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Molecular Cloning and Characterization of CONSTANS-Like cDNA Clones of the Fuji Apple

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Two cDNA clones, *Md*COL1 and *Md*COL2, encoding CONSTANS (CO)-like proteins were isolated from an apple (*Malus domestica* cv. Fuji) fruit cDNA library. Both proteins contain two zinc finger motifs at the amino terminal end and a putative nuclear localization domain at the carboxyl terminal end. Genomic DNA blot analysis suggests that the *CO*-like genes are members of a small multigene family. RNA blot and RT-PCR analyses revealed that these genes are expressed in every organ that was examined. However, the expression levels were higher in floral buds and fruits at their early developmental stages compared to late reproductive stages or vegetative organs. Such expression patterns are quite different from those of the *CO*-like genes from *Arabidopsis*, which show strong organ specificity in either roots, cauline leaves, or flowers. These results indicate that the apple *CO*-like genes are significantly different from the *Arabidopsis* genes and that they appear to play important roles in reproductive organ development.

Keywords: apple, CONSTANS, development, fruit, zinc finger motifs

Early fruit development is characterized by many biochemical and physiological changes, including rapid cell division, cell expansion, and strong sink activity exerted by expanding cells (Gillaspy et al., 1993). A number of genes that reflect the nature of the dynamic changes that occur during early fruit development have been identified. These genes encode a variety of proteins, including tomato anionic peroxidase (*tap*; Sherf and Kolattukudy, 1993), pectin methyl esterase (Ray et al., 1988; Harriman et al., 1991), putative cell wall structural protein (Salts et al., 1991), proline-rich protein (Tfm7; Santino et al., 1997), and glycine-rich protein (Tfm5; Santino et al., 1997). From *Arabidopsis*, it has recently been reported that RBP37 (RNA-binding protein) is expressed in dividing cells during early silique development (Hecht et al., 1997). The regulation and induction of these genes during fruit development are probably due to the activity of specific transcription factors encoded by regulatory genes. However, very little is known about these regulatory factors involved in early fruit development. To this end, it is necessary to isolate and characterize the genes that are required for the regulation of early fruit development (Sung and An,1997).

DNA-binding transcription factors contain conserved domains. These domains include the homeobox, MADS-box, zinc finger and leucine zipper, and many transcription factors appear to have a variety of combinatory arranged functional domains (Mitchell and Tjian, 1989). Zinc finger motifs, which were originally identified in the TFIIIA transcription factor from *Xenopus*, can be classified into several subgroups, according to the number and position of cysteine or histidine residues available for coordination to zinc (Evans and Hollenberg, 1988). Standard zinc finger proteins contain tandem repeats of the Cys_2 / His_2 finger motif, which have been shown to participate in interactions at single target sites (Pavletich and Pabo, 1991). However, members of the GATA-1 multigene family, which play important roles in vertebrate development, contains two Cys_2/Cys_2 zinc finger motifs.

In plants, the majority of the reported zinc finger proteins belong to the standard Cys_2/His_2 type (Tague et al., 1995; Tague and Goodman, 1995; Meissner and Michael, 1997). However, GATA-1 family zinc finger proteins have recently been isolated from *Arabidopsis* and tobacco. The members of this subfamily that have been described to date include the CONSTANS (CO), which controls transition to flowering (Putterill et al., 1995), NTL1, which regulates nitrogen metabolism (Daniel-Vedele and Caboche, 1993), a set of related DNA-binding proteins (Yanagisawa, 1995; De Paolis et al., 1996), and STO, which confers salt tolerance (Lippuner et al., 1996).

The *Arabidopsis CO* gene encodes a protein with two putative zinc finger motifs which are similar in structure to those found in the GATA-1 family (Sanchez-Garcia and Rabbitts, 1994; Putterill et al., 1995). The zinc finger motifs appear to be critical to

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its function, as co mutant alleles carry alteration in this region (Putterill et al., 1995). The *CO* gene controls flowering time, and its expression is induced by long day conditions. It was reported that forced expression of *CO* promotes flowering through the transcriptional activation of *LEAFY*, a flower-meristem-identity gene (Simon et al., 1996). Although there are several genes that are closely related to the *CO* gene in *Arabidopsis*, function of these CO-like genes remain unknown (Ledger et al., 1996; Putterill et al., 1997). In this paper, we report the isolation of *CO*-like genes from the Fuji apple and the examination of their expression patterns during flower and fruit development.

MATERIALS AND METHODS

Plant Materials

Leaves, floral organs, and fruit were collected from Fuji apple trees (*Malus domestica* cv. Fuji), frozen in liquid nitrogen, and stored at −70°C until use. *Arabidopsis thaliana* (Columbia) plants were grown in a growth chamber with 24 h continuous light.

Nucleic Acid Isolation

Total RNAs from leaves and floral organs were obtained by the guanidine isothiocyanate - CsCl method (Sambrook et al., 1989). Total RNA from fruit was isolated by the procedure described by Wang and Vodkin (1994). Poly (A) RNA was purified from total RNA by affinity chromatography in an oligo-dT-cellulose column using a poly (A) quik mRNA isolation kit (Stratagen). Genomic DNA was isolated from young leaves using the CTAB (cetyltrimethylamin onium bromide) method as described by Rogers and Bendich (1988). Plasmid DNA was isolated according to the alkaline lysis method (Sambrook et al., 1989).

Preparation and Screening of the cDNA Library

A cDNA library was constructed using poly (A) RNA isolated from fruit of the Fuji apple tree. Double-stranded cDNAs were synthesized from 5 μg of poly (A) RNA using the moloney murine leukemia virus reverse transcriptase, cloned into the *Eco*RI/*Xho*I site of the Uni-ZAP vector, and packaged in vitro by the Gigapack III Gold packaging extract (Stratagene). Plaque lifts and phage works were done according to standard procedure (Sambrook et al., 1989) using Hybond C membranes (Amersham). The *XL1-Blue MRF'* bacteria were infected with 450,000 pfu of the phage and plated on agar plates. After overnight growth at 37°C, the plaques were transferred to nitrocellulose membranes and hybridized with a cDNA clone that was P^{32}-labeled using random primers (Amersham). After washing, the filter was autoradiographed overnight.

PCR Cloning

A set of primers was designed to isolate the CO and COL2 clones from *Arabidopsis*. For the CO clone, the sense primer (5'-GATCAAGAGGAAGGTGAAG-3') was located 408 bp downstream from the translation initiation site and the antisense primer (5'-GTCCCT-GAGGAGCCATATTTG-3') was located 394 bp upstream from the translation termination codon. For the COL2 clone, the sense primer (5'-GAGAGTGG-TACTTGGGCACG-3') was located 20 bp downstream from the translation start site and the antisense primer (5'-AGCTCCTTTAAGGAGTTG-3') was 370 bp upstream of the translation termination codon. PCR was performed using *Thermus aquaticus* DNA polymerase (Takara) in 50 μL of PCR buffer, containing 10 mM dNTPs 0.2 μM of each PCR primer, and 5 μg of *Arabidopsis* genomic DNA.

Nucleotide Sequence Analysis

Overlapping subclasses of the cDNA clones were created in a pBluescript SK(-) vector and nucleotide sequences of these clones were determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a thermo sequenase cycle sequencing kit (USB). DNA sequence comparison was performed using the Genbank databases.

RNA Blot and Genomic DNA Blot Analysis

For RNA hybridization, 20 μg of total RNA was separated in a 1.3% (w/v) agarose-formaldehyde denaturing gel. The gel was blotted onto a Hybond N + nylon membrane (Amersham) and hybridized with a P^{32}-labeled cDNA insert in Church buffer (Church and Gilbert, 1984) at 60°C for 18 h. A posthybridization wash was done at 25°C for 10 min in 2× SSPE and 0.1% SDS, followed by a wash at 55°C for 10 min in 0.2× SSPE and 0.1% SDS. Nylon membranes were wrapped in plastic and placed against an x-ray film with two intensifying screens at −70°C. For genomic DNA hybridization, 10 μg of DNA was digested with *Eco*RI, *Bam*HI, or *Hind*III restriction

enzymes, separated on a 0.7% agarose gel, and transferred to a nylon membrane Hybond N+. The blot was hybridized at 65°C for 12 h in Church buffer using a P^{32}-labeled probe. Membranes were washed once with 2× SSC, 0.1% SDS at 25°C for 10 min and once with 0.2× SSPE, 0.1× SDS at 65°C for 10 min. The washed membranes were exposed to an x-ray film with intensifying screens at −70°C for 3 days.

RT-PCR Analysis

For reverse transcriptase-mediated polymerase chain reaction (RT-PCR), first strand cDNA synthesis was done using the SuperScript pre-amplification system (GIBCO-BRL). Total RNA was treated with RNase-free DNase I at 37°C for 20 min and extracted with DEPC-saturated phenol. Then, 2.5 µg of DNA-free RNA was reverse transcribed in a total volume of 20 µL, containing 25 ng of oligo $(dT)_{12\text{-}18}$ primer, 2.5 mM dNTPs, and 200 units of SUPERSCRIPT II reverse transcriptase (GIBCO-BRL) in an RT buffer supplied by the company. The PCR reaction was performed in a 50 µL solution containing a 0.5 µL aliquot of the cDNA reaction, 0.2 µM primers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs, and 2.5 units of *T. aquaticus* DNA polymerase (Takara). After denaturation at 95°C for 3 min, the reaction mixture was incubated by a step program (95°C, 1 min; 58°C, 40 s, 72°C, 2 min) of 35 cycles, followed by a 10 min final extension at 72°C. Ten microliters of the reaction mixture was analyzed on a 1.2% agarose gel.

RESULTS

Isolation and Sequence Analysis of CONSTANS-Like cDNA Clones from an Apple Fruit Library

A cDNA clone that shows significant homology to the *CONSTANS* (*CO*) gene of *Arabidopsis* was isolated during expressed sequence tag (EST) analysis of apple fruits at early developmental stages (Sung et al., 1998). The clone encodes for a peptide that shows over 80% identity to the carboxyl terminal region of the CO protein. This region contains clusters of lysine and arginine residues, suggesting that this region may act as a nuclear localization domain (Raikhel, 1992). To obtain full-length cDNA clones, the apple fruit cDNA library was screened with the partial clone as a probe. After screening 450,000 plaques, 15 that showed positive signals were selected for further characterization. These clones could be divided into two groups by restriction enzyme mapping and partial sequencing. The longest clones from each group that contained entire coding regions were designated *Md*COL1 (*Malus domestica* CONSTANS-LIKE 1) and *Md*COL2.

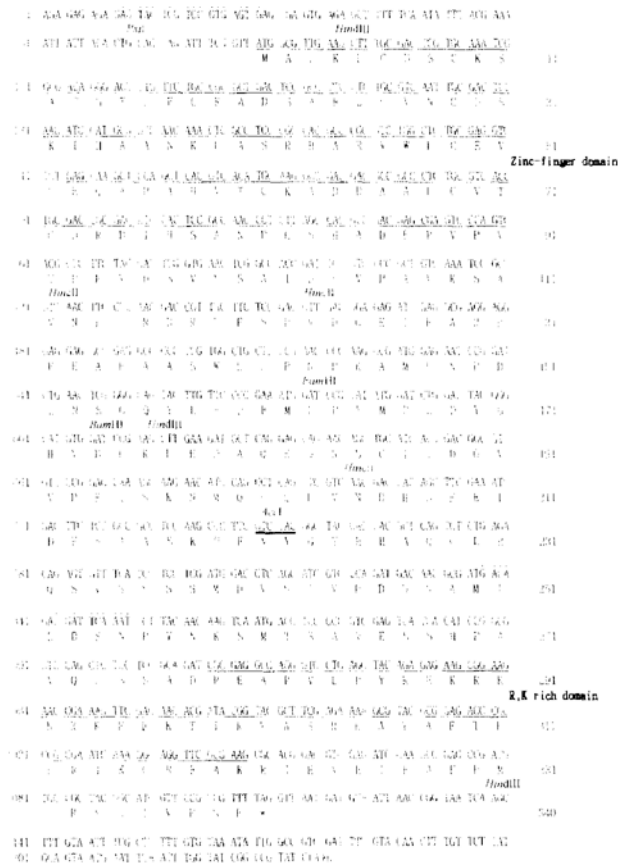

Figure 1. Nucleotide and deduced amino acid sequences of *Md*COL1. The zinc finger domain and putative nuclear localization region (R, K rich domain) are underlined. The restriction enzyme site, *Acc*I, used for the generation of the probe is double underlined. The termination codon is marked with an asterisk. The primer sequences used for the RT-PCR experiment are represented as dot-lines above the sequences. The restriction enzyme sites are shown above the nucleotide sequence. The positions of nucleotides and amino acids are shown on the left and right, respectively. The GenBank accession number for the sequence is AF052584.

The nucleotide sequences of these two clones were determined (Figs. 1 and 2). The cDNAs of *Md*COL1 and *Md*COL2 contain open reading frames of 340 and 329 amino acid residues, respectively. They share an 88% nucleotide sequence identity and 85% amino acid sequence identity within the coding region. Both proteins contain a pair of zinc finger motifs (C-X2-C-X16-C-X2-C) in the amino terminal region and a putative nuclear localization domain in the carboxyl

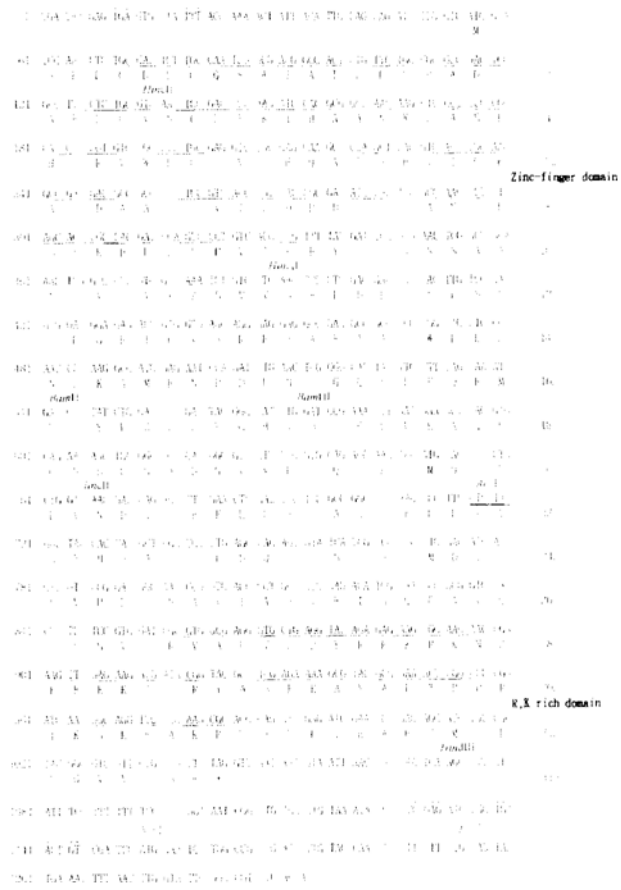

Figure 2. Nucleotides and deduced amino acid sequences of *Md*COL2. The zinc finger domain and putative nuclear localization region (R, K rich domain) are underlined. The restriction enzyme site, *Acc*I, used for the generation of the probe is double underlined. The termination codon is marked with an asterisk. The primer sequences used for the RT-PCR experiment are represented as dot-lines above the sequences. The restriction enzyme sites are shown above the nucleotide sequence. The positions of nucleotides and amino acids are shown on the left and right, respectively. The GenBank accession number for the sequence is AF052585.

terminal region (Figs. 1 and 2). Figure 3 presents an alignment of the amino acid sequences of apple *Md*COL1 and *Md*COL2 with CO, COL1, and COL2 proteins of *Arabidopsis*. The *Md*COL1 and *Md*COL2 proteins were 41% identical to CO. The homology was higher with COL1 and COL2. Among all five genes, the most conserved regions are the zinc finger domains and putative nuclear localization domain, indicating the functional importance of these regions.

Genomic DNA Blot Analysis

Apple genomic DNA was digested with *Eco*RI, *Bam*HI, or *Hin*dIII restriction enzymes and hybridized under high stringency conditions with the 3′ regions of the *Md*COL1 and *Md*COL2 (Fig. 4). Since the 5′ region contained the zinc finger motifs, which may have hybridized to a number of unrelated genes, this region was not included in the probe. For both clones, two strong bands appeared for *Bam*HI. The hybridization intensity indicated that the upper 2.9 kb band contains *Md*COL2 and the lower 1.2 kb band contains *Md*COL1. In addition to the strongly-hybridizing bands, there were several bands that weakly hybridized to the probes, indicating the presence of related genes in apple chromosomes. The *Eco*RI digestion revealed two bands, 8.6 kb and 2.0 kb. The upper band strongly hybridized with both probes, suggesting that both genes may be located in this fragment. It appears that other genes that are related to *MdCOL1* and *MdCOL2* may also be present in the 8.6 kb band since the hybridization pattern is much simpler than other digestions. The *Hin*dIII digestion indicated that the *MdCOL1* gene is divided by the enzyme, resulting in two fragments of 1.4 kb and 0.7 kb, while most of the *MdCOL2* gene is located in the 2.1 kb fragment.

Expression Pattern Analysis during Development

Total RNAs were extracted from various tissues at different developmental stages and the transcript levels of the apple genes were determined by RNA blot analysis. The probe contained the 3′ region of the *Md*COL1 clone that was used for the genomic DNA blot analyses. Since the apple *MdCOL1* and *MdCOL2* genes are highly homologous to each other throughout the entire gene, the *Md*COL1 probe had to hybridize to both transcripts. Therefore, the results in Figure 5A should represent the expression levels of both genes together. The data showed that the transcripts were detected in all the tissues examined. However, the transcript level changed as plants developed. Floral samples were harvested at four development stages. In stage 1, the floral bud emerged on the flank of the branch and the bud length was 4-5 mm. In stage 2, ten days after stage one, bud length was 7-8 mm long. Stage 3 was at the mature flower stage. At this stage, all of the floral organs had completed morphological differentiation. Stage 4 was at post-anthesis of the flower. The anther wall was opened and stigma began to senescence. The transcript amount was higher in stages 1 and 2 (lanes 2 and 3), and the levels decreased as the flowers developed to stages 3 and 4 (lanes 4 and 5). In fruits, higher levels

Figure 3. Comparison of *Md*COL1 and *Md*COL2 with CO (GenBank accession number X94937), COL1 (Y10555), and COL2 (L81120) from *Arabidopsis*. The alignment was done using the multiple sequence alignment program (http://www.ibc.wustl.edu/ibc/msa.html). Shaded boxes represent identical amino acids. Dashed lines (-) are included to assist in alignment of sequences.

of transcripts were detectable thoughout the early stages of development (lanes 6-8). The blot was reprobed with a cDNA encoding a putative apple ribosomal protein L29 (Fig. 5B) to show the presence of intact mRNA in each lane and an approximately equal loading of total RNAs.

Since the *MdCOL1* and *MdCOL2* genes are highly homologous, RNA blot analyses were unable to reveal the expression pattern of each gene independently. Therefore, the RT-PCR approach was performed to study the transcript level of the *MdCOL* genes. Two sets of specific primers (shown in Fig. 1) were synthesized to amplify the 847-bp and 1147-bp DNA fragments of the *Md*COL1 and *Md*COL2 transcripts, respectively. These studies revealed that the transcript level of *Md*COL2 was stronger than that of *Md*COL1 in all the samples examined (Fig. 5C). However, both genes showed similar expression patterns in various organs. Although the intensity of the amplified bands in leaf and seeds was relatively stronger, the overall expression pattern was similar to that of the RNA blot analysis. The different levels of the transcripts between RNA blot and RT-PCR in young leaves seemed to be due to the unequal loading of RNA. As shown in Figure 1B, the ribosomal protein L29 mRNA level in young leaves is lower than those in the other tissues. These data suggest that both genes are important for flower and fruit development, especially during cell division and early growth stages.

Differential Expression between Arabidopsis CO and COL2 Clones and Apple MdCOL1 and MdCOL2 Clones

The sequence similarity between the apple and *Arabidopsis CO* and CO-like genes suggests that the

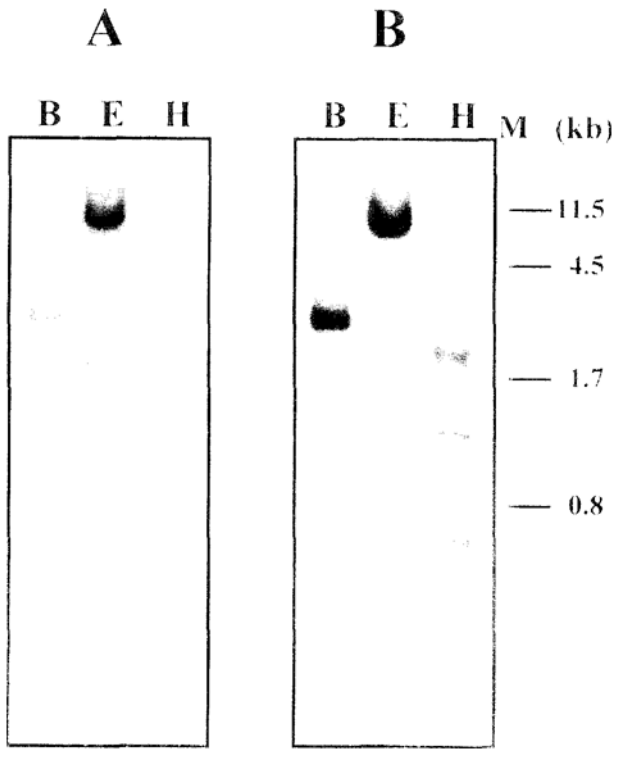

Figure 4. Genomic DNA blot analysis of *MdCOL1* (**A**) and *MdCOL2* (**B**) Apple DNA was digested with *Eco*RI (E), *Bam*HI (B), or *Hin*dIII (H), and hybridized with a probe prepared from the *Acc*I-*Xho*I fragments carrying the 3′ regions of *Md*COL1 and *Md*COL2. The positions of *Pst*I-digested λ DNA size markers are also indicated.

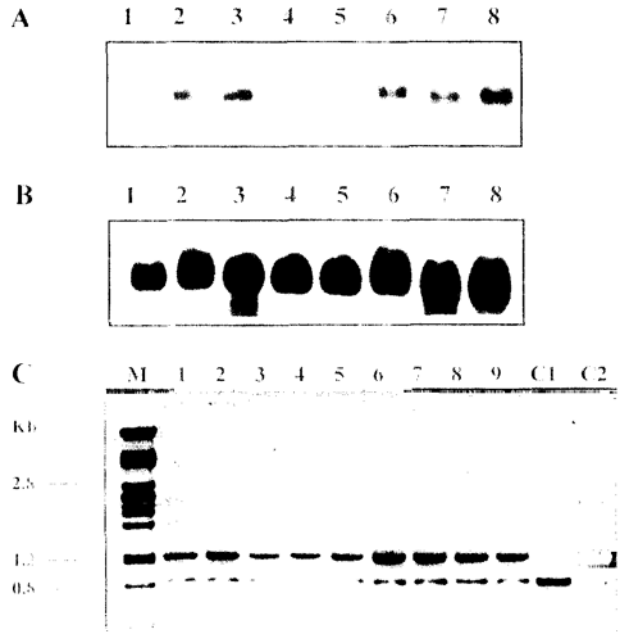

Figure 5. Analyses of the *Md*COL1 and *Md*COL2 transcripts. **A.** RNA blot assay using the *Acc*I-*Xho*I fragment carrying the 3′ regions of *Md*COL1 as a probe. Lane 1, young leaves; 2, floral buds at stage 1 (length ~ 4 to 5 mm); 3, floral buds at stage 2 (length ~ 7 to 8 mm); 4, mature flowers; 5, post-anthesis flowers; 6, early fruits at stage 1 (7-10 days after anthesis); 7, early fruits at stage 2 (8-10 mm in length); 8, early fruits at stage 3 (15-20 mm in length). **B.** The RNA blot assay using a putative apple ribosomal protein, L29, as a probe to demonstrate relatively equal amount of RNA in different lanes. **C.** The RT-PCR analysis. Lanes 1-7, same as A; 8, flesh of stage 3 fruits; 9, seeds of stage 3 fruits. M is markers, λ DNA digested with *Pst*I. C1 and C 2 are RT-PCR products of the *Md*COL1 and *Md*COL2 cDNA clones.

apple *MdCOL1* and *MdCOL2* genes might be functionally similar to the *Arabidopsis* genes. If this is the case, expression patterns of the genes should be similar to each other. To this end, *Arabidopsis* CO and *COL2* genes were amplified using primers deduced from the published DNA sequences. Using these clones, the transcript levels of the *Arabidopsis* genes were examined from a variety of tissues harvested at different developmental stages. At the young seedling stage, whole seedlings, roots, or rosette leaves were collected. After growth in continuous light for six weeks, cauline leaves, stems, floral buds, whole flowers, and siliques were sampled. Silique development was divided into two stages; stage one had siliques 3-5 mm in length and stage two, 7-10 mm in length. As shown in Figure 6A, two distinctive transcripts were hybridized with the CO probe. The transcript level of upper bands was stronger in mature flowers (lane 7) and floral buds (lane 6), and it was lower in young seedlings (lane 1), rosette leaves (lane 3), cauline leaves (lane 4), stems (lane 5), and siliques (lanes 8 and 9), and undetectable in roots (lane 2). In contrast, the level of the lower bands was strongest in roots (lane 2) and lower in other tissues. It was reported that the CO transcript was rare and could not be detected by RNA blot analysis (Putterill et al., 1995). Therefore, the bands visualized by the CO probe are likely cross-hybridizing bands of CO-like transcripts. RNA blot analysis with the COL2 probe showed that the transcript level was highest in cauline leaves (lane 4) and not detected in seedling, roots, and young leaves. This expression pattern is different from those of the bands hybridized with the CO probe, indicating that there are at least three CO-related genes in *Arabidopsis*, and their expression patterns are different from each other. The results also show that the expression patterns of the *Arabidopsis* CO-related genes are different from those of the apple CO-related genes. Taken together, it appears that the apple CO-like genes are different from the *Arabidopsis* genes, playing a major role during flower

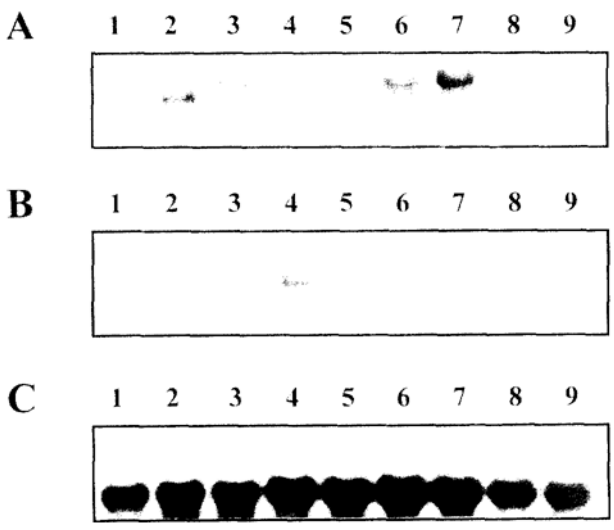

Figure 6. Expression patterns of the CO-like genes in *Arabidopsis*. **A.** RNA blot analysis with the CO gene as a probe. **B.** RNA blot analysis with the *COL2* gene as a probe. **C.** RNA blot analysis with 18S rDNA as a probe to show equal loading in each lane. Lane 1, young seedlings; 2, roots; 3, young leaves; 4, cauline leaves; 5, stems; 6, floral buds; 7, whole flowers; 8, siliques at stage 1 (3-5 mm in length); 9; siliques at stage 2 (7-10 mm in length).

and fruit developments.

DISCUSSION

In this paper, we report the isolation of two CO-like genes from the Fuji apple. Since the clones were isolated from the fruit cDNA library, it appears that the protein encoded by these genes should play an important role in the development of apple fruits. The *MdCOL1* and *MdCOL2* genes share a significant homology throughout the entire coding region and the homology extends to the 5′ and 3′ untranslated regions.

Sequence homology studies identified the two cDNAs as members of the family of CO-like proteins and provided insight into their possible activities as transcription factors. The most striking feature of the deduced proteins from both clones is the presence of two zinc finger domains as well as the arginine- and lysine-rich basic region, which might act as a putative nuclear localization domain, further supporting the view that these proteins are transcription factors. Although there is no demonstration that the *Arabidopsis* CO protein is a DNA-binding factor, the significant similarity in the zinc finger motifs between CO and the GATA-1 protein family suggests that the CO protein is likely a transcription factor. Although *Md*COL1 is highly similar to *Md*COL2, the *Md*COL2 protein lacks 11 amino acids between residues 251 and 261.

Genomic DNA blot analysis revealed that, in addition to *MdCOL1* and *MdCOL2*, there are several genes that share homology to the CO gene. At least 12 ESTs from *Arabidopsis* and five from rice showed significant homology to the CO gene (Coupland., 1997). These observations indicate that the CO-related genes are members of a large gene family in the plant kingdom.

The function of the *Md*COL proteins is unknown, but they seem to play an important role in fruit development since the maximum levels of the transcripts appear during the early fruit developmental stages. In addition, the higher expression in young floral buds suggests that the *MdCOL* genes may also play a role in regulating floral organ development. Although the level was different, expression patterns of both *MdCOL1* and *MdCOL2* genes are similar, suggesting the correlative functions in association with each other.

Although the importance of the *Arabidopsis* CO gene in controlling the switch between vegetative and floral stages has been demonstrated (Blazquez, 1997), there has been little information about the expression patterns of the CO and CO-like genes during plant development. Our results revealed that *Arabidopsis* CO-like genes are more preferentially expressed in either vegetative organs or the flowers. Therefore, the roles of CO-like genes are different in apples (a woody species) and *Arabidopsis* (herbaceous dicot species).

The expression patterns of the *MdCOL* genes appear to be similar to those of the *MdMADS1* gene, which appears to play a major role in the initiation of reproductive organ developments (Sung and An, 1997). Both *MdCOL* and *MdMADS* genes were highly expressed in the early developmental stages of reproductive organs, and the expression decreased as they matured. This suggests that the two classes of transcription factors may function together to control development of reproductive tissues. It is possible that there is a hierarchical relationship between *MdCOL* and *MdMADS* genes, that is, *MdCOLs* may be a target gene of the gene products of MADS-box genes. Alternatively, the *Md*COL proteins may regulate the expression of MADS-box genes as negative regulators. In the petunia, it was hypothesized that a gene for a zinc finger protein (*EPF1*) is a downstream transcription factor of a MADS-box gene through the observation that a putative binding site of a MADS-box protein is present in the 5′ upstream region of

the *EPF1* gene (Takatsuji et al., 1994).

In conclusion, we have identified two apple clones that encode CO-like proteins, which appear to be transcription factors playing important roles during flower and fruit development. The differences found in the amino acid sequences and the expression patterns between *Arabidopsis* CO-like genes and apple *MdCOL* genes suggest that they are functionally different from each other.

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