerprints (DAFs) and arbitrary signatures from amplification profiles (ASAPs) of chrysanthemum cultivars. (A) Fingerprints of the Charm series and outgroup generated by primer GAGCCTGT. Lane M = molecular weight in bp, 1 = 'Charm', 2 = 'Coral Charm', 3 = 'Dark Bronze Charm', 4 = 'Dark Charm', and 5 = 'Salmon Charm', 6 = 'Davis', and 7 = 'Pomona'. Note the monomorphic profiles among the cultivars of the 'Charm' series. (B) Reproducibility of three ASAP profiles. Replicate DNA samples obtained from different plants were subjected sequentially to DAF and ASAP analyses in independent experiments. Lane M = molecular weight in bp, 1 = profiles of 'Dark Bronze Charm' generated by GAGCCTGT $\times$ HCAT, 2 = profiles of 'Davis' produced by GTAACGCC $\times$ HCAT, and 3 = profiles of 'Charm' made by AATGCAGC $\times$ HGCA. (C) ASAP profile of the Charm series generated by AATGCAGC $\times$ HCAT. M = molecular weight in bp, 1 = 'Charm', 2 = 'Coral Charm', 3 = 'Dark Bronze Charm', 4 = 'Dark Charm', and 5 = 'Salmon Charm'. Arrowheads indicate polymorphic character loci.

Results and Discussion

DAF fingerprints of chrysanthemum obtained with standard octamer primers were clear and reproducible (Fig. 2A). The primers failed to detect any polymorphic regions in the genomic DNA of the closely related cultivars of the Charm family; all 87 character loci examined were monomorphic. Similar results for these primers were obtained by Scott et al. (1996) and lack of polymorphisms within different chrysanthemum families was also noted with RAPD decamer primers (Wolff et al., 1995). However, DAF using primers GATGCAGG and GTTACGCC produced six (2.2%) polymorphic characters for the Charm family, although not all cultivars could be distinguished from one another with unique products (Scott et al., 1996). Since all but one of these cultivars were selected as sports from an irradiated parent (Fig. 1), ionizing (gamma) radiation appears not to have significantly altered their genomes as targeted by individual primers and expressed in DAF patterns. However, changes within the primer-flanked sequences of the DAF amplicons were possible and were therefore examined with the ASAP technique.

Identical ASAP banding patterns were generated in separate experiments from DNA isolations that were collected from leaf tissue of different plants using conditions determined following iterative optimization of plant ASAP analysis (Caetano-Anollés and Gresshoff, 1996). Figure 2B shows reproducible ASAP patterns generated from independent DAF profiles originally produced from separate DNA isolations, with some minor differences in staining intensity. The number of scorable ASAP bands produced by the different primer combinations ranged from 27 to 40 with a mean of 32. ASAP revealed 142 polymorphisms out of a total of 384 character loci (Fig. 2C). The high levels of polymorphic DNA (37%) detected in this study are in contrast with a meager 4.4% polymorphic loci previously detected by six primers in four families including Charm (Scott et al., 1996). The 17-fold increase in detection of polymorphic DNA in this ASAP study of the Charm family compares favorably to that reported in the study of the relatively homogenous genome of *D. destructiva*, a fungus

that causes dogwood anthracnose (Caetano-Anollés et al., 1996). ASAP results lend support to the contention that the technique allowed similar identification of gamma radiation-induced and spontaneous sequence changes within amplicons and that these changes were not the result of chromosomal loss in all six homologues. This is because variation between radiation-induced and spontaneous mutants was observed with an invariant (allelic) set of DAF amplicons.

Among the polymorphic loci studied, 46 (32%) were diagnostic for specific cultivars (Table 1). Although a single combination of primers could not positively identify all five cultivars in the Charm family, two combinations, GAGCCTGT × HGCA and AATGCAGC × HCAT, could unequivocally distinguish four of them. A minimum of two primer pairs were necessary to differentiate all of the members of the family. In a previous DAF study of chrysanthemum, only 11 unique polymorphisms were generated using 6 primers examining 16 cultivars in 4 families. Furthermore, three to six DAF primers were necessary to uniquely identify all cultivars of families composed of either three or five members, respectively (Scott et al., 1996).

Phenetic analysis of DAF data using UPGMA algorithm clearly established the very close genetic relationship between cultivars belonging to the Charm family (Fig. 3A). However, a better elucidation of these relationships was made possible by the increased number of informative characters generated by ASAP, using cluster (Fig. 3A) and principal coordinate analyses (Fig. 3B). The consistent generation of monomorphic DAF profiles revealed an unusually high level of genetic homogeneity. In contrast, ASAP analysis was able to characterize each and every one of the cultivars examined (Table 1). However, phenetic relationships failed to correlate with the individual origin of the cultivars. For example, irradiation was expected to produce greater changes in sequence than spontaneous mutation. Wolff (1996) detected the absence of a single RAPD band in the two cultivars of a family that were derived by irradiation and suggested that irradiation may induce major chromosomal aberrations and even loss of chromosomal segments. Nevertheless, in our study, the closest individual

Table 1. Uniquely identifying arbitrary signatures from amplification profile (ASAP) markers for cultivars in the Charm family of chrysanthemum.

ASAP primer combinations ²	P/T ³	Cultivars in the Charm family				
		Charm	Coral Charm	Dark Bronze Charm	Dark Charm	Salmon Charm
GAGCCTGT	0/34	---	---	---	---	---
× HGCA	9/28	175 ^w , 150	200	310	380, 300	---
× HGGT	11/37	450, 200	---	675	---	140
× HCAT	17/32	700, 290, 185, 150	---	---	---	---
× HCTA	18/37	625, 175	700, 385, 290	---	750	---
GTAACGCC	0/26	---	---	---	---	---
× HGCA	7/30	---	---	---	---	---
× HGGT	9/29	---	300	---	---	140
× HCAT	7/29	---	260	700, 190	---	---
× HCTA	11/28	525, 300, 295, 120	---	---	---	405
AATGCAGC	0/27	---	---	---	---	---
× HGCA	21/40	450	620, 390, 275, 130	110	---	---
× HGGT	9/31	---	---	175	---	---
× HCAT	7/29	---	130	310	155, 140	320, 160
× HCTA	15/32	---	210	280, 130	---	---

²All primer sequences are 5' to 3'. Minihairpin primers have the core sequence GCGAAGC (H).

³P/T = number of polymorphisms/total number of characters.

^{*}Unique products not found using this primer or primer combination.

^wNumber equals bp in length of unique product.

Fig. 3. Cluster and principal coordinate analyses of the relationship between cultivars of the Charm series. (A) UPGMA analysis of DNA amplification fingerprint (DAF) and arbitrary signatures from amplification profile (ASAP) data. Note that the analysis using ASAPs more clearly delineated the relationship between the cultivars. (B) Graphic representation using principal coordinate analysis.

to the parent 'Charm' at the genetic level was the radiation-induced mutant 'Coral Charm'. In contrast, the spontaneous sport, 'Dark Bronze Charm', which was expected to be the most similar to 'Charm', was relatively distant in comparison. These results show there is no selective influence of irradiation on the genetic makeup of DAF fingerprints. Since our DAF studies with over 270 character loci, potentially distributed 5 per chromosome, showed genetic homogeneity (Scott et al., 1996), the ASAP data suggests that, at best for the Charm family and the few sports examined, sporting and somaclonal variation produces a sizable number of mutations. These mutations result predominantly from discrete changes of sequence that give rise to new amplification products capable of uniquely identifying individual sports. Furthermore, mutations cannot be attributed to gross chromosomal aberrations or rearrangements within the regions of DNA examined, at least expressed concordantly in the hexaploid chromosomal set.

The ASAP technique detected nucleotide changes between the parent and each individual sport within an average of 18,815 base pairs (bp) of DAF sequence, basically composed of 50- and 3,000-bp fragments with most of them falling in the 100 to 3000 bp range (Fig. 4). These changes were expressed as an average of 5.7 ± 2.9 (SD) DNA polymorphisms per ASAP primer in individual parent-sport combinations (Fig. 4). This represents a minimum of one nucleotide change detected by each ASAP primer in 3,300 bp of

DAF sequence. This value is comparable to the gamma radiation-induced mutation rates (0.02%) observed for example in the *Triplo-lethal* locus of *Drosophila melanogaster* Meigen (fruit fly) (Keppy and Denell, 1979). However, the number of sites targeted by the minihairpin primers is limited and can be calculated to be as low as 6 or as high as 127, assuming that the fractional GC content of DAF fragments is average (40%) and the system tolerates 5 or 4 bp mismatching at the primer 5' termini (as shown by the amplification of plasmid DNA; Caetano-Anollés and Gresshoff, 1994), respectively. Therefore, while minihairpin primers have the potential to target the overall DAF product sequence, the actual generation of an average of 32 ASAP character loci indicates there must 4.5 bp mismatching occurring at the primer 5' termini. Under this scenario, a maximum mutation rate can be calculated to represent one nucleotide change per 62 bp of DAF product sequence. This maximum value compares with the 1% nucleotide differences observed in the analysis of genomic clones containing distinct white gene alleles of *Anopheles gambiae* Giles (mosquito), and 90% of which were confined to noncoding regions (Besansky et al., 1990). This value is also consistent with the high rates of phenotypic radiation-induced somatic mutation in the *dumpy* locus of the fruit fly (0.1% to 0.2% at 2000 rad doses) (Inagaki et al., 1974) and in *Tradescantia ohiensis* Raf. stamen hairs (3.4 to 3.8×10^{-4} mutations from blue to pink hair/rad; i.e., 68% or about less than one mutation every two events at 2000 rad doses) (Ichikawa, 1972). The high number of possible nucleotide changes in DAF sequence, somewhere within the 0.03% to 1.6% range, can arise from either base substitutions or strand inversions, but not from deletions or insertions in these amplified regions. Point mutations could result from direct oxidative damage to bases or from double-strand breaks, believed to occur with increased frequency when DNA is subjected to hydroxyl radicals produced from the interaction of ionizing radiation with water (Britt, 1996). While our analysis insinuates high incidence of point mutations, a detailed

Fig. 4. Distribution of fragment length of DNA amplification fingerprint (DAF) products used to evaluate mutation frequency in chrysanthemum. The closed circles below the bars indicate the occurrence of arbitrary signatures from amplification profile (ASAP) polymorphisms within DAF fragments of that size range. Products >1000 bp were not included, but were sparsely distributed.

characterization of radiation-induced mutations in *Arabidopsis* flavonoid biosynthetic genes showed that they resulted preponderantly from chromosomal rearrangements (Shirley et al., 1992). This discrepancy may represent disparities between coding and noncoding genomic regions, believed to be subjected to different selective evolutionary pressures.

While the average number of ASAP polymorphisms per minihairpin primer strongly correlated with the total length of DAF products by linear regression ($R^2 = 0.998$) (Fig. 4), no differences in mutation rates were observed between the individual mutants (one-way ANOVA, $F = 0.093$, $p > 0.963$). This again indicates that radiation-induced and somatic mutations occur at similar paces in chrysanthemum, when expressed as discrete changes in DNA sequence.

Our calculated mutation rates in chrysanthemum cannot be explained by primer nesting. Based on sequence analysis of minihairpin primer amplification of subgenomic DNA (Caetano-Anollés and Gresshoff, 1994), fingerprinting of plasmid pUC18 (2,686 bp) with primer GCGAAGCCTG produced products that resulted from primer annealing to 12 sites, 67% of which were used more than once to produce nested amplification products. In contrast, amplification of plasmid pBR322 (4,363 bp) involved primer annealing to 23 sites, 22% of which produced nested amplicons. Regression analysis of template size versus primer nesting indicated that ASAP products generated from the average 18,815 bp DAF template cannot result from nested amplification events. Therefore, our putatively high calculated mutation rate can only be the result of nucleotide changes within the DAF sequence analyzed, combined with perhaps the way how ASAP selects for amplification sites and is influenced by amplification kinetics.

This study demonstrates that ASAP is well-suited for distinguishing radiation-induced mutations and spontaneously occurring sports and has provided a preliminary estimate of the mutation rates induced by gamma radiation in selected chrysanthemum cultivars. Unique amplification products can be reliable and efficiently produced using only a very few primer combinations. The ability to unequivocally identify individual cultivars will be useful in proprietary protection, marker assisted breeding and genetic mapping applications.

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