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The Phenotype of the Soybean Disease-Lesion Mimic (*dlm*) Mutant is Light-Dependent and Associated with Chloroplast Function

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The *dlm* (disease lesion mimic) mutant of soybean (*Glycine max* L. Merr) shows the similar lesion of a soybean disease caused by a fungus, *Corynespora cassilcola*. The lesion was examined at cellular and molecular level. Trypan blue staining result indicated that cell death was detectable in the entire region of leaves excluding veins when the lesions had already been developed. We found that the mesophyll cells of palisade layer in the *dlm* mutant appeared to be wider apart from each other. The chloroplasts of the *dlm* mutant cells contained bigger starch granules than those in normal plants. We also found that the lesion development of *dlm* plant was light-dependent and the starch degradation during the dark period of diurnal cycle was impaired in the mutant. Three soybean pathogenesis-related genes, *PR-1a*, *PR-4*, and *PR-10*, were examined for their expression patterns during the development of disease lesion mimic. The expression of all three genes was up-regulated to some extent upon the appearance of the disease lesion mimic. Although the exact function of DLM protein remains elusive, our data would provide some insight into mechanism underling the cell death associated with the *dlm* mutation.

Keywords : cell death, chloroplast, defense, disease lesion mimic, soybean

Cellular damage may be incurred when a plant is stressed by a pathogenic infection. This can lead to further damage and/or uncontrolled cell death if it is not repaired or contained. Controlling the extent of cell damage is an important feature of the plant defense response. Rapid localized cell death is the predominant feature of the hypersensitive cell death response elicited by avirulent pathogens during a resistance response (Kim and Martin, 2004). The hypersensitive response involves rapid necrosis of infected cells and may include the formation of a visible lesion. Cell death also occurs during susceptible response. In this case, cell death may be deleterious to the host, since nutrients can be released and pathogen can subsequently multiply and spread in the plant.

The soybean mutation, *dlm* (disease lesion mimic), is characterized by the formation of small necrotic spots surrounded by chlorotic halos (Chung et al., 1998). This phenotype began to appear on aging leaves as the mutant plants developed into floral transition and expands continuously to almost all of the leaves. This causes the mutant plant to undergo senescence about a week earlier than its wild-type plants. Its seed yield was also lower than that of its wild-type plant. Some of the larger necrotic spots consisted of concentric yellow and brown rings of dead tissues. This is similar to a typical lesion of a soybean disease caused by the fungus *Corynespora cassiicolar* (Athow, 1987). Finally, this mutation is strictly inherited in a recessive fashion, showing a Mendelian segregation ratio. Based on the phenotype of the *dlm* mutant, it appears to show some of the characteristics of the disease lesion mimicry mutation. The disease lesion mimic mutants have been used to elucidate the molecular mechanisms of plant cell death (Lorrain et al., 2003). The lesion mimic genes encode a variety of functions, including membrane receptors (Buschges et al., 1997; Collins et al., 1999; Devoto et al., 1997; Hu et al., 1996; Sun et al., 2001), a putative transcription factor regulating superoxide dismutase (Kliebenstein et al., 1999), and salicylate and sphingolipid signaling (Broderson et al., 2000; Rate et al., 1999). The genes underlying other lesion mimic phenotypes appear to play more direct roles in the maintenance of cellular homeostasis. For example, the blockage of metabolic processes, such as the synthesis or degradation of chlorophyll results in the accumulation of porphyrin intermediates, which become toxic free radicals when cells are exposed to excess light (Hu et al., 1998; Ishikawa et al., 2001). A deficiency in fatty acid biosynthesis caused pleiotropic effects on plant growth and resulted in premature cell death (Mou et al., 2000).

Although studies of lesion mimics have identified genes, only a few studies have revealed the detail mechanisms on cell death, especially on programmed cell death (PCD)

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(Greenberg et al., 1997). Thus far, although it has been observed in animals, no example of apoptotic-type cell death has been observed in plants (Doorn and Woltering, 2005). Our knowledge regarding plant cell death remains rudimentary.

In this study, we report that the formation of disease lesion mimic on the leaves of soybean *dlm* mutant is light-dependent and also we show cytological characteristics of cell death in *dlm* mutant and unique expression patterns of biochemical markers with potential roles in systemic acquired resistance.

Materials and Methods

Plant materials and growth conditions. Etiolated soybean seedlings (cotyledons and hypocotyls) were obtained by germinating seeds in darkness at 28°C for 3 days in a growth chamber. For the microscopy study, soybean seedlings were grown in a greenhouse for 3 weeks, and were then grown in the growth chamber at 28°C under short-day conditions (10 h light/14 h dark) for 2 more months. Leaves were harvested at 30 min before and 8 h later after the subjective sunrise. To examine the expression of the PR genes, the leaves from the *dlm* and Sinpaldal2 plants grown in a greenhouse were harvested. Samples from all treatments were frozen in liquid nitrogen immediately after harvesting. They were used immediately or were stored at −70°C until use.

Trypan blue staining. Trypan blue staining was performed according to the method of Vogel and Somerville (2000). Whole leaves were examined by treating them in the LPTB solution for 5 min at 70°C. Samples were then cooled at room temperature for 1 h after replacement of the LPTB without trypan blue. Leaves were cleared of chlorophyll by incubating in 70% ethanol.

Light microscopy and Electron microscopy. After vacuum infiltration for 4h, soybean leaves at relevant stages were fixed overnight at 4°C in a solution containing 2.5% glutaraldehyde and 4% para-formaldehyde in 100 mM phosphate buffer (pH 7.4). They were then rinsed in 0.1 M phosphate buffer (pH 7.4) and further fixed in 1% (w/v) osmium tetroxide (OsO_4) for 4 h at 4°C. After rinsing in 0.1 M phosphate buffer, the samples were dehydrated and embedded in LR white resin (London Resin Company, UK). Thin sections (40-50 nm thickness) were prepared with an ultra-microtome (LKB, Bromma 2088) and were collected on nickel grids (1-GN, 150 mesh). The tissue sections were then observed under a light microscope. These sections were stained with uranyl acetate and lead citrate and were then examined under a transmission electron microscope (JEM-100CX-1).

PR gene expression. Total RNA was isolated using Tri reagent (MRC Inc, OH, USA) from one gram of soybean leaf, stem, or root according to the instructions of the manufacturer. The cDNA synthesis was prepared using total RNA as previously described (Kim et al., 2005). Semi-quantitative RT-PCR was performed according to the method of Shin et al. (2004). As a control, the PCR was performed for soybean ubiquitin mRNA (Kim et al., 2005). The amplification conditions were: 5 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at the annealing temperature of each primer set, and 1 min at 72°C. Annealing temperatures were 64°C for WIN-like gene (*PR-4*, 246 bp) and SAM gene (*PR-10*, 377 bp), and 58°C for *PR-1a* gene (365 bp). The primer sets for each gene were published previously (Graham et al., 2003).

Results

***Dlm* allele mutant shows a disease-like cell death phenotype.** The *dlm* allele mutant was isolated by screening mutant lines of soybean that had been mutagenized by gamma-irradiation for a lesion mimic phenotype showing spontaneously activated cell death. The *dlm* phenotype segregated as a recessive trait. The homozygous *dlm* mutant did not display a freckling on the leaves (Chung et al., 1998). The *dlm* phenotype followed a developmental gradient, with lesions forming first on old, aging leaves, and then progressively forming on every leaf up the body of plant when the plant begins to flower. These necrotic spots surrounded by chlorosis merged to form larger lesions later that were observed throughout the leaves, and eventually spread to whole soybean plant (Fig. 1B). Leaf senescence with chlorophyll breakdown was also accelerated in this mutant(data not shown). These results suggest that lesions on the *dlm* mutant are developmentally regulated. However, within a leaf that has attained developmental competence, *dlm* lesions appear to form in a random pattern.

To examine whether these appearance of lesion mimic phenotype was a result of cell death in *dlm* mutant or not, the leaves with apparent lesion of *dlm* mutants were stained with lacto-phenol-trypan blue solution, a histochemical indicator of cell death or membrane damages. Figure 1D shows deep blue staining in cells at the site of necrosis in *dlm* mutant. Blue staining was not observed in young leaves of *dlm* mutant in which lesion mimic could not be seen. These phenotypes of cell death were similar to those that are seen during the HR or disease symptom caused by the soybean pathogen, *Corynespora cassiicolor*. However, the veins were not affected by staining with trypan blue. This suggests that non-photosynthetic vascular cells were

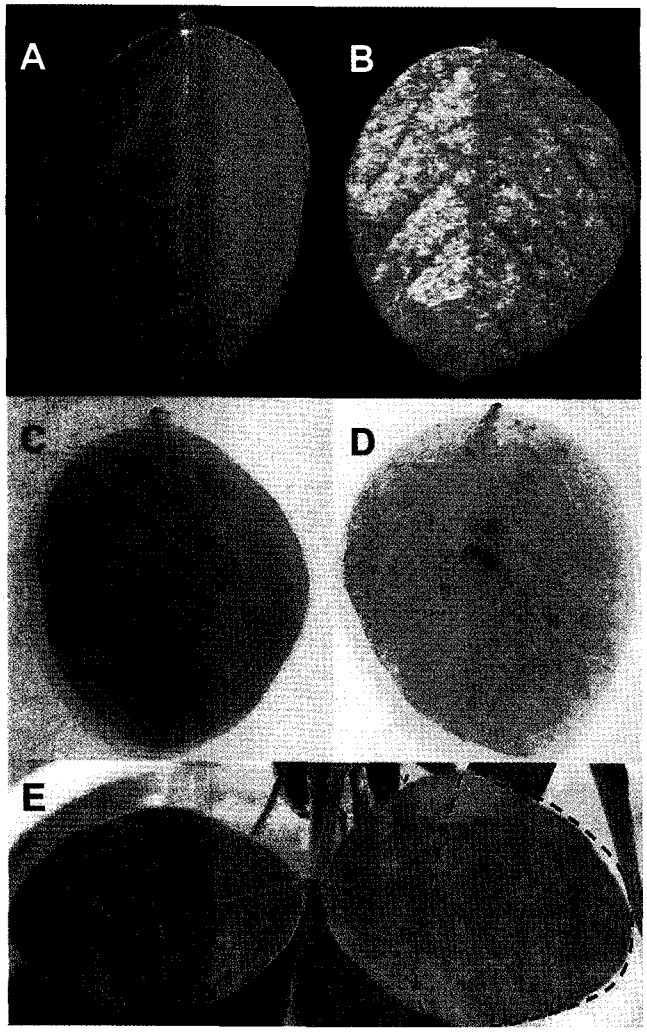

Fig. 1. Phenotypic features of the soybean *dlm* mutation. (B) Typical pattern of the dlm mutation on an 4-week-old soybean plant. (D) Trypan blue staining of the *dlm* mutant leaf before and after the appearance of its phenotype. (E) Light-dependent phenotype of the soybean disease lesion mimic. The dot-marked area of the right side of a leaf was covered with aluminium foil. Both the leaves showing the phenotype or not showing the phenotype were taken from the same *dlm* mutant plant. The phenotype began to appear on old leaves at approximately the same time as the floral transition occurred. The leaf of Sinpaldal 2 cultivar was used as control.

not affected by the *dlm* mutation.

Light is required for spontaneous cell death on the leaves of *dlm* mutant. To characterize the spontaneous cell death on *dlm* mutant, we identified environmental factors such as light, day length, and relative humidity to affect the development of cell death phenotype. When parts of soybean leaves were protected from light by covering aluminum foil prior to the appearance of *dlm* lesions, soybean leaves failed to develop disease lesion mimics (Fig. 1E). The lesions did not form on covered parts of the leaf, and the lesions which had already formed did not expanded into the covered regions. This result indicates that light plays a role in the induction of the *dlm* lesions, in addition to their expansion. The potential involvement of relative humidity in *dlm* lesion development was also investigated. However, high relative humidity (95%) did not prevent phenotype development in the *dlm* mutant (data not shown).

The *dlm* mutant shows a defect in starch degradation of chloroplasts during diurnal fluctuations. To characterize the cellular events which may be causally involved in the death of *dlm* cells, cells in and around the lesions were examined by light and electron microscopy. We examined cells around the lesions of the *dlm* mutant grown under greenhouse conditions (Fig. 2A). One difference between the *dlm* mutant and the wild-type was that the intercellular spaces of the *dlm* mutant expanded wider, especially among mesophyll cells in the palisade layer, which made it appear that the mutant contained fewer mesophyll cells overall, as compared to the wild-type. The other differences were that there were small vesicles in the chloroplasts of the mutant, and the size of starch granules in its chloroplasts appeared bigger (Fig. 3A).

To investigate whether the chloroplast was involved in the development of disease lesion mimic, we have examined the cellular structures of the *dlm* mutant grown under diurnal conditions (14 h light and 10 h dark, 28°C) (Fig. 2B and 3B). The size and number of starch granules in the

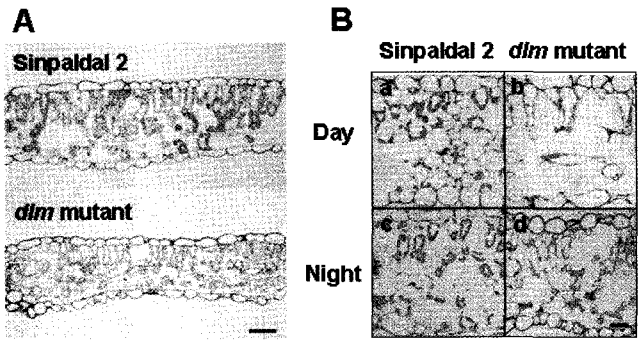

Fig. 2. Cellular characteristics of the *dlm* mutant leaf tissue. A. The leaves of Sinpaldal 2 cultivar (A upper) and the *dlm* mutant (A lower) were examined under a light microscope. In case of the *dlm* mutant leaf, the intercellular spaces seemed wider, especially among mesophyll cells in the palisade layer and the size of starch granules in its chloroplast appeared bigger. B. Morphological changes of the *dlm* mutant leaf cells under diurnal condition. The leaf cells were examined at 30 min before (a, b) or 8 h after (c, d) the subjective sunrise under long day condition (14 h light and 10 h dark). When the *dlm* mutant developed its phenotype, deformed cells were observed (b, d) whereas cells looked normal when it did not show its phenotype yet (a, c).

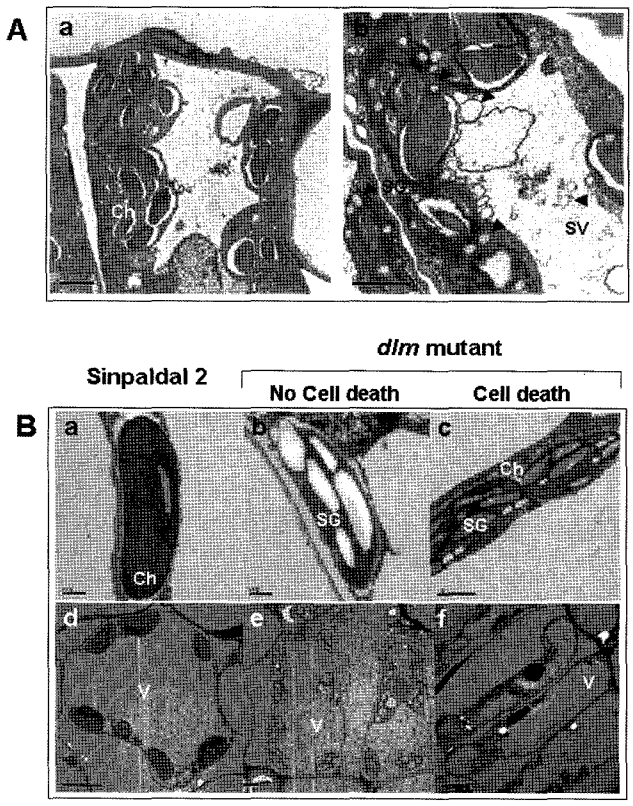

Fig. 3. Transmission electron microscopy of mesophyll cell chloroplasts of the *dlm* mutant. Cells were examined at 30 min before (a, b, c) or 8 h after (d, e, f) the subjective sunrise under long day condition (14 h light and 10 h dark). The *dlm* mutant without (b and e) or with (c and f) its phenotype. Sinpaldal 2 cultivar undergone through normal senescence (d) or before senescence (a). Before the *dlm* mutant showed any lesions, large starch granules remained in chloroplasts during the dark period (b). After the lesion appeared, however, chloroplasts of those un-deformed cells still seemed to maintain their integrity during the dark period, although the size and number of their starch granules seemed to decrease (c).

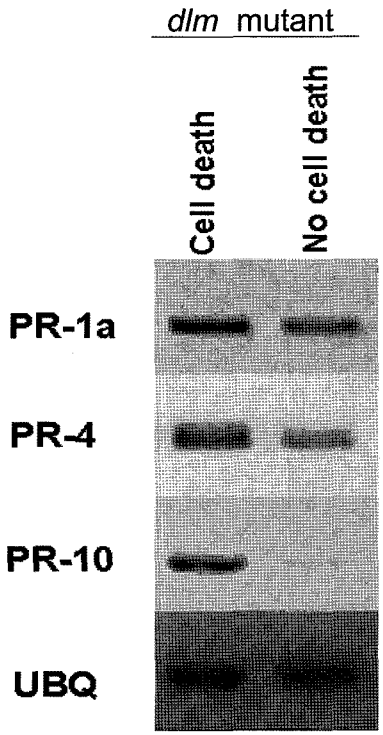

Fig. 4. Expression profiles of the soybean PR genes in the *dlm* mutant. Total RNA were isolated from leaves. Semi-quantitative RT-PCR analysis was applied to measure the relative mRNA abundance of three soybean PR genes, *PR-1a* (accession no. AF136636), *PR-4* (WIN-like, accession no. X60043), and *PR-10* (SAM22, accession no. Z11977). The soybean ubiquitin (UBQ) mRNA level was examined as control.

chloroplast decrease at the end of the dark period and increase again during the light period of the diurnal fluctuation. The most prominent change in the *dlm* mutant during the diurnal fluctuation was that very little decrease, if any, was observed in the size or number of starch granules in its chloroplasts during the dark period. After the *dlm* lesion appeared, the cells in and around the lesion seemed to undergo the cell death process regardless of light and dark periods (Fig. 3B). For example, some cells showed deformed under a light microscope. Electron microscopy more clearly revealed morphological changes at the cellular level (Fig. 3B). Before the *dlm* mutant showed any lesions, large starch granules remained in chloroplasts during the dark period. After the lesion appeared, chloroplasts of the un-deformed cells still maintain their integrity during the dark period, although the size and number of their starch granules decreased. During the light period, nuclei seemed to maintain their morphology, whereas the thylakoid membranes of chloroplasts appeared to be disintegrated, although starch granules still existed. The cellular phenotype of the *dlm* mutant was different from that of leaf senescence (Fig. 3B). During leaf senescence, leaf cells were highly vacuolated and their cell organelles, such as chloroplasts, were already degraded; only small volumes of cytoplasm remained. These data are consistent with some characteristics that could be observed in plant programmed cell death (Jones, 2001).

Three PR genes are induced in the *dlm* mutant. mRNA accumulation of PR genes was strongly correlated with the onset of systemic acquired resistance (SAR) in dicot as well as in lesion mimic mutants in both dicots and monocots (Dietrich et al., 1994). To test whether spontaneous lesion formation correlated with the expression of PR genes, total RNA was isolated from leaves with lesion or without lesion of *dlm* mutant. The *dlm* mutant was analyzed for their expression of the soybean PR-genes, PR-1a, PR-4, and PR-10 using RT-PCR, all analysis took place on 3-weeks old

plants. As shown in Fig. 4, these PR genes were activated in the *dlm* mutant at different levels during the lesion development. For the PR-10 gene, the lesion area in the leaf of *dlm* mutant showed highest expression whereas the same mutant before lesion formation showed very low expression. Little or no PR-1a, PR-4 and PR-10 expression was detected in the same stage of wild type Sinpaldal 2 (data not shown). Expression of PR genes in *dlm* mutant during lesion formation suggests that these genes were correlated with spontaneous cell death.

Discussion

Light is known to aggravate cell death in plants, as documented in the case of hypersensitive response (HR), in many lesion mimic mutations, in barley aleurone cells, and in fumonisin-induced cell death in Arabidopsis (Hu et al., 1998; Jabs et al., 1996; Mach et al., 2001; Mock et al., 1998; Shirasu and Schulze-Lefert, 2000; Stone et al., 2000). One way that cell death was promoted was through the production of free radicals by light (Hipeli et al., 1999; Jabs, 1999). For instance, in the case of *les22* plants, the production of excess porphyrin free radicals is the precipitating cause of cell death (Hu et al., 1998; Mock et al., 1998). Similarly, in *acd2* plants, it is the photo-activation of the red chlorophyll catabolite that triggers free radical production and subsequent cell death (Mach et al., 2001). The fact that *lls1* lesions do not develop in cells lacking chlorophyll is consistent with the idea that *lls1* cell death occurs in a similar fashion. The inability to remove photo-activatable chlorophyll intermediates could explain the existence of the developmentally regulated formation of lesions only in green tissue.

In animals, mitochondrial membrane changes which result in mitochondrial swelling have been noted in apoptotic cells that assist in the generation of reactive oxygen species (ROS), and in the release of a number of apoptogenic factors (such as cytochrome c and apoptosis-inducing factor) from the intermembrane space of mitochondria to the cytosol (Hengartner, 2000; Simon et al., 2000; Von Ahsen et al., 2000). We could not observed any mitochondrial changes in dying *dlm* mutant cells. Although trypan blue staining was performed to examine the dead cells, diaminobenzidine staining to detect hydrogen peroxides would have revealed the involvement of ROS generation in the development of the *dlm* mutant phenotype (data not shown).

A key finding of this study is that a defect in the starch degradation of chloroplasts during the subjective night is the most conspicuous feature of dying *dlm* cells. Additionally, the *dlm* cells contained larger starch granules in chloroplasts, as compared to Sinpaldal 2 cultivar cells. Distortion of the chloroplast has been reported in other plant cell deaths (Mou et al., 2000). Chloroplasts of tobacco plant cells undergoing TMV-induced PCD in the HR also had starch granules of increased size (Weintraub and Ragetli, 1964). With a few exceptions, the morphology of mitochondria appeared more stable than chloroplasts during the HR cell death process (Goodman and Novacky, 1994). It is not yet known whether the increased size of starch granules in tobacco chloroplasts during the HR cell death process is due to a defect in starch degradation. Another PCD feature in the *dlm* cells is lack of or slight cytoplasmic degeneration prior to the collapse of vacuoles. In senescing cells, however, chloroplast degradation occurs well prior to vacuole collapse. During the plant cell death process, the cell must be "metabolically active" to synthesize the hydrolases it needs to process its corpse; therefore, plant cells saves the hydrolases in vacuoles and releases them when the vacuole collapses. In order to synthesize more hydrolases, cells would need to produce more starch. This may result in the change in chloroplast starch granules in *dlm* cells, as well as the tobacco cells undergoing TMV-induced PCD.

Many lesion mimic mutants exhibit a state of increased disease resistance, referred to as systemic acquired resistance, and show high, constitutive levels of pathogenesis-related (*PR*) gene expression (Dietrich et al., 1994; Park et al., 2004). However, disruptions of cellular physiology apparently unrelated to disease defense can also trigger cell death and SAR (Mock et al., 1999; Molina et al., 1999). This can make it difficult to determine whether or not genes defined by lesion mimic mutants play a direct role in the signaling and control of cell death. Additionally, many Arabidopsis mutants (*cpr1*, *cpr5*, *edr1*, *mpk4*) have been identified to show constitutive expression of defense-related genes in the absence of cell death (Bowling et al.; 1994, 1997; Clarke et al., 1998; Frye et al., 2001; Petersen et al., 2000). This indicates that, although the HR can trigger systemic *PR* gene expression and SAR, cell death is not always required for *PR* gene expression and SAR to occur. In this study, we have observed the up-regulation of the three *PR* genes in *dlm* plants. However, we must postpone interpreting the significance of this result until we are able to identify the gene responsible for the *dlm* phenotype.

The developmental pattern of lesion formation in the *dlm* plant raises the question of why the lesions always initially appear in old leaves, and never in young leaves. Manzano et al. (2004) showed that the lesion mimic phenotype which appeared only in the mature leaves of *Arabidopsis thaliana* was triggered by a developmental decline in endogenous 3-hydroxy-3-methylglutaryl-CoA reductase activity. Further possible study would be an investigation of whether the *dlm* mutant lesion is also triggered by the developmental incline or decline of endogenous activity of 3-hydroxy-3-methyl-

glutaryl-CoA reductase.

Acknowledgments

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