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# Possible involvement of DNA methylation on expression regulation of carrot *LEC1* gene in its 5′-upstream region

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ABSTRACT

DNA methylation plays important roles in various developmental processes in many organisms. In carrots, the treatment of embryogenic cells (ECs) with DNA methylation inhibitors induces hypomethylation and blocks somatic embryogenesis. *CARROT-LEAFY COTYLEDON 1* (*C-LEC1*) is an important transcription factor for embryo development that shows embryo-specific expression in ECs and somatic and zygotic embryos. However, the regulation of embryo-specific transcription factor genes such as *C-LEC1* in plants is not well understood.

In this study, we used embryogenic carrot cells (*Daucus carota* L. cv. US-Harumakigosun) to investigate the DNA methylation status of the embryogenesis-related genes *C-LEC1*, *Carrot ABA INSENSITIVE 3* (*C-ABI3*), and *Daucus carota Embryogenic cell protein 31* (*DcECP 31*) during the transition from embryogenesis to vegetative growth. The *C-LEC1* promoter region showed a reduced level of DNA methylation during somatic embryogenesis followed by an increase during the transition from embryonic to vegetative growth. To test whether the increased level of DNA methylation down-regulates *C-LEC1* expression, RNA-directed DNA methylation (RdDM) was used to induce the hypermethylation of two segments of the *C-LEC1* 5′-upstream region: Regions 1 and 2, corresponding to nucleotides −1,904 to −1,272 and −896 to −251, respectively. When the hypermethylation of Region 1 was induced by RdDM, *C-LEC1* expression was reduced in the transgenic ECs, indicating a negative correlation between DNA methylation and *C-LEC1* expression. In contrast, the hypermethylation of Region 2 did not greatly affect *C-LEC1* expression. Based on these results, we hypothesize that DNA methylation may be involved in the control of *C-LEC1* expression during carrot embryogenesis.



## 1. Introduction

DNA methylation is an epigenetic regulatory mechanism of gene expression. DNA methylation causes X-chromosome inactivation (Mohandas et al., 1981), the silencing of transposons (Miura et al., 2001) and other multicopy genes, and genomic imprinting (Bird, 2002). Genomic imprinting, i.e., the modification of a single allele of a particular gene, shows parental dependency. As mentioned above, DNA methylation plays a major role in gene silencing. In mammals, 5-methylcytosine is found almost exclusively as part of CG dinucleotides. DNA methylation may be divided into two types (Li, 2002). The first, known as maintenance methylation, maintains the original methylation pattern in the new strand of DNA upon replication. In the second, known as *de novo* methylation, unmethylated cytosines are methylated. DNA methylation is associated with development and cellular differentiation. In particular, dynamic changes in DNA methylation have been demonstrated during early embryogenesis in mammals. DNA methylation decreases depending on cellular proliferation after fertilization and actively increases as soon as differentiation begins in mice (Jähner et al., 1982). Moreover, DNA methylation regulates the expression of a key factor that is essential for the establishment of pluripotency, which is important for early embryogenesis in mice (Hattori et al., 2004). On the other hand, a mutation in DNA methyltransferase causes abnormal embryogenesis and embryo lethality in mice (Li et al., 1992; Lei et al., 1996; Okano et al., 1999). Based on these findings, DNA methylation is believed to regulate the expression of important genes for embryo development in mammals.

DNA methylation also occurs in plants, not only at CG dinucleotides but also at sites containing CNG (*N* = A, T, G, or C) and CHH (*H* = A, T, or G); in fact, *de novo* methylation is often observed at CNG and CHH sites (Cao and Jacobsen, 2002a). Genes encoding the methyltransferases for CNG and CHH sites, *DOMAINS REARRANGED METHYLTRANSFERASE* (*DRM*) and *CHROMOMETHYLASE3* (*CMT3*), have been

*Abbreviations:* 2,4-D, 2,4-dichlorophenoxyacetic acid; EC, embryogenic cells; NC, non-embryogenic cells; SE, somatic embryo; aza-C, 5-azacytidine; RdDM, RNA-directed DNA methylation; siRNA, small interfering RNA; D-SE, days after induction of the SEs.

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isolated in *Arabidopsis* (Bartee et al., 2001; Lindroth et al., 2001; Cao and Jacobsen, 2002a,b). The *drm1 drm2 cmt3* triple mutant shows a dwarf phenotype and shortened siliques (Chan et al., 2006). In addition, it was reported that the gene responsible for *drm1 drm2 cmt3* triple mutant developmental phenotype has curled leaves and reduced stature. It was suggested that the gene was regulated by DNA methylation via siRNAs (Henderson and Jacobsen 2008). Furthermore, a decrease in DNA methylation influences development and morphogenesis in some plants, such as maize and tobacco (Brown, 1989). In *Arabidopsis*, *decreased in DNA methylation1* (*ddm1*), a hypomethylation mutant, exhibits pleiotropic phenotypes during development, including early flowering, dwarfism, and irregular numbers of organs (Kakutani et al., 1996). In addition, hypomethylation mutant shows reduced seed viability (Xiao et al., 2006). In contrast, it was reported that *FWA*, *FERTILIZATION-INDEPENDENT SEED 2* (*FIS2*), and *MEDEA* (*MEA*) are controlled by genomic imprinting via DNA methylation (Kinoshita et al., 2004; Gehring et al., 2006; Jullien et al., 2006). *FWA* is involved in flowering time (Kakutani, 1997; Soppe et al., 2000) while *FIS2* and *MEA* regulate endosperm development (Preuss, 1999). Recently, it was revealed in *Arabidopsis* that the mutant of *METHYLTRANSFERASE1* (*MET1*), which encodes the methyltransferase for CG sites, shows abnormalities during embryogenesis (Xiao et al., 2006). The expression of *YODA* (*YDA*), which controls embryo and suspensor cell identity, is thought to be affected by DNA methyltransferases during early embryogenesis (Lukowitz et al., 2004; Xiao et al., 2006). These data suggest that DNA methylation in plants regulates the expression of genes with important roles in morphogenesis and development, including embryogenesis. However, the mechanism of regulation for each gene by DNA methylation in relation to physiological events remains to be clarified.

Embryogenesis is one of the most interesting physiological events in plants. The expression of embryogenesis-related genes changes dramatically throughout embryo development and is spatio-temporally regulated in embryonic tissues. To understand the mechanism of embryogenesis, it is necessary to clarify how the expression of embryogenesis-related genes is regulated. In *Arabidopsis*, the genes encoding several embryogenesis-related transcription factors, such as *ABSCISIC ACID INSENSITIVE3* (*ABI3*) and *LEAFY COTYLEDON1* (*LEC1*), have been isolated (Koornneef et al., 1984; Meinke et al., 1994); however, the mechanism of expression regulation is unclear. It is difficult to analyze gene expression during embryo development since embryogenesis proceeds in very limited areas within the seed.

Somatic embryogenesis may allow for the circumvention of the above problem. Somatic embryos (SEs) can develop into young plantlets through morphological changes that are similar to those that occur in zygotic embryos (Zimmerman, 1993). Carrot is one of the most useful model plants for somatic embryogenesis. Numerous researchers have used somatic embryogenesis in carrot, and simple and efficient experimental procedures have been established (Zimmerman, 1993). Since SEs in carrot can develop synchronously, embryos at the same developmental stage can be obtained in large quantities. For this reason, the expression analysis of embryogenesis-related genes during embryo development may be possible using somatic embryogenesis in carrot (Nomura and Komamine, 1985; Satoh et al., 1986). Recently, *C-LEC1* and *C-ABI3*, the *LEC1* and *ABI3* homologs in carrot, were isolated and shown to regulate the expression of *late embryogenesis abundant protein* (*LEA*) genes (Yazawa et al., 2004; Shiota et al., 1998; Shiota and Kamada, 2000).

Several reports have suggested that DNA methylation is important for the development of carrot SEs. The total DNA methylation level is altered during SE development (LoSchiavo et al., 1989). In addition, treatment with hypomethylation-inducing drugs such as 5-azacytidine (aza-C) or 2-amino 5-ethoxy-carbonyl-pyrimidine has been shown to arrest somatic embryogenesis (LoSchiavo et al., 1989; Yamamoto et al., 2005). These data suggest that DNA methylation regulates the expression of embryogenesis-related genes. However, few reports have shown that the expression of embryogenesis-related genes is regulated directly by DNA methylation in plants. To examine the association between the level of DNA methylation and expression of embryogenesis-related genes, we analyzed the status of DNA methylation at *C-LEC1*, *C-ABI3*, and *Daucus carota Embryogenic cell protein 31* (*DcECP31*), which belongs to the *LEA* gene family (Kiyosue et al., 1992, 1993) using somatic embryogenesis in carrot.

## 2. Materials and methods

### 2.1. Plant materials

Carrot (*D. carota* L. cv. US-Harumakigosun) seedlings were grown at 25 °C under a 16-h light/8-h dark cycle (white light at 50 μmol photons $m^{-2}$ $s^{-1}$). Embryogenic cells (ECs) and SEs were isolated as described by Satoh et al. (1986). SEs were induced from the ECs by transferring them from 2,4-D-containing medium to 2,4-D-free medium. Mature leaves were collected from plants that had been grown for about 2 months at 25 °C under the above light conditions.

### 2.2. Southern blot analysis

Carrot genomic DNA was isolated from the leaves of 2-month-old plants, non-embryogenic cells (NCs), ECs, and 14 days after induction SE (14D-SE) using nucleon™ Phytopure (Amersham Pharmacia Biotech, Buckinghamshire, UK). Aliquots (10 μg) of carrot genomic DNA were digested with *Eco*RII; fractionated by electrophoresis on a 0.7% agarose gel; transferred to a Biodyne B nylon filter (Pall BioSupport); and hybridized with [$^{32}$P]-labeled fragments of *C-LEC1* [nt −1963 to −1282], *C-ABI3* [nt +477 to +1190], and *DcECP31* [nt +45 to +815]. Hybridization was performed at 60 °C in a hybridization solution containing 5 × Denhardt's solution, 6 × SSC, 0.5% SDS, and 100 μg of herring sperm DNA. Hybridization signals were detected with a bio-imaging analyzer (BAS-2000; Fuji, Tokyo, Japan).

### 2.3. Methylation analysis using the bisulfite sequencing method

The DNA methylation status of *C-LEC1* and *C-ABI3* was investigated using the bisulfite sequencing method (Figs. 2 and 5A). A total of

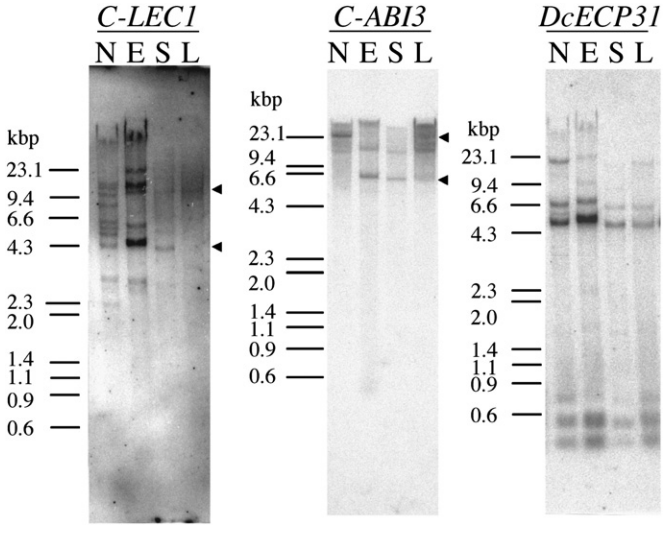


**Fig. 1.** Analysis of the DNA methylation pattern for embryogenesis-related genes using methylation-sensitive restriction enzyme. Southern blotting using *Eco*RII (methylation-sensitive restriction enzyme at $CC^{A}/_{T}GG$)-digested genomic DNAs was performed using the embryogenesis-related genes *C-LEC1* (left), *C-ABI3* (middle), and *DcECP31* (right) as probes. Genomic DNA was extracted from non-embryogenic cells (N), embryogenic cells (E), 14-day-old somatic embryos (S) induced from the same EC cell line, and leaves (L). Arrowheads indicate different bands between the tissues.