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Overexpression of the *Downward Leaf Curling* (*DLC*) Gene from Melon Changes Leaf Morphology by Controlling Cell Size and Shape in *Arabidopsis* Leaves

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A plant-specific gene was cloned from melon fruit. This gene was named *downward leaf curling* (*CmDLC*) based on the phenotype of transgenic *Arabidopsis* plants overexpressing the gene. This expression level of this gene was especially upregulated during melon fruit enlargement. Overexpression of *CmDLC* in *Arabidopsis* resulted in dwarfism and narrow, epinastically curled leaves. These phenotypes were found to be caused by a reduction in cell number and cell size on the adaxial and abaxial sides of the epidermis, with a greater reduction on the abaxial side of the leaves. These phenotypic characteristics, combined with the more wavy morphology of epidermal cells in overexpression lines, indicate that *CmDLC* overexpression affects cell elongation and cell morphology. To investigate intracellular protein localization, a CmDLC-GFP fusion protein was made and expressed in onion epidermal cells. This protein was observed to be preferentially localized close to the cell membrane. Thus, we report here a new plant-specific gene that is localized to the cell membrane and that controls leaf cell number, size and morphology.

INTRODUCTION

The morphology of leaves is regulated by the extent and orientation of the division and elongation of cells (Tsukaya, 1995). From the mid-1990s, anatomical analyses on leaf morphogenesis using *Arabidopsis* and T-DNA insertional mutagenesis have helped to decipher the molecular mechanisms determining leaf morphology. So far, about 40 genes involved in leaf morphogenesis have been identified (Cho et al., 2007). These genes are involved in such functions as leaf primordium formation, establishment of dorsoventrality, cell proliferation, and cell elongation, including polar cell elongation (Cho et al., 2007; Horiguchi et al., 2005; Kim et al., 1998a; 1998b; 2002; Lee et al., 2006). Among many morphological alterations, changes in the orientation of leaves, leaf epinasty and hyponasty have frequently been observed. It is known that both hyponastic and epinastic curvatures are caused by differential growth of either the abaxial or the adaxial sides of leaves (Kang, 1979; Keller and Van Volkenburgh, 1997). Plants sometimes use these nastic responses to adapt to environmental stresses. For example, flooding induces hyponastic leaf movement, which contributes to increased survival by enabling leaves to reach the water surface (Voesenek et al., 2003). Plant hormones such as ethylene, auxin, abscisic acid and brassinosteroid (BR) are involved in leaf curling phenomena (Cox et al., 2004; Horiguchi et al., 2005; Qin et al., 2005; Schlagnhaufer and Arteca, 1985).

Previously, we found expressed sequence tags (ESTs) that are involved in fruit development in melon (Choi et al., 2004). Subsequently, we isolated a full-length cDNA by screening a melon fruit cDNA library with those ESTs. The cDNA encoded a plant-specific protein with unknown function. In the present study, we introduced this cDNA into *Arabidopsis* and showed that transgenic *Arabidopsis* overexpressing this gene developed an abnormal leaf morphology. Thus, we attempted to understand the function of this gene by analyzing the phenotypes and molecular characteristics of overexpression lines. Here, we report on this new plant-specific gene and its involvement in cell proliferation and cell enlargement in leaf morphogenesis.

MATERIALS AND METHODS

Plant materials and growth conditions

Charentais melon (*Cucumis melo* L cv. Reticulatus) seeds were planted and grown in a greenhouse. Plants were maintained at 30-35°C and 15-20°C during the day and night, respectively. Fruits were harvested at anthesis, 9, 18, 27 and 36 days after

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pollination (DAP), representing the unfertilized ovary, actively growing stage 1, growing stage 2, mature green fruit, and mature ripened fruit, respectively. Seeds of *Arabidopsis* ecotype Col-0 and transgenic plants were sterilized and placed in the dark at 4°C for 2 d, and sown on agar plates of one-half MS with 2% Suc (Murashige and Skoog, 1962) or in soil. Plants were grown at 23°C in a growth room under a long day (16 h light/8 h dark) light regime.

RNA extraction, melon cDNA library construction and library screening

PolyA$^+$ RNA was isolated from nine DAP melon fruits using the PolyATtract mRNA Kit (Promega). We constructed a unidirectional *Eco*RI/*Xho*I cDNA library using a ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII gold packaging extracts (Stratagene) according to the manufacturer's instructions. To find full length cDNA clones expressed during the fruit development process, we screened excised cDNA libraries *in vivo* with the ^{32}P labeled EST clones previously identified. Hybridization, membrane washing and autoradiography were performed as described (Choi et al., 2004). Total RNAs from various tissues of Arabidopsis were extracted using Trizol reagent.

Reverse transcript-mediated polymerase chain reaction (RT-PCR)

First strand cDNA was synthesized from total RNA (5 μg) by reverse transcription using the oligo (dT)-primer as the antisense primer. The first strand reaction was used for subsequent PCR reactions to detect gene expression using gene-specific primers designed from the coding sequences of each gene. RT-PCR was performed for 15 to 40 cycles at 5-cycle intervals, and the best RT-PCR results showing non-saturating levels of amplified gene expression were chosen to quantify the transcript level of each gene. RT-PCR of *Ubiquitin* was used as an internal standard. The primers used for RT-PCR were as follows: At3g07760-F (5′-CCTCTTGATTTCTTCATCTG-3′), At3g07760-R (5′-CTTTCAAGCAAGCCACTTTA-3′); CmDLC-F (5′-CACCTCATAGGGAGATG-3′), CmDLC-R (5′-GATCTCGGTTCTATGGT-3′); UBQ10-F (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′), UBQ10-R (5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′).

DNA sequence analyses

Sequences were determined using a DNA sequencer (Applied Biosystems). DNA and predicted amino acid sequences were searched against DNA and protein databases using the BLAST program available at the National Center for Biotechnology Information (NCBI).

Construction of transgenic Arabidopsis overexpressing CmDLC

The complete *CmDLC* ORF was PCR-amplified from the *CmDLC* cDNA isolated by library screening. The primers used were CmDLC-F (5′-ACCTCTAGAGGAGATGAACA-3′) and CmDLC-R (5′-GTTGAGCTCGGTTCTATGGTT-3′), which contained sites for the restriction enzyme *Xba*I and *Sac*I, respectively. The PCR product was ligated into the pGEN-T easy vector (Promega). The pGEN-T-CmDLC *Xba*I/*Sac*I fragment was then recloned into the *Xba*I and *Sac*I sites of the pBI121 vector, and the construct was introduced into the *Agrobacterium tumefaciens* GV3101 strain, which was used to transform *Arabidopsis* ecotype Columbia by the floral-dipping method (Clough and Bent, 1998). The transformed seeds were selected on one-half MS agar plates containing 30 μg/ml kanamycin. The transcripts of *CmDLC*-overexpressing lines were detected by RT-PCR.

Measurements of leaf length, and microscopic observation of epidermal cells

Fully grown 3rd leaves of 5 week old plants were collected, and leaf lengths and widths were measured. For observations of epidermal cells, leaves were fixed in FAA solution (5% acetic acid, 45% ethanol, and 5% formaldehyde), immersed in 5% NaOH, and then boiled for 1 min. After rinsing with dH_2O, the leaves were incubated in bleach until they become clear (Iwakawa et al., 2007). Samples were photographed under bright field illumination with DIC optics (Axioscope2, Zeiss, Germany). The area and number of photographed epidermal cells were measured as described (Kim et al., 1998).

Intracellular localization of CmDLC in onion cells

We made a CmDLC-GFP fusion construct, *CmDLC-GFP*. The construct was made by inserting *CmDLC* cDNA into the 326GFP-3G vector (Clontech) using the *Xba*I and *Bam*HI sites. The construct was introduced into onion epidermal cells as described (Scott et al., 1999). Expression of constructs was monitored using an Axioskop2 fluorescence microscope (Zeiss).

RESULTS

Cloning and expression of CmDLC cDNA

We previously isolated several cDNAs involved in fruit development (Choi et al., 2004). One of those clones was investigated in this study. The gene was named d*ownward leaf curling* (*CmDLC*) based on the phenotype of its overexpression in *Arabidopsis*. The *CmDLC* cDNA ORF is composed of 378 bp and encodes a predicted polypeptide of 125 amino acids (Fig. 1A). The expected molecular mass and pI are 14.9 kDa and 9.51, respectively. Homologous sequences of the *CmDLC* were found from other plants, but not from any other organisms. The deduced amino acids of *CmDLC* shared 88% identity with those from rice and *Arabidopsis*. It showed at least 75% identity with those cloned from other plants, indicating that *CmDLC* is a highly conserved plant-specific gene (data not shown). Except for its sterile alpha motif (SAM) domain, which is an evolutionarily conserved protein binding domain involved in the regulation of numerous developmental processes (Schultz et al., 1997), the sequence provides no clue as to the function of the predicted protein.

In melon, the *CmDLC* transcript was detected in RNA from roots, young seedlings, mature leaves and fruits. Its levels were especially upregulated during fruit enlargement, but not during fruit ripening (Fig. 1B, upper panel). The *Arabidopsis* orthologue of *CmDLC*, *At3g07760*, is a single copy gene. Its expression was relatively evenly detected from all tissues examined, including leaves, flowers, siliques, inflorescence stems and roots (Fig. 1B, lower panel).

Characterization of CmDLC-overexpressing transgenic plants

To investigate its function, The *CmDLC* ORF was cloned under control of the 35S promoter of pBII21 and introduced into *Arabidopsis*. We isolated several transgenic lines. Transgenic plants showed pleiotropic phenotypes, including semi-dwarfism, leaf epinasty and narrowing of leaves (Figs. 2A and 2B). The most severe phenotype was semi-dwarf plants with epinastic leaves (Fig. 2A, lane 2). Those phenotypic variations were dependent on the extent of *CmDLC* expression (Fig. 2C). Transgenic plants with more severe phenotypes showed a higher level of expression, while plants with milder phenotypes

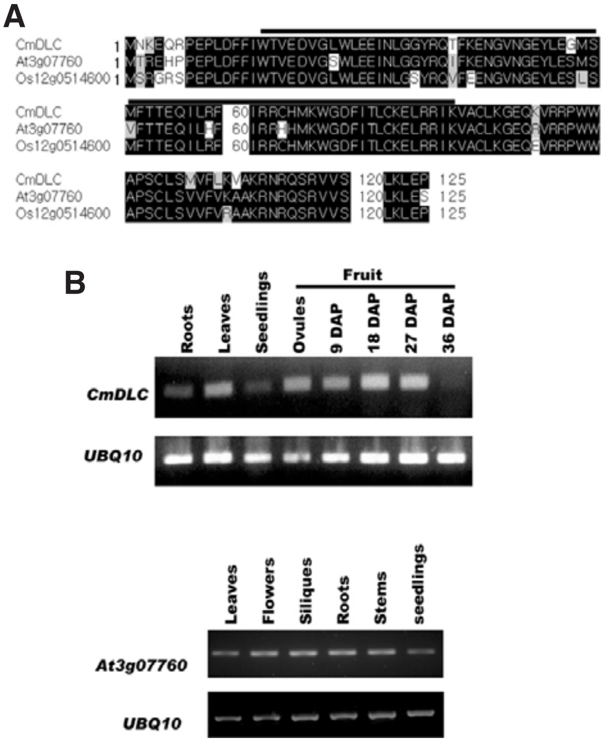

Fig. 1. Cloning and expression of *CmDLC*. (A) Comparison of deduced amino acid sequence of *CmDLC* with its orthologues *Os12g0514600* (*Oryza sativa*), *At3g07760* (*Arabidopsis thaliana*). Identical amino acids are illustrated as white letters on black background; similar amino acids are indicated as white letters on gray background. Black bars indicate the sterile alpha motif (SAM) domain known to be involved in diverse protein-protein interactions. Numbers indicate amino acid sequence length. The accession numbers of *CmDLC* cDNA, *At3g07760, Os12g0514600* in GenBank are AB375061, NM_180207, NM_001073415, respectively. (B) RT-PCR analyses showing the expression of *CmDLC* and its *Arabidopsis* orthologue. Upper panel shows *CmDLC* expression in melon organs and during fruit developmental stages. Roots, roots of 9 day old seedlings; Leaves, mature leaves of 28 day old plants; Seedlings, 9 day old seedlings; Ovules, unfertilized ovules; DAP, days after pollination. Lower panel shows *At3g07760* expression in various organs in *Arabidopsis*. The *UBQ10* RT-PCR was used as an internal standard.

exhibited less or no expression of transgenes (Fig. 2C). To examine the effects of *CmDLC* overexpression on leaf morphology, we measured leaf lengths and widths of 3rd leaves from 5-week-old (fully expanded stage) Col-0 and *CmDLC*-overexpressing plants, s5-3 and s4-3. Leaf widths were reduced both in s5-3 and s4-3, and were 87% and 76% that of wild type (wt), respectively (Fig. 3A). Leaf lengths were also reduced both in s5-3 and s4-3 transgenic plants, and were 73% and 67% that of Col-0, respectively (Fig. 3A). Thus, the leaf length/width ratios of s5-3 and s4-3 were slightly reduced relative to Col-0 (Fig. 3A). Due to the downcurled morphology and no clear distinction of leaf from petiole, the leaves of *CmDLC*-overexpressing transgenic plants appeared to be even narrower than they actually measured (Fig. 2B).

To determine whether the reduced size of leaf blades in the transgenic plants were attributable to a change in cell elongation or cell number, we performed anatomical analysis of epidermal cells. The sizes of epidermal cells on both adaxial and abaxial sides of the leaf were significantly reduced in the central and marginal regions of transgenic plants (Fig. 3B and Table 1). However, the abaxial epidermal cells showed more reduction in size than adaxial cells in transgenic plants (Table 1), indicating that the downcurling leaves of *CmDLC*-overexpressing plants were due to a discriminative reduction in epidermal cell size between the adaxial and abaxial sides.

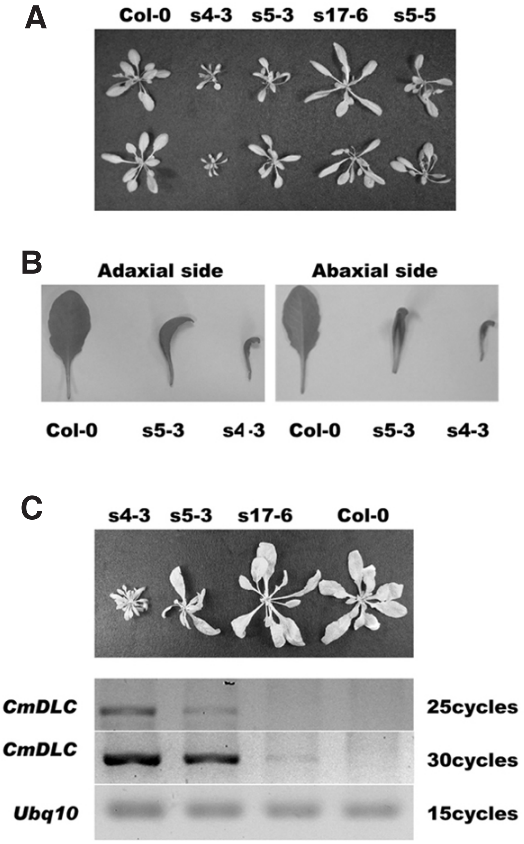

Fig. 2. Phenotypes of transgenic *Arabidopsis* overexpressing *CmDLC*. (A) Picture of whole plants showing mild to severe phenotypes of 4 transgenic lines (s4-3, s5-3, s17-6, s5-5). Some transgenic lines showed severe phenotypes such as retarded growth, narrowed leaves and downward leaf curling (Lanes 2 and 3), while other lines showed a milder phenotype (Lane 4). Lane 1, Col-0; lanes 2-5, transgenic lines: lane 2, s4-3; lane 3, s5-3; lane 4, s17-6; lane 5, s5-5. (B) The enlarged pictures of individual leaves of Col-0 and transgenic lines, s5-3 and s4-3. (C) The severities of the phenotype corresponded to the level of *CmDLC* expression. Plants showing a higher level of *CmDLC* expression showed more severe phenotypes such as stunting and narrowed leaves (lanes 1 and 2). Plants expressing a lower level of transcript showed a milder phenotype. Gene expression was surveyed using semi-quantitative RT-PCR analyses. Cycle numbers indicate # of PCR cycles producing non-saturation levels of the amplified gene.

We also measured the epidermal cell number in wt and transgenic plants. The number of epidermal cells on both the adaxial and abaxial sides along the direction of width was also reduced in transgenic plants compared to those of wt (Table 1). The epidermal cell numbers on the adaxial sides of leaves from the transgenic plants s5-3 and s4-3 were reduced to 87% and 72% that of wt, respectively (Table 1). The epidermal cell num-

Table 1. Anatomical analyses of the rosette leaves of wt and *CmDLC* overexpressing transgenic plants.

	Cell size in central region[a]			Cell size in marginal region[a]			Cell number along the direction of leaf width[a]	
	Adaxial epidermis (μm^2)	Abaxial epidermis (μm^2)	Ratio (Ad:Ab)	Adaxial epidermis (μm^2)	Abaxial epidermis (μm^2)	Ratio (Ad:Ab)	Adaxial epidermis	Abaxial epidermis
wt (Col-0)	6188.8 ± 1377.8 (100%)	5390.7 ± 10.5 (100%)	1:0.87 (100%)	5875.7 ± 1086.6 (100%)	4881.0 ± 1034.1 (100%)	1:0.83 (100%)	151.0 ± 9.9 (100%)	185.0 ± 4.2 (100%)
s4-3	4729.7 ± 40.2 (76%)	3423.0 ± 102.6 (64%)	1:0.72 (63%)	4761.9 ± 36.0 (81%)	3775.9 ± 247.8 (77%)	1:0.79 (95%)	108.0 ± 8.5 (72%)	124.0 ± 2.8 (67%)
s5-3	4609.9 ± 549.2 (74%)	3814.2 ± 93.6 (71%)	1:0.83 (95%)	4894.9 ± 557.3 (83%)	3937.2 ± 319.8 (81%)	1:0.80 (96%)	131.0 ± 12.7 (87%)	145.0 ± 9.9 (78%)

[a] The epidermal cells of fully expanded third leaves were examined. At least seventy epidermal cells were used for cell area calculation for each line. ( ) indicates the percentage of transgenic plants relative to wt. ± indicates standard deviation.

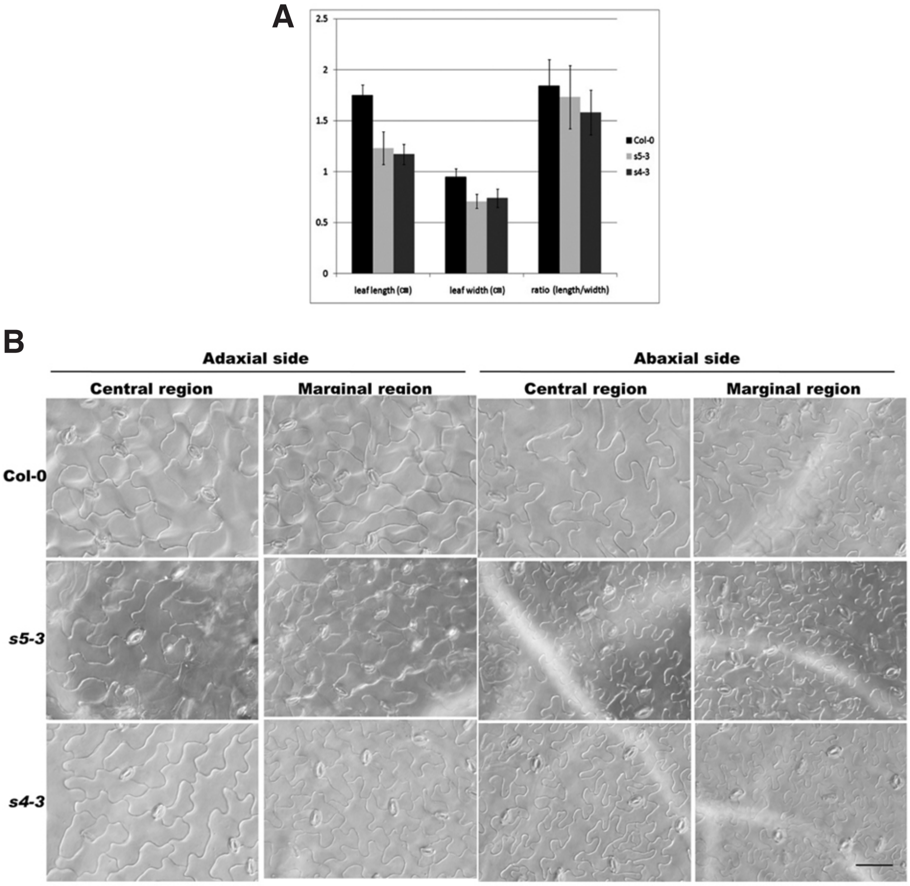

Fig. 3. Leaf phenotypes of *CmDLC* overexpressing transgenic plants. (A) Comparison of leaf length and width of wt and transgenic plants. For measurement, the 3rd rosette leaves of 5 week-old plants were used. (B) The morphology of epidermal cells in wt and transgenic plants. Central (middle half region of each right or left side of leaf blades) and marginal (near the edge of each right or left side of leaf blades) regions of each adaxial and abaxial side of leaves were observed and measured. Scale bar, 50 μm.

bers on the abaxial sides of leaves from the transgenic plants s5-3 and s4-3 were 78% and 67% that of wt, respectively. As with cell size, the epidermal cell number on the abaxial side of the leaves in transgenic plants to that of wt showed a greater reduction than on the adaxial side (Table 1). Taken together, Overexpression of *CmDLC* caused a reduction in leaf size via regulation of cell proliferation and cell expansion in the epidermis of leaves. Furthermore, overexpression of *CmDLC* caused greater reductions in cell proliferation and cell expansion in the abaxial side of leaves, resulting in epinasty. With regard to cell shape, the outlines of pavement cells of transgenic plants were more lobed and wavy than those of wt leaves (Fig. 3B).

We isolated a homozygous knock-down mutant of At3g 07760 (SALK 135261), an orthologue of *CmDLC* from the SALK mutant pool, showing more than 10-fold reduction in gene expression (supplementary data). No phenotypic change in leaf morphology was observed from knock-down mutant (data not shown).

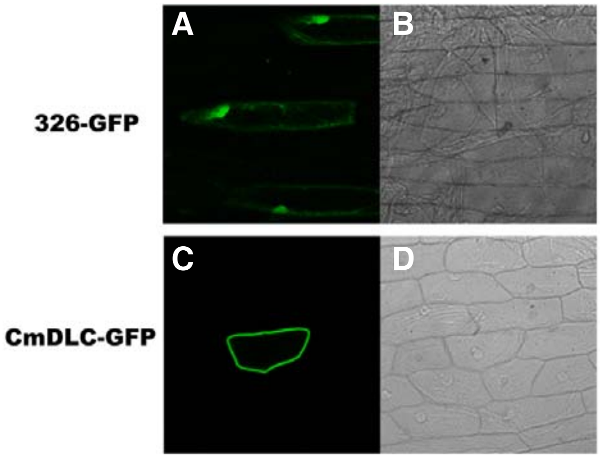

Fig. 4. Fluorescence (A, C) and bright-field (B, D) images of onion epidermal cells expressing CmDLC-GFP fusion and 326-GFP proteins. The 326-GFP was used as a control for GFP localization in the nucleus and cytoplasm.

Subcellular localization of CmDLC protein

To identify the subcellular localization of CmDLC, we made a *CmDLC-GFP* fusion construct and transfected it into onion epidermal cells by biolistic bombardment. As shown in Fig. 4, CmDLC-GFP fluorescence was exclusively observed in the area close to the cell membrane, whereas in control cells, fluorescence for GFP alone was found throughout the cytoplasm and nucleus (Fig. 4).

DISCUSSION

Overexpression of *CmDLC* resulted in pleiotropic phenotypes such as semi-dwarfism, epinastic leaf curling and narrowing of leaves (Fig. 2). Because it is generally considered that epidermal cells have an important role in the process of cell expansion (Dale, 1988), analyses of unfolded leaf shape and epidermal cell morphology and size were carried out to determine the cellular basis of leaf shape changes in the transgenic plants. The overexpression of the *CmDLC* caused reductions in the widths and lengths of leaves, and resulted in a slight reduction in leaf index (the ratio of length/width), indicating that the narrow leaf phenotype was due to curled leaves, and not to reduced width. Similar leaf narrowing phenotypes have been observed in the *lng1-1D* (Lee et al., 2006) and *angustifolia* mutants (Kim et al., 2002; Tsuge et al., 1996). The opposite phenotype, which showed a short petiole and wide and round leaves, was observed in *rot3* mutants (Kim et al., 1998a). In all of these mutants, the genes responsible affected polar cell elongation. In our transgenic plants, reduced leaf size was caused by a reduction not only in the number but also in the size of the epidermal cells (Table 1). The epidermal cell size and number of s4-3 transgenic plants were more reduced that those of s5-3, indicating that higher expression of *CmDLC* caused a greater reduction in cell size and number on both sides of the leaf epidermis. Thus, it is probable that *CmDLC* overexpression might disturb the elongation of cells in both width and length, whereas the *lng1-1D* mutant (Lee et al., 2006) and *angustifolia* mutant showed defects in the polar elongation of cells in the leaf width direction rather than in the leaf length direction (Kim et al., 2002; Tsuge et al., 1996). In addition to a reduction in cell size, transgenic lines had more lobed epidermal cells with a thinner neck relative to wt lines. These changes were more evident when the level of transgene expression was high (Fig. 3B). This alternation of cell morphology also indicates that CmDLC plays an important role in the regulation of cell expansion. Similar changes were also observed in mutants that showed polarity-specific cell elongation (Barry et al., 2001; Tsuge et al., 1996).

To identify its subcellular localization, we expressed a CmDLC-GFP fusion protein in onion epidermal cells. The CmDLC-GFP fusion protein was preferentially localized close to the cell membrane (Fig. 4). Thus, we expect that CmDLC negatively regulates cell size and cell elongation at the cell membrane. In addition, the ratio of abaxial/adaxial cell size in transgenic plants was reduced compared with Col-0 (Table 1). This indicates that the primary reason for epinastic leaf curling was a greater reduction in cell size and number on the abaxial side, rather than the adaxial side, of the leaves.

Several genes involved in leaf curling have been identified recently. Overexpression of *ASYMMETRIC LEAVES2 (AS2)* resulted in upcurling of leaves by reducing the adaxial/abaxial cell number ratio (Iwakawa et al., 2007). Other genes that regulate leaf curling are *Indole-3-acetic acid carboxyl methyltransferase (IAMT1)*, which modifies IAA to methyl-IAA ester (MeIAA), *SPOROCYTELESS (SPL)*, which modulates auxin action, and genes that are related to the formation or function of miRNA, including *HASTY (HST), HYPONASTIC LEAVES1 (HYL1),* and *ARGONAUTE1 (AGO1)* (Li et al., 2008; Qin et al., 2005). Research on these genes has suggested two hypothesizes. One theory is that genes involved in the formation or functioning of miR165/166 regulate leaf flatness (Li et al., 2008); the other possibility is that IAA homeostasis modified by IAMT1 or SPL determines leaf flatness by regulating the expression of leaf developmental genes such as *TCP* and *HASTY* (Li et al., 2008; Qin et al., 2005). Recently, it has also been reported that reduced expression of AUXIN BINDING PROTEIN1 (ABP1) resulted in small epinastically curled leaves, very similar in phenotype to those of our CmDLC overexpressing transgenics (Braun, 2008). In ABP1 anti-sense transgenics, epidermal cell size in leaves was reduced and shape was altered. Additionally, the greater reduction in cell size of abaxial epidermal cells resulted in epinasty (Braun, 2008). Although it is not clear whether CmDLC affects expression or activation of miRNA-related genes and leaf developmental genes, we suggest that CmDLC is connected with one or both of these processes, and regulates the shape and polarity of leaves by adjusting cell division, elongation, and morphology.

There is only one orthologue of *CmDLC* in Arabidopsis, At3g 07760. No knock-out mutant was isolated so far from Arabidopsis and a knock-down mutant showing more than 10-fold reduction in gene expression showed no visible phenotype. Thus, it is probable that At3g07760 is essential gene and it can do normal function with 1/10 of gene expression compared to wt. Considering high amino acid sequence homology among *CmDLC* orthologues, we think they might be involved in similar cell proliferation and cell enlargement process in other plants**.** Due to lack of knock-out mutant plant, studies using gain-of-function plants of orthologues from various plants will help to find how each orthologue function in plant development. We speculate that at least CmDLC and its Arabidopsis orthologue may be involved in whole plant developing process because of its ubiquitous expression. However, we speculate CmDLC is more involved in fruit development of melon than in other plant developing process because it was highly expressed during fruit enlargement process. Thus, it will be interesting to study function of each orthologues using different gain-of-function systems.

For CmDLC, no signal peptide was predicted; instead, a cytoplasmic protein was predicted by PSORT II computer analysis (Nakai, 1999). Therefore, we speculate that CmDLC is a plant-specific protein localized close to the cell membrane. It may negatively regulate cell size and cell number, and alter leaf morphology by changing cell elongation during leaf development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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