Isolation of a *Leucoanthocyanidin Dioxygenase (LDOX)* Gene from a Spray-type Chrysanthemum (*Dendranthema × grandiflorum*) and Its Colored Mutants

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**Abstract.** A full-length cDNA and genomic DNA of a *leucoanthocyanidin dioxygenase (DgLDOX)* gene was isolated from the petals of chrysanthemum ‘Argus’, and comparative features of the gene among three flower color mutants derived from a gamma-ray mutagenesis were characterized. The cDNA coding region of the gene was 1068 bp and was translated into 356 amino acids accordingly. The genomic DNA size was 1346 bp for ‘Argus’, while three mutants revealed ranges of 1363 to 1374 bp. A single intron between two coding exons for the *DgLDOX* gene was found, of which size was 112 bp for ‘Argus’, but 128 or 137 bp for three flower color mutants, indicating that a genomic insertion in the intron occurred during the gamma-ray mutagenesis. DNA blot analysis revealed the *DgLDOX* gene presenting as a single copy in the chrysanthemum genome. The *DgLDOX* gene was expressed in both ‘Argus’ of light-pink color and two purple color mutants (AM1 and AM3) but had very weak expression in only white color mutant (AM2). The results demonstrated that variations in the flower color of the mutants might be associated with changes in the amino acid moieties in the coding exons or fragment insertions in the intron of the *DgLDOX* gene, which potentially resulted in less expression of the gene in the white colored mutant.

**Additional key words:** anthocyanin, flower colored mutant, full-length cDNA, gamma-ray mutagenesis, RACE

**Introduction**

Chrysanthemum is the second most traded cut-flower following rose among the ornamental plants in the flower market in Korea, and is available in a wide range of flower color, flower type, and plant size. Three major pigments (betalain, carotenoid, and anthocyanin) constitute the natural display of the flower color (Grotewold, 2006). The anthocyanin pigments are responsible for the majority of the orange, red, purple, and blue colors of flowers and their biosynthetic pathways are well studied in other plant species (Harborne and Baxter, 1999; Tanaka et al., 2005).

Mutation breeding by a radiation has been widely utilized to improve well-adapted plant varieties by modifying one or two major traits, or to develop new varieties with improved agricultural characteristics (Sudhir et al., 2006; van Harten, 1998). A mutation in the biosynthetic pathway of the structural or regulatory genes results in changes in various target traits including flower color (Hongmei et al., 2009; Mol et al., 1998; Nakatsuka et al., 2005). Mutation blocking in the early steps of the anthocyanin pathway results in white flowers; a blockage in later steps leads to different colors of flowers through an accumulation of a particular anthocyanin (Mato et al., 2000).

Previously, some genes (3MaT, F3H, F3’H and F3’5’H) related to the anthocyanin biosynthesis were characterized in chrysanthemum and *Osteospermum*. (Kim and Park, 2002; Seitz et al., 2006; Suzuki et al., 2004) and transformation efforts have been made in the Asteraceae family (Lema-Rumińska and Zalewska, 2005; Seo et al., 2007). Natural mutants for a flower color have been selected from wild genetic resources of chrysanthemum, but nowadays artificial mutagens such as ionizing radiations or chemicals are preferred (Datta et al., 2005; Lema-Rumińska and Zalewska, 2005; Nagatomori et al., 1995; Park et al., 2007). Flower color mutants have been used for characterization of genes and regulatory

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Received 29 April 2010; Accepted 5 July 2010. This study was supported by the Biogreen 21 Program, Rural Development Administration (RDA), Korea (Code No. 20070301034033) and from the Nuclear R&D Program, Ministry of Education, Science and Technology (MEST), Korea.
factors related to anthocyanin synthesis (Martin et al., 1993). In most cases, visible changes are caused by differences in the amount and components of flower pigments (Nakatsuka et al., 2005).

The \textit{LDOX} (also known as anthocyanidin synthase - \textit{ANS}, E.C. 1.14.11.19) is a 2-oxoglutarate-dependent oxygenase enzyme that catalyses the conversion of leucoanthocyanidin to anthocyanidin, an essential step in the formation of colored metabolites in the anthocyanin biosynthesis (De Carolis and De Luca, 1994; Nakajima et al., 2001). The \textit{LDOX} is a direct precursor enzyme of one of the most conspicuous flavonoid classes, anthocyanins, which are found in fruits, flower petals, and leaves exhibiting a wide range of functions such as the attraction of pollinators and seeds dispersers, UV light damage protection, and a plant defense against a pathogen attack (Lucheta et al., 2007; Tanaka et al., 2002). Many reports have identified and characterized the \textit{LDOX} gene isolated from arabidopsis, peach and carrot plants (Hirner et al., 2001; Ogundiwin et al., 2008; Pelletier et al., 1997), but the \textit{LDOX} gene of chrysanthemum (designated as \textit{DgLDOX} hereinafter) has not been reported. The objectives of this study, therefore, were to isolate the \textit{DgLDOX} gene from a spray-type chrysanthemum ‘Argus’ and to characterize structural changes of DNA sequence and differential expression pattern of the gene in flower colored mutants. This is the first report on the \textit{DgLDOX} isolation and structural comparisons associated with the shifting of flower color in the chrysanthemum mutants induced by a gamma irradiation.

\section*{Materials and Methods}

\subsection*{Plant material}

A spray-type chrysanthemum \textit{[Dendranthema grandiflorum (Ramat.) Kitam.]} ‘Argus’ was used in this experiment. Three flower color mutants were initially induced by a 40 Gy (AM1 and AM2) and 50 Gy (AM3) gamma-irradiation on regenerated plantlets under in vitro conditions (Park et al., 2007). After acclimatization of the plantlets following the culture in a controlled room, plants were propagated vegetatively and grown for two years under natural conditions in a plastic greenhouse in Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute. Three flower color mutants (AM1, AM2, and AM3) with visually distinguishable inherited color mutation for two years were used for the \textit{DgLDOX} isolation and structural comparisons of gene structure and expression pattern with the original plant ‘Argus’ (Fig. 1A).

\subsection*{Extraction and analysis of total Anthocyanin}

Ray florets of the ‘Argus’ and three mutants were ground in a liquid nitrogen, and then crude extracts were induced by overnight at 4°C in a solution of 7% HCl (v/v) in methanol (Nissim-Levi et al., 2007). Absorption of the extracts at wavelengths of 530 and 657 nm was determined by using a spectrophotometer (UVIKON 923, Bio-Tek, USA), and the resulting absorbance value was normalized to the fresh weight for each sample. At least three independent measurements were performed to obtain representative average of the anthocyanin contents.

\subsection*{RNA extraction and Cloning of full length cDNA of \textit{DgLDOX}}

Total RNA was prepared from the ray florets of the ‘Argus’ and three flower color mutants by using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcription polymerase chain reaction (RT-PCR) was performed by using Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara, Shiga, Japan) according to the manufacturer’s instruction. To obtain the \textit{DgLDOX} gene sequence, the partial fragment was amplified with a degenerated primer (LDOXdeF: 5’-CAG CT(T/G/C) GA(A/G) TGG GA(A/G) GAC TA(T/C) TT-3’; LDOXdeR:: 5’- CTC (T/C)TT (A/T/G/C)GG (A/T/C)GG CTC (A/G)CA (A/G)AA AAC -3’), which was designed from the conserved DNA sequences of several plants including gentian, grape, arabidopsis, perilla and carrot by using a ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2).

The partial fragment about 500 bp length from the amplified sequence was subsequently cloned into an RBC T&A cloning vector and HIT competent cells (RBC Bioscience, Taipei, Taiwan), and sequenced extensively. To determine the 3’-end of a sequence of the \textit{DgLDOX} gene, a rapid amplification
of 3'-cDNA ends (3'-RACE) was performed with a primer (LDOX3race: 5'-AGC ATC CTA CAT AGA GGA CTT GT-3') and oligo dT primer by using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara), which allowed a 400 bp extended sequence of DgLDOX cDNA from the chrysanthemum. A 5' rapid amplification of cDNA ends (5'-RACE) based on the designed primer (LDOX5race: 5'-CTC AAG TCC TAA GCC GGA C-3') was performed to determine the full-length cDNA sequence with a SMART™ RACE cDNA Amplification Kit (CLONTECH Laboratories, USA) according to the manufacturer’s instruction, which allowed a 500 bp extended sequence. Each full length RT-PCR (1068 bp) was performed with a primer as follows: LDOXcDNA-F: 5'- ATG GTG ATT CCA ACA ACA AG -3'; DgLDOX-R: 5'-TCA TTT TGG GTC AAC GGA ATC AC -3' by using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara) and cloned into an RBC T&A cloning vector (RBC Bioscience, Taiwan), and the fully amplified fragments were sequenced. All Sequencing was performed by using an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster, USA). The obtained sequences were compared with sequence database using BLASTN and annotated on the basis of the exiting annotation of non-redundant databases at the NCBI. Sequence analysis and alignments of the wild type and three mutants were performed by using GENETYX-WIN Software Ver. 5.0 (Genetyx, Tokyo, Japan) and a ClustalW2.

Isolation of the genomic DNA sequence of DgLDOX

A genomic DNA was isolated from the flower tissues of ‘Argus’ and three flower color mutants by using a cetyltrimethylammonium bromide (CTAB) method. A set of primers from the full-length cDNA [DgLDOX-F (5'-GGA GAA TAC AAT TAC AAA AT-3') and DgLDOX-R (5'-TCA TTT TGG GTC AAC GGA ATC AC-3')] was designed to amplify the DgLDOX gene. The PCR conditions were as follows: initial denaturation at 94 ℃ for 5 min, followed by 35 cycles for amplification (94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 1 min 30 s) and 72 ℃ for 5 min. The PCR products were cloned into the RBC T&A cloning vector (RBC Bioscience), and the inserts were sequenced (about 500 clones). All sequencing was performed by using an ABI 3130 XL Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed using GENETYX-WIN Software Ver. 5.0 (Genetyx).

RNA blot analysis

A denatured RNA (5 μg) was separated on 1.2% (w/v) agarose-formaldehyde gel using a 1 X MOPS buffer (Sambrook et al., 1989). The separated RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences Corp, NJ, USA) with 20X SSC, and crosslinked by using an ultraviolet crosslinker (UV Inc., California, USA). The DgLDOX probe was labeled using the PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences were as follows: LDOXblos-F: 5'-ACC TGC TAC CGC GGA GTA TGC TA-3', DgLDOX-R: 5'-TCA TTT TGG GTC AAC GGA ATC AC-3'. The PCR amplification conditions were 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 50 s. About 600 bp labeled probe was precipitated using 100% ethanol and 0.3 M NaOAC, and the probe was denatured at 100 ℃ for 10 min before use.

Hybridization and membrane washing procedures were performed following the supplier’s instruction (Roche Diagnostics GmbH). After a washing, the membranes were incubated in a CSPD Chemiluminescent Substrate (Roche Diagnostics GmbH) and subsequently exposed to an x-ray film (AGFA, Mortsel, Belgium).

DNA blot analysis

Ten micrograms of the genomic DNA digested with the appropriate restriction enzyme was separated on 1.0% agarose gel and then transferred to a Hybond-N+ membrane (Amersham Biosciences), followed by crosslinking using an ultraviolet crosslinker (UVP Inc.). A DgLDOX probe was labeled using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH). The primer sequences were as follows: HaeIII (LDOXcDNA-F: 5'-ATG GTG ATT CCA ACA ACA AG -3'; DgLDOX-R: 5'-TCA TTT TGG GTC AAC GGA ATC AC -3') and XmnI (LDOXblos-F: 5'-ACC TGC TAC CGC GGA GTA TGC TA-3', DgLDOX-R: 5'-TCA TTT TGG GTC AAC GGA ATC AC-3'). The PCR amplification conditions were 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 50 s. The labeled probes about 600 bp were precipitated using a mixture of 100 % ethanol and 0.3M NaOAC, and the probes were denatured at 100 ℃ for 10 min before use. Hybridization, membrane washing and detection procedures were performed as the same procedure as the above RNA blot assay above.

Comparison of the amino acid sequence and phylogenetic analysis

Multiple alignments of LDOX amino acid sequence were made using the program BioEdit software (Ver. 7.0.9.0, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic tree of DgLDOX was constructed using the neighbor-joining methods (Saitou and Nei, 1987) in Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0, http://www.megasoftware.net/index.html) (Tamura et al., 2007).
Results

Analyses in Anthocyanin contents

The LDOX encodes the enzyme for an essential step in the formation of colored metabolites in the anthocyanin biosynthesis (De Carolis and De Luca, 1994; Nakajima et al., 2001). The three flower color mutants and an ‘Argus’ chrysanthemum used in this study is shown in circles in Fig. 1A.

Concentration of total anthocyanin pigments in the ray florets differed among ‘Argus’ and three flower color mutants (Fig. 1B). The total anthocyanin content of ‘Argus’ was 0.16 mg·g⁻¹ fresh weight. Two purplish mutants (AM1, 1.87 mg·g⁻¹ fresh weight; AM3, 2.31 mg·g⁻¹ fresh weight) accumulated higher levels of total anthocyanin than white-flowered AM2 (0.04 mg·g⁻¹ fresh weight).

Cloning and structural characterization of the DgLDOX gene

We initially implemented an isolation of the DgLDOX gene by a RT-PCR with the DNase treated total RNA and degenerate primer which was extracted from each plant. The obtained partial cDNA fragments were about 500 bp from those chrysanthemum materials used in this study (Data not shown). Nucleotide sequences of the PCR products showed that the amplified fragments were 71 to 85% identical to the corresponding region of the sequences of the LDOX gene of other plant species.

The partial cDNA of 400 to 500 bp size in the chrysanthemum materials were used to design primers to obtain a full-length DgLDOX cDNA based on the 3'- and 5'-RACE method. The fully amplified cDNA fragments of the DgLDOX gene were about 1,068 bp in length including a 5' UTR of 21 nucleotides and a 3' UTR of 145 nucleotides contained in poly A (Fig. 2A). To compare the genome structure of the DgLDOX gene, the primers designed from the isolated full-length DgLDOX cDNA were used to amplify the genomic DNA containing a DgLDOX ORF (Fig. 2A). The amplicon of the genomic DNA was successfully amplified and was found to have a size of 1.3 kb, which was sequentially compared to identify the gene structure (Fig. 3A). The result of sequence comparison indicated only one intron region (~200 bp) between two exon ends (Fig. 2B). First exon is located at position from 22 to 524 bp and the second exon is located at position from 637 to 1201 bp, respectively. An intron, at position from 525 to 636 bp, is located between the two exons in ‘Argus’ (Fig. 2B). The structure of the ‘Argus’ DgLDOX indicated that the single intron region between two coding exons was 112 bp long, which was different from the introns of AM1 and AM3 with 137 bp,
and AM2 with 128 bp (Fig. 2B). Also, the 3′-noncoding upstream regions of the polyA tail of the gene differed in its sequence (Data not shown).

Expression pattern of the DgLDOX gene

In order to determine the expression pattern of the DgLDOX gene in ‘Argus’ and three flower color mutants of chrysanthemum, total RNAs were prepared from petals and subjected to RNA blot analysis using DgLDOX cDNAs as probes. Result of the RNA blot analysis showed that the mRNA of the DgLDOX gene was transcribed properly in ‘Argus’ and two mutants (AM1 and AM3), but only one mutant (AM2) showed low transcription (Fig. 3B). Thus, the expression level of the DgLDOX gene was well correlated with the anthocyanin contents (Fig. 1B) and RT-PCR (Fig. 2A) of the ‘Argus’ and three flower color mutants of chrysanthemum. These results suggest that a different regulation of the structural DgLDOX gene involved in the anthocyanin pathway was associated with a variation in the chrysanthemum flower color by the gamma irradiation. It was found that the mRNA accumulation of the DgLDOX gene was well associated with anthocyanin contents in the ray florets, and expression pattern of other enzymes of anthocyanin pathway were compared among the ‘Argus’ and flower color mutants by RT-PCR (Fig. 3A).

DNA analysis of the DgLDOX gene

The copy number of DgLDOX was determined by using a genomic DNA among ‘Argus’ and three flower color mutants (Fig. 4B). We chose the endonuclease EcoRI without a restriction site and BanII, HaeIII and XmnI with one restriction site in the DgLDOX gene (Fig. 4A). We designed the amplification probes containing either side of the without or one restriction site, and the hybridization trial showed only a single band (EcoRI) or two bands (BanII, HaeIII and XmnI) for all the materials treated with the enzyme, indicating that

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**Fig. 3.** Gel electrophoresis and RNA expression patterns in the ‘Argus’ and three flower color mutants. (A) Gel electrophoresis patterns of the amplified fragments from an RT-PCR and genomic PCR analysis. RT-PCR analyses were performed to amplify ORF of each gene using total RNA (1 ug/ul) isolated from the ray florets. The actin gene (AB205087) was used as a control. Amplified products (20 cycles) were size fractionated on 1.0 % agarose gel. (B) Differential expression of ‘Argus’ and flower color mutants of the DgLDOX gene. Ribosomal RNA bands stained with SYBR-Green are shown as the control.

**Fig. 4.** Restriction map and genomic DNA blot analysis of the DgLDOX gene from ‘Argus’ and three flower color mutants. (A) The selected restriction enzyme sites in DgLDOX cDNA sequence. Unboxed regions are N-terminal sequence (N), Intron (INT) and the 3′ untranslated region (UTR). Arrows indicate restriction enzyme sites for HaeIII (H), XmnI (X) and BanII (B). (B) Genomic DNA blot analysis of the DgLDOX gene from ‘Argus’ and three flower color mutants. Genomic DNAs (10 ug each) were digested with EcoRI, HaeIII, BanII and XmnI, respectively.
the DgLDOX gene was only one copy in the genome of chrysanthemum (Fig. 4B). The size standard indicated that the detected two fragments were the exact parts of the corresponding DgLDOX gene.

Sequence comparison of the DgLDOX between ‘Argus’ and three flower color mutants

The isolated full-length cDNAs were compared with the genomic DNA, which allows to identify the sequences variation in the DgLDOX gene between ‘Argus’ (GenBank accession no. EU980538) and its three flower colored mutants (GenBank accession no. EU980539, EU980541 and EU980542, respectively). The sequence similarity level between ‘Argus’ and three mutants reached to 95%. The amino acid sequences of the DgLDOX showed 78% identities with the *Vitis vinifera* (XP_002282039, 355 aa), 77% with *Petunia x hybrida* (P51092, 430 aa) (Weiss et al., 1993) and 76% with *Eustoma grandiflorum* (BAD34462, 362 aa) (Noda et al., 2004) and *Arabidopsis thaliana* (CAD91994, 356 aa) (Abrahams et al., 2003), *Prunus persica* (ABX89941, 357 aa) (Ogundiwin et al., 2008) and *Malus x domestica* (P51091, 357 aa) (Davies, 1993), 74% with *Perilla frutescens* (O04274, 362 aa) (Saito et al., 1999), 72% with *Clitoria ternatea* (BAF49295, 354 aa), 50% with *Zea mays* (P41213, 395 aa) (Menssen et al., 1990) and 48% with *Oryza sativa* (Os06g0626700, 373 aa) (Ohyanagi et al., 2006) (Fig. 5). But all the DgLDOX and other reported LDOX were conserved for all the characteristic amino acids of the typical 2-oxoglutarate-dependent dioxygenases and isopenicillin N synthase (Roach et al., 1997) (Fig. 5).

**Fig. 5.** Sequence alignment of the deduced amino acid sequences of the DgLDOX gene from ‘Argus’ and three flower color mutants with LDOX from other dicot and monocot species. Grape, *Vitis vinifera* (XP_002282039); *Arabidopsis*, *Arabidopsis thaliana* (CAD91994); *Eustoma*, *Eustoma grandiflorum* (BAD34462); Apple, *Malus x domestica* (P51091); *Perilla*, *Perilla frutescens* (O04274); *Petunia*, *Petunia x hybrida* (P51092); *Prunus*, *Prunus persica* (ABX89941) Rice, *Oryza sativa* (Os06g0626700); Maize, *Zea mays* (P41213). Identical amino acid residues are shown as black shading. LDOX active site which all the DgLDOX and other reported LDOX were conserved for all the characteristic amino acids of the typical 2-oxoglutarate-dependent dioxygenases and isopenicillin N synthase (Roach et al., 1997) are marked with a black arrowhead below the sequences. These sequences showed that a limited number of amino acid residues revealed a DgLDOX difference from those of the three flower color mutant sequences, but 2-oxoglutarate-dependent dioxygenases were conserved well between not only all the DgLDOX genes from the chrysanthemum but also the other dicot and monocot plant species.
Phylogenetic relationships of LDOX

Phylogenetic tree of LDOX in amino acid level carried out from various plant species were aligned using the BioEdit program (Ver. 7.0.9, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree of full-length LDOX was constructed by using the neighbor-joining methods (Saitou and Nei, 1987). LDOX amino acid sequences of monocot and dicot were clearly separated in the tree (Fig. 6). DgLDOX of ‘Argus’ and three mutants were clustered with the other dicot LDOX than to monocot LDOX. Especially, DgLDOX was more closely grouped to the Petunia and Eustoma LDOX than to the Arabidopsis and Prunus LDOX.

Discussion

In this study, we attempted to characterize the molecular difference of the DgLDOX gene from chrysanthemum ‘Argus’ and its flower color mutants derived by a gamma-irradiation. A phylogenetic analysis based on the amino acid of the DgLDOX also revealed a genomic relationship between the DgLDOX and the LDOX of the other plant species (Fig. 6). A comparison of other LDOX sequences in the GenBank database with DgLDOX showed high sequence identities and positives on whole protein level with the LDOX of other plant species. The DgLDOX gene consists of one intron between two exons, and the intron resides at the position of 525-636 bp in ‘Argus’. Currently, the intron sequences of a LDOX gene have been reported from V. labrusca x V. vinifera (Kobayashi et al., 2002), P. persica and A. thaliana (Abrahams et al., 2003). However, no report was presented on the structure of the LDOX gene including the intron region. The genomic DNA sequences of the ‘Argus’ DgLDOX showed more than 95% homology to the sequences from three flower color mutants, but sequence and length difference was evidently indicated in the noncoding insertion (Fig. 2B).

An LDOX is one of the enzymes involved in anthocyanin biosynthesis, which is catalyzed by 2-oxoglutarate-dependent dioxygenases (Saito et al., 1999). In addition to the 2-oxoglutarate-dependent dioxygenases, two histidine residues and an aspartic acid residue are essential for an Fe$^{II}$ coordination, and an arginine residue is involved in a 2-oxoglutarate binding (Lukačin and Britsch, 1997). The DgLDOX is also present in two transcripts, which contain 2-oxoglutarate-dependent dioxygenases and isopenicillin N synthase, an essential step in the formation of colored metabolites in the anthocyanin biosynthesis. The DgLDOX from ‘Argus’ and three mutants had these conserved amino acid residues, but the deduced amino acid sequence revealed a DgLDOX difference among the three flower color mutants (Fig. 5).

In chrysanthemum, the LDOX gene copy number was still unknown because not yet reported and the result from the DNA blot analysis performed in this study showed that the LDOX gene had a single copy in the chrysanthemum genomic.
DNA (Fig. 4B). Saito et al. (1999) reported that LDOX is quite similar to flavonol synthase of Arabidopsis. In A. thaliana, except flavonol synthase, all of the key enzymes involved in the anthocyanin pathway were believed to be encoded by single-copy genes (Winkel-Shirley, 2001). Daucus carota (Hirner et al., 2001) and P. persica (Unpublished) were reported to have two or three copies of the LDOX genes, respectively, but the other plant species have only a single LDOX gene (Kobayashi et al., 2002; Noda et al., 2004; Sparvoli et al., 1994).

Shikazono et al. (2003) created A. thaliana mutants lines with no pigment in the leaves and stems (tt mutants) by using carbon-ion beam, which was found to have a small deletion within the LDOX gene. Rather than fragment deletions, small insertions in the intron (Fig. 2B) and changes in amino acids (Fig. 5) of the DgLDOX gene were considered the reason for color mutation. The RT-PCR and RNA blot analysis indicated a weak transcript of the DgLDOX gene from the only white colored mutant (AM2) (Fig. 3). These results suggested that the color mutation of those mutants was not simply caused by a mutation in the structural LDOX gene, but other regulatory genes or transposable elements are assumed to be involved (Matsubara et al., 2005). Some mobile genetic elements such as MELSs (mobile element-like sequences) and DRs (direct repeats) were found in the introns and intergenic regions of the anthocyanin biosynthesis genes (Hoshino et al., 2001). In a white-flowered chrysanthemum, extremely high accumulation was found in the transcripts of a carotenoid cleavage dioxygenase (CCD) gene which accounts for a white flower, but is not a structural gene involved in the carotenoid biosynthesis (Ohmiya et al., 2006).

Although more evidences remain to be discovered, the relatively low accumulation of the transcripts of DgLDOX in the white flowered mutant (AM2) is assumed to be associated with a fragment insertion in the intron region, or activation of other regulatory genes controlling the structural LDOX gene. In this study, however, the first time identified novel DgLDOX gene from a chrysanthemum is believed to provide the genetic mechanisms for a color mutation in chrysanthemum in the near future. Currently, we are constructing cDNA library from flower color mutants of chrysanthemum by using suppression subtractive hybridization (SSH). A more detail analysis of the promoters and the characterization of regulators for anthocyanin biosynthesis will provide further understanding of the regulatory mechanism of anthocyanin biosynthesis in the chrysanthemum.

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스프레이형 국화와 화색변이체로부터 *Leucoanthocyanidin dioxygenase (LDOX)* 유전자 분리

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초 록. 스프레이 국화 ‘Argus’의 꽃잎으로부터 DgLDOX의 전장 cDNA와 genomic DNA를 분리하였고, 감마선 변이원으로부터 유래된 3가지 화색변이체 사이의 다양한 유전자 특성을 밝혀냈다. cDNA 영역은 1068bp이고 356 amino acid로 변환되었다. Genomic DNA의 크기는 ‘Argus’에서 1346bp이었고, 3가지 화색 변이체에서는 1363부터 1374의 크기를 나타내었다. DgLDOX 유전자는 두 개의 엑손 사이에 하나의 인트론을 갖고 있는 구조이고, 그 크기는 ‘Argus’에서 112bp 이지만 3가지 화색 변이체에서는 128 혹은 137bp였다. 이것은 감마선 조사에 의해 인트론 부분에 유전자가 삽입됐다는 것을 나타낸다. DNA 분석 결과 국화의 게놈 내에서는 하나의 LDOX 유전자를 갖는 것이 확인되었다. DgLDOX 유전자의 발현 정도를 분석한 결과, 연분홍의 ‘Argus’와 두 개의 보라색 변이체(AM1 and AM3)에서 높게 발현되었으나 흰색 변이체(AM2)에서는 매우 약하게 발현되었다. 이러한 결과들은 DgLDOX 유전자의 인트론에 삽입된 유전자 조각 혹은 엑손 부위의 일부 아미노산의 변화에 의해서 변이체의 화색이 변할 수 있다는 것을 보여주고 있다.

추가 주요어 : 안토시아닌, 화색 변이체, 전장 cDNA, 감마선, RACE