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Variation in the Phenotypic Features and Transcripts of Color Mutants of Chrysanthemum (*Dendranthema grandiflorum*) Derived from Gamma ray Mutagenesis

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We investigated the structural genes and their transcripts for anthocyanin synthesis in *Dendranthema grandiflorum* 'Argus'. Color variations in chrysanthemum mutants were obtained through gamm ray irradiation to regenerated plants from an *in vitro*. Normal florets were pinkish, but the mutants had white or purple ray florets and white, purple, or yellow-green disc florets. Irradiation modified both flower size and the number of ray florets. Compared with the control, levels of total anthocyanins in the mutants ranged from 4 times lower to 6 times higher for the disc florets. This disparity was even more evident, up to 14-fold greater, in the ray florets. Expression of the CHI, F3'H, F3'5'H, DFR, and LDOX genes varied among the mutants, but no dramatic changes were detected in CHS and F3H transcripts in either leaf or floret tissues. Sequence homology to known anthocyanin genes from other plant species was 61 to 84%, 62 to 74%, and 71 to 76% for CHI, F3'H, and LDOX, respectively. Our results support the proposal that such radiation-induced mutations in genes within the anthocyanin pathway are associated with variations in chrysanthemum flower color.

Keywords: anthocyanin pigments, chrysanthemum, flower color, gamma ray irradiation, mutagenesis

One method for obtaining new cultivars of ornamental plants is mutation breeding (Ahloowalia and Maluszynski, 2001; Broertjes et al., 1976; Zalewska and Jerzy, 1997). This technique is especially efficient for creating sterile, inter-specific hybrids (Miyazaki et al., 2006); native ornamentals with limited gene pools in a given species (Maluszynski et al., 1995); or plants with long juvenile periods before flowering and seed production begin (Predieri, 2001). Because the mutagens employed can cause large DNA alterations that encompass transversion as well as transition, those derived mutants have become increasingly important to functional, structural, and comparative genomics (Emmanuel and Levy, 2002; Levin et al., 2004). Thus, such an approach continues to be a viable tool in plant improvement and genetics studies (Ahloowalia and Maluszynski, 2001; Sanjay, 2007; Schum, 2003).

More than 2500 mutant varieties have now been registered, with 625 representing ornamental and floral plants. Among those major flowering species are 267 varieties of chrysanthemum (http://www-mvd.iaea.org). Physical radiation, e.g., from gamma rays and X-rays, has been widely used for inducing mutations (Jain, 2005; Park et al., 2007), and several physical ion beam mutagens (i.e., heavy ions or fast neutrons) have been reported to induce a wider spectrum of mutants in flowering plants (Miyazaki et al., 2006; Okamura et al., 2003).

Three major pigments (betalain, carotenoid, and anthocyanin) are responsible for visual reflection of flower color (Grotewold, 2006). Among them, anthocyanin is associated with the majority of orange, red, purple, and blue hues (Kim et al., 2007). Betalain and anthocyanin pigments rarely coexist in a particular plant species, and are more likely to be mutually excluded, whereas the anthocyanin and carotene pigments can occur together (Grotewold, 2006; Kimler et al., 1970). A mutation in the biosynthetic pathway of structural or regulatory genes causes a change in flower color (Nakatsuka et al., 2005). When the blockage occurs in the early steps of anthocyanin synthesis, white flowers result; a blockage in later steps leads to different flower colors because of the accumulation of a particular anthocyanin (Mato et al., 2000).

Researchers have attempted to correlate the phenotypic characteristics of mutants to the particular physical, chemical, or biological mutagens used as well as to the doses that were applied by which those inductions were originally prompted. Further investigations into the molecular changes in genes of interest can facilitate our understanding, at the genomic level, of the phenotypic features when such induced mutants are available. Therefore, our objectives here were to characterize flowering features quantitatively, and to verify the difference in expression by target genes that control anthocyanin pigmentation among chrysanthemum mutants derived through gamma ray irradiation. This we combined with an *in vitro* culture approach.

MATERIALS AND METHODS

Plant materials and morphological evaluation

We have previously described our methods for mutant induction and gamma ray irradiation treatment, with the flower-color mutants used in this study selected from that earlier mutant population (Park et al., 2007). Briefly, stem segments of *Dendranthema grandiflorum* (Ramat.) Kitam. 'Argus', with purplish flowers in a white background (Figure

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Table 1. Degenerate primers designed from the conserved regions of different plant species for reverse-transcription (RT) analysis.

Gene	Primer set	PCR conditions	
	Forward (above) and reverse (below) sequences	Annealing temperature (°C)	Extension time
CHS	5′-ATG GCT TCC TTA ACT GAC ATT GC-3′ 5′-TTA TGC AAC CGA TAT AGT GGT TGG-3′	56	1 min 20 s
CHI	5′-TCC THG SHG GYG CWG GKS WKA GR-3′ 5′-GGR GAA ACD SCN TKC TYB CCR AT-3′	62	30 s
F3H	5′-ATG GAC GAT AAT TCG CTG CAT G-3′ 5′-CTA AGC CAA GAT ACT TTC AAT G-3′	59	1 min
F3′H	5′-TVG GAA ACY TNC CNC AYM TSG GC-3′ 5′-GGR TCW CGR GMW ATG GCC CAH AY-3′	62	1 min 10 s
F3′5′H	5′-GAY ATG GTK GTD GAG YTV ATG AC-3′ 5′-TYC CAR TCA AAD GMD TGM AYC AA-3′	54	1 min
DFR	5′-GTG GCC ACT CCT ATG GAC TTT GA-3′ 5′-GAA GTC GTC TAA GTG CAC GTA TT-3′	56	1 min 20 s
LDOX	5′-CAG CTB GAR TGG GAR GAC TAY TT-3′ 5′-CTC YTT NGG HGG CTC RCA RAA AAC-3′	57	30 s
Actin	5′-TGG GAT GAT ATG GAG AAA ATC TGG-3′ 5′-ATC GGC TAT GCC GGG GAA CCT AGT-3′	59	50 s

1), were used to regenerate plants on a Murashige and Skoog (MS) medium containing 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} Kinetin (Park et al., 2007). Regenerated plants were irradiated with 30, 40, or 50 Gy of gamma rays. After the plants were acclimatized following four courses of sub-culturing, they were propagated vegetatively and grown under natural conditions in a plastic greenhouse. In the ensuing two-year period, no other cuttings were made for propagation. Color mutations were individually marked in the greenhouse, and five mutants that were visually distinguished as having inherited and maintained that mutated color over those two years were sampled. Their data were recorded for flower color and morphological traits, including flower diameter and number of ray florets at the full-bloom stage. We assigned the colors for ray and disc florets based on the code from the Color Chart of the Royal Horticultural Society (RHS; London).

Extraction and Quantification of Total Anthocyanin

Ray and disc florets were collected in separate tubes containing liquid nitrogen, then transferred to the laboratory where samples were immediately ground and stored at –80°C. Their anthocyanin pigment was extracted overnight at 4°C in a solution of 7% HCl (v/v) in methanol (Nissim-Levi et al., 2007). After the crude extracts were centrifuged at 15,000 rpm for 15 min, the optical density of the supernatants was measured at 530 and 657 nm with a spectrophotometer (UVIKON 923, Bio-Tek, USA) before relative anthocyanin concentrations were calculated as described by Martin et al. (2002).

RNA preparation

Leaf and floret tissues were collected from the mutants and the original 'Argus' control. Total RNA was isolated with Trizol® reagent according to the manufacturer's instructions (Invitrogen, Carlbad, CA, USA). All extracted RNA was treated with RNase-free DNase I at 37°C for 3 h (Promega, Madison, WI, USA) to digest contaminant DNA. The yield of total RNA was quantified with a Nanodrop (NanoDrop Technologies, USA).

For RT-PCR analysis, we designed sets of primers by using *in silico* information on chrysanthemum for chalcone synthase (CHS; NCBI Accession No. DQ521272), dihydroflavonol 4-reductase mRNA (DFR; NCBI Accession No. EF094936), and flavanone hydroxylase mRNA (F3H; NCBI Accession No. U86837). Because other genes involved in the anthocyanin pathway have not yet been reported for chrysanthemum, their degenerated primers were designed based on the most conserved regions known in other plant species to amplify chrysanthemum-specific cDNA fragments (Table 1).

For reverse transcription, 1 μg of DNase-treated total RNA was subjected to cDNA synthesis in 5 μL of reaction mixture incubated at 42°C for 60 min. This was followed by amplification with a mixture containing RT 1X buffer, 1.25 U of AMV reverse transcriptase, 5 U of RNase inhibitor, 1 mM dNTP, 5 mM $MgCl_2$, and 0.5 pmol gene-specific primer. The resulting cDNA served as templates for subsequent PCR amplification using primers specific for the anthocyanin structural genes. PCR conditions were modified for those individual genes depending on the amplification length and base compositions (Table 1). Differential display of reverse-transcribed amplifications was carried out on 1% agarose gels, fused into a T&A cloning vector (Real Biotech Corporation, USA), then sequenced to confirm amplification of the predicted genes. Multiple sequence alignments were finally made to determine homology similarity, using a CLUSTALW

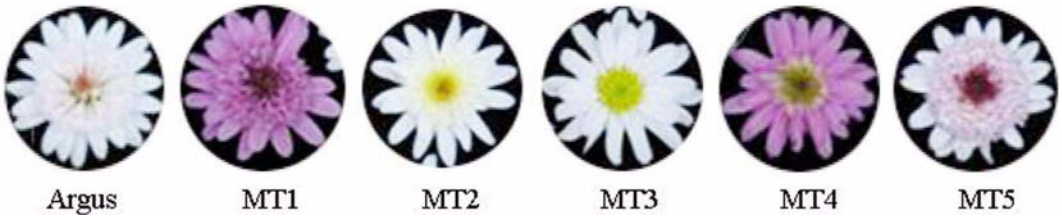

Figure 1. Variations in color of ray and disc florets from wild-type 'Argus' control and five mutants.

Table 2. Phenotypic features of selected chrysanthemum mutants and original cultivar 'Argus'.

	Diameter of whole flower (cm) [a]	Diameter of disc floret (cm)	Number of ray florets	RHS index of flower color	
				Ray floret	Disc floret
'Argus'	4.1 ± 0.1[b]	2.1 ± 0.1	21.4 ± 0.2	69-D	65-B
MT1	4.4 ± 0.2	2.4 ± 0.1	21.0 ± 0.5	72-C	60-A
MT2	4.5 ± 0.2	2.4 ± 0.2	19.4 ± 0.2	69-D	150-A
MT3	4.0 ± 0.1	1.4 ± 0.1	19.8 ± 0.3	155-B	158-B
MT4	4.3 ± 0.1	1.5 ± 0.1	26.4 ± 1.9	72-C	13-B
MT5	4.5 ± 0.1	2.6 ± 0.1	20.8 ± 0.2	69-D	59-A

[a]Comprises disc (inside) and ray (outside) florets
[b] Mean (n=5) ± SE

program.

RESULTS

Variations in Flower Color and Morphology

Color variations were found in flowers from mutants of 'Argus' chrysanthemums (Figure 1; Table 2). After consecutive propagation events, all mutants were found to inherit and maintain a mutated flower color and shape that was distinguishable from the original. 'Argus' control flowers had light-pink disc and ray florets while the mutants produced white and purple ray florets and white, purple, and yellow-green disc florets (Figure 1). Except for Mutants 3 and 4, all others had wider flowers. Diameters of the disc florets from Mutants 3 and 4 were statistically smaller than the control. Mutant 4 had statistically more ray florets than all other mutants and the original. RHS indices also indicated that the ray colors from Mutants 1, 3, and 4 differed from 'Argus' while those of Mutants 2 and 5 were the same (Table 2). All of these mutants produced new disc colors that were unique from the original.

Differences in Anthocyanin Contents

Total anthocyanin pigments in the disc and ray florets differed among 'Argus' and our color mutants (Figure 2). A quantitative comparison showed that the pinkish or purplish mutants, either in the disc or ray florets, accumulated more anthocyanin while white-flowered individuals had lower detectable amounts, as would be expected based on their colors. In the disc florets, total anthocyanin levels for the mutants ranged from 4 times lower to 6 times higher compared with 'Argus'. In the ray florets, those relative levels were more evident, with a difference of up to 14 times more.

Sequence comparisons among 'Argus' and flower-color mutants

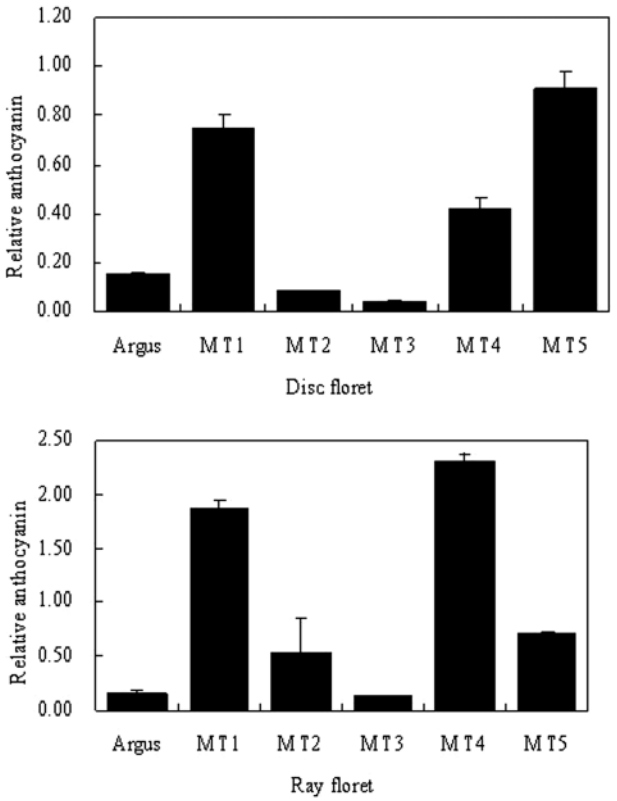

Figure 2. Total anthocyanin content in ray or disc florets from chrysanthemum color mutants.

Our visualized reverse transcriptions of mRNA indicated that the anthocyanin structural genes were expressed differently depending on tissue or mutant type (Figure 3). Degenerate primers designed from the conserved regions of other species were used to obtain chrysanthemum-specific cDNA

Table 3. Sequence homologies of partial fragments from anthocyanin-synthesizing genes of chrysanthemum compared with other plant species.

Gene	Common name	Partial cDNA sequence		Homology (%)
		Length (bp)	Accession no.	
CHI	Chrysanthemum	500	-	-
	Verbena	650	AB234907	67
	Petunia	654	AF233637	67
	Camellia	660	DQ120521	72
	Eustoma	651	AB078955	69
	Saussurea	654	AF509335	84
	Callistephus	654	Z67980	84
	Lotus	636	AJ548840	61
	Soybean	645	AF276302	64
F3'H	Chrysanthemum	1100	-	-
	Osteospermum	1235	DQ250711	74
	Gerbera	1223	DQ218417	71
	Soybean	1229	AB061212	62
	Ipomoea	1250	AY333419	64
	Arabidopsis	1226	NM_120881	63
LDOX	Chrysanthemum	500	-	-
	Carrot	942	AF184274	76
	Grape	948	X75966	74
	Eustoma	936	AB078959	73
	Perilla	948	AB003779	74
	Arabidopsis	930	AK226417	71

fragments. The mRNA of CHS, CHI, F3H, F3'H, F3'5'H, DFR, and LDOX genes was amplified by 1.2, 0.5, 1.1, 1.1, 0.8, 1.2, and 0.5 kb, respectively, with an average of 843 bp. Sequence analysis of those partial fragments indicated that the chrysanthemum color genes were sequentially and highly homologous to known sequences from other plants, with ranges of 61 to 84%, 62 to 74%, and 71 to 76% for CHI, F3'H, and LDOX, respectively (Table 3). Those sequences for LDOX and ANS were identically aligned, and we considered them to be the same as those explained previously (Marles et al., 2003; Turnbull et al., 2004).

Expression Patterns for Anthocyanin Pathway Genes in Flower and Leaf Tissues

We compared expression patterns for anthocyanin structural genes (CHS, CHI, F3H, F3'H, F3'5'H, DFR, and LDOX) among the mutants and found that they varied depending on the gene and tissue used (Figure 3). Some, such as CHS and F3H, showed no difference in expression while CHI, F3'H, F3'5'H, DFR, and LDOX were either enhanced or repressed among the mutants. In addition, genes such as CHI, F3'H, and LDOX were not amplified (i.e., in Mutant 5, Mutant 4, and Mutants 4 and 5, respectively; Figure 3).

DISCUSSION

Irradiation-induced changes in flower color and shape were diverse in this study: white, red-purple and yellow-green disc florets; ivory to white, red-purple, and red ray florets; and variations in the lengths and widths of ray florets. When 50 Gy of gamma rays was applied as the mutagen, 17% of all individuals differed from the 'Argus' control in their flower color and shape (Park et al., 2007). a gamma ray irradiation has previously been proven to be useful for inducing color changes in chrysanthemum (Mandal et al., 2000).

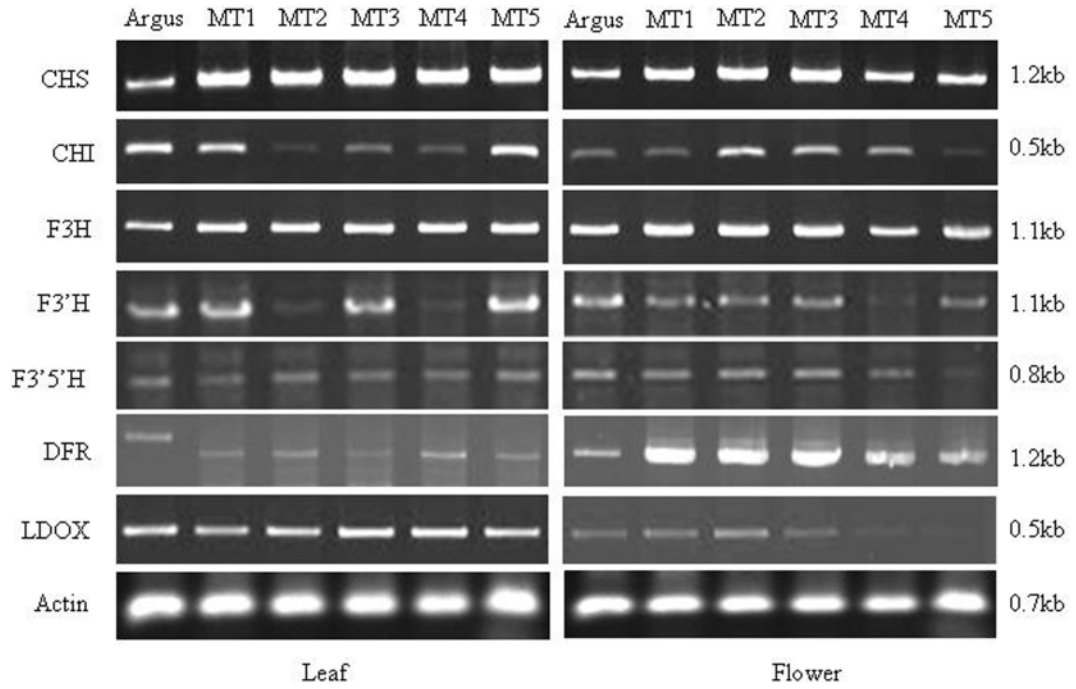

Figure 3. Transcriptional pattern of genes involved in anthocyanin pathway in leaf and flower tissue of 'Argus' (control) and five color mutants.

Materials that were vegetatively propagated maintained their mutated colors and shapes over consecutive cuttings (Park et al., 2007). The anticipated bottleneck when using radiation, including gamma rays, ion beams, and X-rays, to induce mutants in ornamental species is a reversion of those chimeric traits to the original form when the original and mutated cells co-exist (Mandal et al., 2000; Sun et al., 2007). Interestingly, Mutants 2 and 5, where RHS indices for ray florets were the same as for 'Argus', had higher total anthocyanin contents than did the control. Therefore, this demonstrates that screening of color mutants that relies on either an RHS color chart or total anthocyanin content alone is not enough, but that both tools are necessary for clear evaluation.

A suppression or deficiency in the LDOX gene (equivalent to ANS) is associated with a white-colored mutant from a naturally blue gentian plant (Nakatsuka et al., 2005; Turnbull et al., 2004). By comparison, we detected mRNA of LDOX in the leaves and flowers from Mutants 2 and 3, which had white ray florets and yellowish disc florets (Figure 3). However, Mutants 2, 3, and 4, in which yellowish disc florets were common, showed reduced transcripts in their leaves but enhanced mRNA in their flowers for encoding CHI, a regulation gene involved in an early step in the anthocyanin pathway (Mato et al., 2000).

Floral pigments in a pink-colored *Torenia* mutant from the original blue plant exhibit extremely greater amounts of peonidin and cyanidin derivatives for red hues, which results from the strong expression of F3'H and DFR in the anthocyanin pathway (Miyazaki et al., 2006). Our Mutants 1 and 5, with higher total anthocyanin contents in the disc florets, had more mRNA for F3'H in the leaf tissue but not in the flower (Figure 3). Because more than one copy of the anthocyanin genes exists in many plant species, some transcripts of those copies might convey an erroneous association with flower coloration (Holton and Cornish, 1995; Jaakola et al., 2002).

DFR is involved in the accumulation of all three major anthocyanins (cyanidin, pelargonidin, and delphinidin). Therefore, we presumed that the five mutants used here, which exhibited greater or lower amounts of those anthocyanins (Figure 2), had similar levels of the mRNA encoding for DFR (Figure 3) (see also Grotewold, 2006).

Likewise, increased activity for F3'5'H is associated with the predominant synthesis of delphinidin derivatives for lilac to blue hues (Jeong et al., 2006; Seitz et al., 2007). Based on the limited blue-colored flowers from our mutants, such delphinidin derivatives are not likely to predominate. This is further supported by the consistent mRNA levels of F3'5'H among those color mutants (Figure 3). A red discoloration in our transgenic chrysanthemums is, however, is associated with co-expression of F3'H and F3'5'H, as found in Mutants 2 and 3 (Seo et al., 2007).

Ionizing radiation, including from gamma rays, induces fragment deletions or insertions that eventually lead to changes in amino acids and a modification of leaf and stem pigmentations (Shikazono et al. 2003). In addition to the structural genes involved in the anthocyanin biosynthetic pathway, mutations in other regulatory genes or the involvement of transposable elements, e.g., MELSs (mobile element-like sequences) and DRs (direct repeats), might be associated with variations in flower coloration (Hoshino et al., 2001; Kim et al., 2007). For example, in yellow-flowered chrysanthemums, transcripts of a carotenoid cleavage dioxygenase (CCD) gene that accounts for white flowers are accumulated in extremely low amounts. Such a response is a case of less association by a structural gene involved in carotenoid biosynthesis (Ohmiya et al., 2006).

Environmental stresses such as cold can cause pigmentation accompanied by anthocyanin accumulations in the petals or leaves of some species (Christie et al., 1994; Nakatsuka et al., 2005). However, in our study, variations in flower color are thought to have been caused by a mutation in a structural or regulatory gene involved in the anthocyanin biosynthesis pathway, rather than because of environmental factors. Our selected mutants were maintained under greenhouse conditions to avoid extremes that can cause light or temperature stresses. Therefore, all of our results demonstrate that the targeting flower color and shape of selected chrysanthemum plants are inherited from mutated origins.

Among these mutants, transcript profiles indicated that various color features are associated with different mRNA levels for CHI, F3'H, F3'5'H, DFR, and LDOX -- all genes involved in anthocyanin synthesis. Therefore, we assume that a mutation in one or some pathway genes should stop or detour to the downstream pathway, eventually resulting in variations in flower color. We must still investigate the genomic structure of those genes to verify whether their expression patterns result from gain- or loss-of-function because of gamma ray mutagenesis. Although coloration can be re-directed in a few plant species by a transgenic technique that introduces some isolated genes (Shimada et al., 2001), a physical mutagen, e.g., gamma ray radiation, also is effective for acquiring a unique or complicated pigmentation of mutants with favorable morphologies. Such efforts can then meet the increasing demands for diverse color features in horticultural industries.

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