Mol. Cells 2015; 38(3): 243-250
http://dx.doi.org/10.14348/molcells.2015.2297

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Comparative Analysis of the Conserved Functions of Arabidopsis DRL1 and Yeast KTI12

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Patterning of the polar axis during the early leaf developmental stage is established by cell-to-cell communication between the shoot apical meristem (SAM) and the leaf primordia. In a previous study, we showed that the *DRL1* gene, which encodes a homolog of the Elongator-associated protein KTI12 of yeast, acts as a positive regulator of adaxial leaf patterning and shoot meristem activity. To determine the evolutionally conserved functions of DRL1, we performed a comparison of the deduced amino acid sequence of DRL1 and its yeast homolog, KTI12, and found that while overall homology was low, well-conserved domains were presented. DRL1 contained two conserved plant-specific domains. Expression of the *DRL1* gene in a yeast *KTI12*-deficient yeast mutant suppressed the growth retardation phenotype, but did not rescue the caffeine sensitivity, indicating that the role of Arabidopsis Elongator-associated protein is partially conserved with yeast KTI12, but may have changed between yeast and plants in response to caffeine during the course of evolution. In addition, elevated expression of *DRL1* gene triggered zymocin sensitivity, while overexpression of *KTI12* maintained zymocin resistance, indicating that the function of Arabidopsis DRL1 may not overlap with yeast KTI12 with regards to toxin sensitivity. In this study, expression analysis showed that *class-I KNOX* genes were downregulated in the shoot apex, and that *YAB* and *KAN* were upregulated in leaves of the Arabidopsis *drl1-101* mutant. Our results provide insight into the communication network between the SAM and leaf primordia required for the establishment of leaf polarity by mediating histone acetylation or through other mechanisms.

INTRODUCTION

Leaves are dedicated photosynthetic organs in plants, and diverse morphologies in various species allow them to inhabit a wide range of natural environments. Leaf organization is accomplished through serial processes according to specific temporal and spatial demands. Leaves originate postembryonically in a radial pattern from the periphery of the shoot apical meristem (SAM; Reddy, 2008), after losing their stem cell identity as meristem cells in response to a cell differentiation signal. Cells located in the peripheral regions of the SAM grow as leaf primordia (LP) and acquire leaf identity. During the early LP developmental stage, the LP establish three axes of leaf polarity: adaxial-abaxial, proximal-distal, and medial-lateral (Cho et al., 2007; Hasson et al., 2010). Adaxial-abaxial polarity is involved directly in the optimal formation of structures specific for the functions of the upper and lower surfaces of leaves. During the leaf maturation stage, the differentiation of vascular tissues also exhibits adaxial-abaxial polarity. Several studies have identified polarity determinant genes and revealed their cooperation and antagonism in the establishment of adaxial-abaxial polarity. Adaxial-determining homeodomain-leucine zipper (*HD-ZIPIII*) genes, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*), and abaxial-determining genes of the *KANADI* (*KAN*) family in Arabidopsis (*Arabidopsis thaliana*), regulate each other by mutual inhibition of expression and activities (Izhaki and Bowman, 2007; Sarojam et al., 2010; Tsukaya, 2013). In addition, the *YABBY* (*YAB*) gene family integrates a genetic cascade for abaxialization by acting downstream of all abaxial polarity determinants (Eshed et al., 2001; 2004; Siegfried et al., 1999). MicroRNA miR165/166, which accumulates in the abaxial domain of the LP, regulates *PHB/PHV* transcript levels in the abaxial side (Kidner and Martienssen, 2004). In addition, *trans*-acting small interfering RNAs (*ta*-siRNAs) are also reported to downregulate the expression of members of the *AUXIN RESPONSE FACTOR* (*ARF*) gene family, *ETTIN (ARF3)* and *ARF4*, indicating that auxin signaling plays an important role in the patterning of leaf polarity (Chitwood et al., 2009; Griffiths-Jones et al., 2006; Hunter et al., 2006). The Elongator complex, which has histone acetyltransferase (HAT) activity and participates in several processes including RNA polymerase II (RNAPII) transcriptional elongation, tRNA anticodon modification and growth inhibition by the fungal tRNase zymocin toxin complex (Fichtner et al., 2002; Frohloff et al., 2001; Huang et al., 2005; Mehlgarten et al., 2010; Otero et al., 1999; Wittschieben et al., 1999), has been reported to regulate leaf polarity by indirectly repressing abaxial determining families and *class-I KNOX* family in Arabidopsis (Kojima et al., 2011; Nelissen et al., 2010). In addition, *CENTER CITY* (*CCT*)

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Received 5 November, 2014; revised 3 December, 2014; accepted 3 December, 2014; published oneline 16 December, 2014

Keywords: *Arabidopsis thaliana*, *DRL1*, elognator-associated protein, leaf polarity, yeast KTI12

eISSN: 0219-1032

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and *GRAND CENTRAL* (*GCT*) are identified as the Arabidopsis orthologs of *MEDIATOR12* (*MED12*) and *MED13*, respectively and are involved in the regulation of *KAN* expression independently of *PHB* in peripheral-abaxial identity (Gillmor et al., 2010). Collectively, these reports indicate that cell position in plants is vital for determining the cell type and structure, and leaf morphology. Therefore, partitioning of leaves through the establishment of leaf axes plays an important role in overall leaf organization. Although the patterning of leaf polarity is based on complicated communications between the SAM and the LP during the early leaf developmental stage (Sussex, 1954), the mechanism that regulates this process turns out a little bit.

In previous studies, it was reported that the Arabidopsis *Deformed Roots and Leaves 1* (*DRL1*) gene encodes a homolog of Elongator-associated protein, yeast KILLER TOXIN INSENSITIVE12 (KTI12) protein, and acts as a positive regulator of adaxial leaf patterning and shoot meristem activity (Cho et al., 2007; Fichtner et al., 2002; Frohloffet et al., 2001; Nelissen et al., 2003). Although biochemical approaches revealed that KTI12 did not affect the HAT activity of Elongator, deletion of the *KTI12* gene resulted in Elongator-linked phenotypes, indicating that KTI12 overlaps functionally with Elongator (Petrakis et al., 2005). In addition, KTI12 was shown by chromatin immunoprecipitation to bind chromatin throughout the genome. The yeast *KTI12* gene was identified originally from *Saccharomyces cerevisiae* in a screen for resistance towards zymocin (Butler et al., 1994; Fichtner and Schaffrath, 2002; Frohloff et al., 2001), a tRNase ribotoxin from *Kluyveromyces lactis* that inhibits the growth of sensitive yeast species (Jablonowski and Schaffrath, 2007; Jablonowski et al., 2006; Lu et al., 2005; Nandakumar et al., 2008). In addition to zymocin resistance, yeast strains deleted for the *KTI12* gene exhibit hypersensitivity to temperature and caffeine (Fichtner et al., 2002; Frohloff et al., 2001). Elongator (ELP) complex contains six subunit which form the core complex (ELP1-ELP3) and a second module (ELP4-ELP6; Winkler et al., 2001). In Arabidopsis, loss-of-function mutants of ELP showed pleiotropic effects including narrow leaves, disorganized SAM, short roots, high sensitivity to ABA and deficiencies in basal immunity (Chen et al., 2006; DeFraia et al., 2010; Nelissen et al., 2003; 2005; 2010; Zhou et al., 2009).

To further clarify the role of chromatin remodeling and transcription regulation during leaf development, we studied the structural and functional features of DRL1 involved in the patterning of leaf polarity. We analyzed evolutionally conserved functions of DRL1 and alterations in the SAM and leaves of the Arabidopsis *drl1-101* mutant. Our comparative analysis of the function between Arabidopsis *DRL1* and yeast *KTI12* provides insight into the mechanism that regulates communication between the SAM and LP for proper establishment of leaf polarity.

MATERIALS AND METHODS

Plant and yeast materials and growth conditions

The *drl1-101* was isolated from Ds-transposon insertional mutants in Arabidopsis Nossen-0 (No-0) background, as described by Cho et al. (2007). Seeds of plants were surface sterilized, germinated on Murashige and Skoog (MS) media and transferred to soil after 3 weeks. Plants were grown at 23°C under long-day light condition (50-100 $\mu E/m^2s$, 16 h light/8 h dark). All yeast strains are from *Saccharomyces cerevisiae* (Supplementary S1). *KTI12*-deficient mutant (YKL110C) and wild type (WT; BY4741) was used for thermosensitivity and caffeine sensitivity. *KTI12*-deficient mutant (LFY12) used for zymocin sensitivity assay was generated by interruption of *KTI12* region by insertion of fragment from pYF6, YEp213 carrying the LEU2 (Butler et al., 1994). Yeast strains were routinely grown on standard rich and minimal growth media, YPD or SC media at 30°C (Sherman, 1991). To test complementation of thermosensitivity and caffeine sensitivity, yeast strains grew on media at 30°C or 39°C or on media with or without caffeine (Sigma, USA), respectively. For zymocin toxin sensitivity assay, WT (LS20) and *kti12Δ* (LFY12) mutant were transformed with galactose inducible γ-toxin gene expression vector, pHMS14 (Frohloff et al., 2001).

Amino acid sequence alignment

DRL1 and homologs nucleotide and amino acid sequences from various organisms were retrieved from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/"www. ncbi.nlm.nih.gov) and amino acid sequences of DRL1 and homologs were aligned according to CLUSTALW multiple alignment program.

Microscopic observation

For anatomical analysis, samples were fixed in fixation solution under a vacuum, dehydrated by graded ethanol series and clarified, as described by Cho et al. (2007). Samples were observed on stereoscopic microscope (Leica MZ12.5, Leica, Germany) and light microscope (Axioskop2, Carl Zeiss, Germany). For scanning electron microscope, we followed the method described by Cho et al. (2007). Palisade cells localized between in the midvein and in the margin of leaf blades were analyzed for measuring the cell number and size.

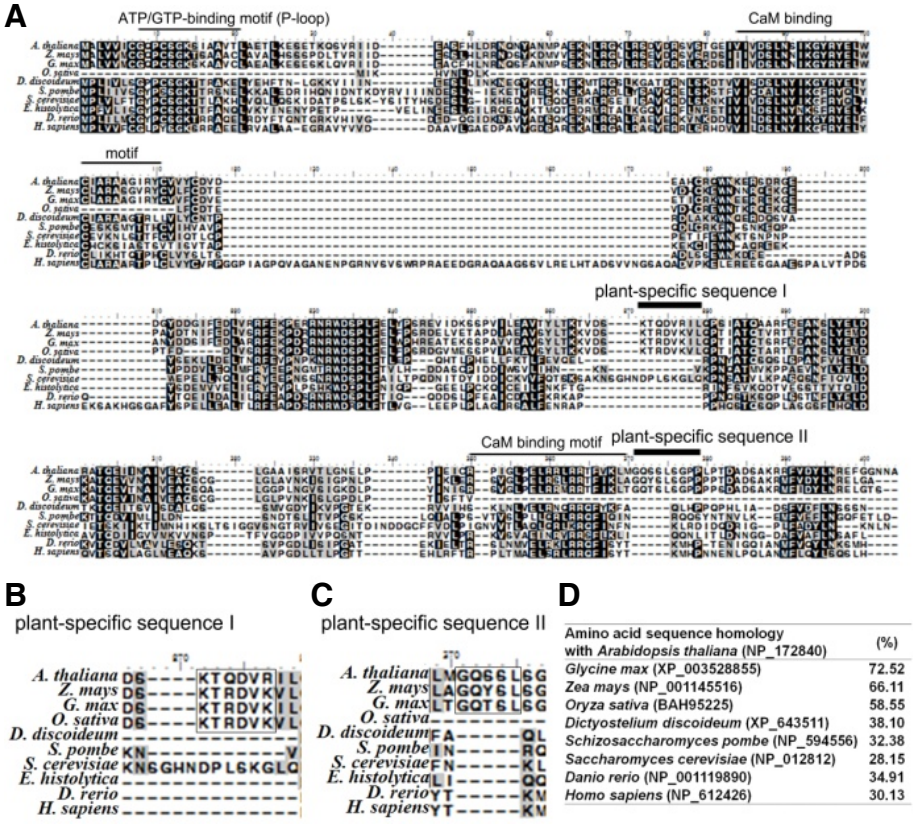
Cloning of Arabidopsis and rice DRL1 and semi-quantitative RT-PCR

Arabidopsis *DRL1* gene and its rice ortholog (*OsDRL1*), At1g13870 and Os11g0312782, respectively, were amplified from Arabidopsis and rice total mRNA using specific primer pairs and cloned, as described by Cho et al. (2007). Cloned *DRL1* genes were transferred into yeast expression vector, pTU1 derived from pRS316 containing CEN6 replicon and ARS associated with histone 4 (ARSH4) (Ueda et al., 2001). *KTI12* and *DRL1* overexpression in multicopy for zymocin toxin sensitivity assay was achieved by YEplac181 vector harboring *KTI12* or *DRL1* ORF with *KTI12* promoter as described previously (Butler et al., 1994). Constructed plasmids were transformed into yeast by LiCl method or electroporation. Total RNA from the shoot apex of 7-day old seedling and leaves of 3-weeks old plants was isolated by RNeasy mini kit (Qiagen, USA) and used as template for cDNA synthesis (TOYOBO, Japan). For semi-quantitative RT-PCR, PCR mixture containing 20 ng cDNA was reacted as follows: one cycle of 30 s at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 53-58°C and 30 s at 72°C. The primer pair sequences of *DRL1*, SAM specific-, leaf polarity related-, cell proliferating regulatory genes, and *β-TUBULIN 4* (*TUB4*) gene for a positive control are described in Supplementary Table S2.

RESULTS

Comparison of the deduced Arabidopsis DRL1 amino acid sequence with homologous sequences from yeast and other species

DRL1 was reported previously to encode a homolog of the yeast Elongator-associated protein, KTI12 (Cho et al., 2007; Nelissen et al., 2003). To examine the evolutionary history and structural features of the DRL1 protein, we aligned the Arabidopsis DRL1 sequence with the sequences of DRL1 homologs



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Amino acid sequence homology with *Arabidopsis thaliana* (NP_172840)	(%)
Glycine max (XP_003528855)	72.52
Zea mays (NP_001145516)	66.11
Oryza sativa (BAH95225)	58.55
Dictyostelium discoideum (XP_643511)	38.10
Schizosaccharomyces pombe (NP_594556)	32.38
Saccharomyces cerevisiae (NP_012812)	28.15
Danio rerio (NP_001119890)	34.91
Homo sapiens (NP_612426)	30.13

Fig. 1. Amino acid sequence alignment and homology analysis of DRL1. (A) Amino acid sequence alignment. Conserved domains include an ATP/GTP-binding motif and two CaM-binding motifs (thin line), two plant-specific sequences (thick lines). The functions of the plant-specific motifs are unknown. Amino acid sequences of DRL1 and its homologs used in the alignment were retrieved from the NCBI GenBank database. The GenBank accession numbers of the amino acid sequences are NP_172840 (*Arabidopsis thaliana*), NP_001145516 (*Zea mays*), XP_003528855 (*Glycine max*), BAH95225 (*Oryza sativa*), XP_643511 (*Dictyostelium discoideum*), NP_594556 (*Schizosaccharomyces pombe*), NP_ 012812 (*Saccharomyces cerevisiae*), NP_001119890 (*Danio rerio*), and NP_612426 (*Homo sapiens*). (B) A portion of the overall alignment containing plant-specific sequences I (box). (C) A portion of the overall alignment containing plant-specific sequences II (box). (D) Amino acid sequence homologies of DRL1 homologs from plants, animals, protozoa and yeasts to Arabidopsis DRL1.

from various other species. Alignment of the amino acid sequences of DRL1 homologs from plants (*Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, and *Glycine max*), yeast (*S. cerevisiae* and *Schizosaccharomyces pombe*), protozoa (*Dictyostelium discoideum*), and animals (*Danio rerio* and *Homo sapiens*) revealed the presence of conserved domains including an ATP/GTP-binding motif in the N-terminus, two calmodulin (CaM)-binding motifs in the N- and C-terminal regions, and domains specific to plant species (plant-specific sequences I and II) (Fig. 1A). The Arabidopsis DRL1 amino acid sequence exhibited the highest similarity to the sequences of other plant DRL1 homologs with similarities of 58.55, 72.52, and 66.11% to the DRL1 homologs of *O. sativa*, *G. max*, and *Z. mays*, respectively, and the lowest similarity to the yeast DRL1 homologs from *S. cerevisiae* (28.15%; Fig. 1D). Based on an amino acid alignment and functional site prediction analyses, we identified a conserved sequence, KTQ(R)DVR(K) designated plant-specific sequence I, in the central region that could form a short α-helix and may have WD40 repeat-binding motif (Fig. 1B; Dinkel et al., 2013). We also identified a second invariant sequence, GQS(Y/T)SL designated plant-specific sequence II, in the C-terminal region that was conserved among dicot plants; could form a shorter α-helix than in the yeast proteins and may have NEK2 phosphorylation motif (Fig. 1C; Dinkel et al., 2013). Among the genes examined in this study, the *H. sapiens DRL1* homolog encoded the longest amino acid sequence (Fig. 1A). A protein secondary structure prediction analysis of the full amino acid sequences of Arabidopsis (DRL1) and *S. cerevisiae* (KTI12) using the PHYRE2 program (www.sbg. bio.ic.ac.uk/phyre2/) showed that the structure of both proteins was very similar (Supplementary Fig. S1). The DRL1 protein contained 13 α-helices and 6 β-sheets, while the KTI12 protein had 11 α-helices and 5 β-sheets (Supplementary Figs. S1A-S1D). Based on hydropathy and transmembrane prediction analysis using TMpred and SPLIT programs, the DRL1 and KTI12 proteins apparently lack any transmembrane domains (Supplementary Figs. S1E and S1F).

Complementation of the yeast *kti12Δ* mutant by the DRL1 protein

In a previous study, we suggested that Arabidopsis *DRL1* gene encodes a homolog of the yeast *KTI12* gene, which interacts with the Elongator complex (Cho et al., 2007; Fichtner et al., 2002; Frohloff et al., 2001; Nelissen et al., 2003). The KTI12 protein assocaites with Elongator, a six-subunit histone acetyltransferase complex with roles in RNAPII transcription and tRNA anticodon modification that are conserved among yeast and plant cells (Nelissen et al., 2003; 2005; 2010; Otero et al., 1999; Wittschieben et al., 1999). The yeast *kti12Δ* mutant exhibited a thermosensitive phenotype above 38°C and growth retardation caused by a delayed G1 phase in the cell cycle (Fichtner et al., 2002; Frohloff et al., 2001). To confirm that *DRL1* might be related to yeast *KTI12*, we performed complementation tests using overexpression of Arabidopsis *DRL1* and rice *OsDRL1* genes in the yeast *KTI12* deletion strain YKL110C (Fig. 2). The YKL110C strain grew normally at 30°C, but exhibited inhibited growth at 39°C, compared to WT BY4741 (Fig. 2). Growth at 39°C was restored in YKL110C harboring both *DRL1* and *OsDRL1* genes inserted into the yeast expression vector pTU1, indicating that the deletion of *KTI12* was, at least

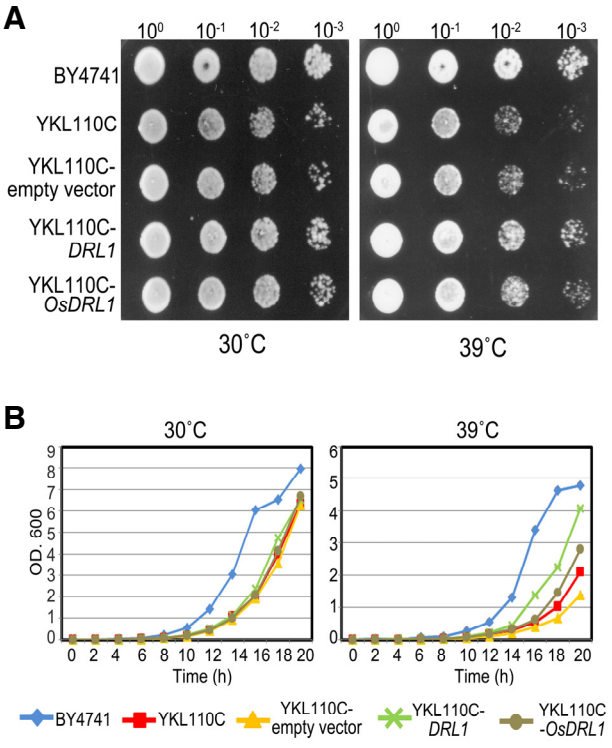

Fig. 2. Complementation of thermosensitivity in yeast *KTI12* mutants expressing plant *DRL1* genes. (A) Thermosensitivity assay. *kti12Δ* mutant (YKL110C) was transformed with pTU1 plasmid harboring Arabidopsis *DRL1* and rice *OsDRL1* genes. Empty pTU1 vector was used as a control. To test the thermosensitivity of yeast strains, serial dilutions of the yeast strains were replica-spotted and incubated on YPD media for 30 h at either permissive (30°C) or non-permissive (39°C) temperature. (B) Time-course experiment for quantitative analysis of yeast growth rate. To determine thermosensitivity, optical densities (ODs) of cell population were measured at 2-h intervals over 20 h at either permissive (30°C) or non-permissive (39°C) temperature, using a spectrophotometer at 600 nm.

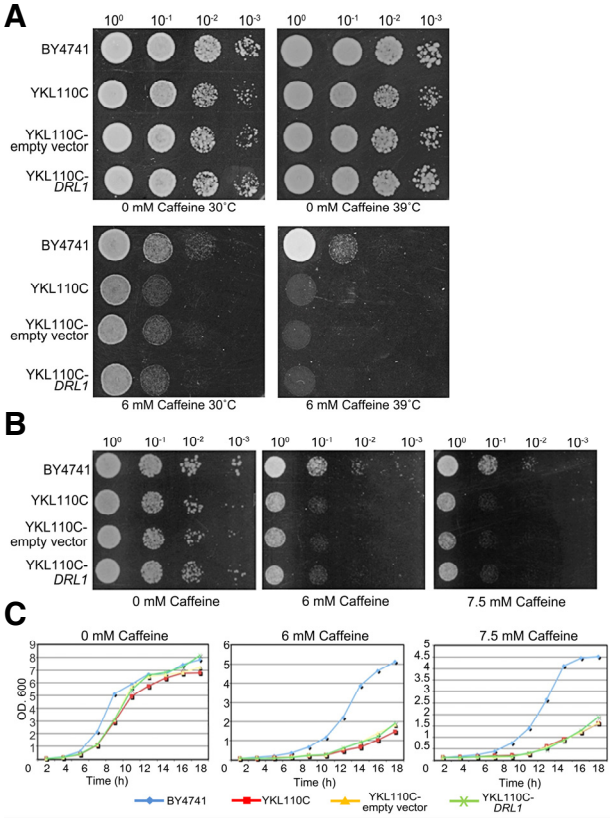

Fig. 3. Complementation of thermosensitivity and caffeine sensitivity of yeast *KTI12* mutants expressing Arabidopsis *DRL1*. (A) Synchronized assay of thermo- and caffeine sensitivities. Serial dilution of yeast strains were replica spotted and incubated on combined conditions of temperature (30°C or 39°C) and growth media (YPD meida without caffeine or with 6 mM caffeine) for 30 h. (B) Caffeine sensitivity assay. Serial dilution of yeast strains were replica-spotted and incubated on YPD media containing 0, 6, and 7.5 mM caffeine at 30°C for 30 h, respectively. (C) Growth curve of yeast strains on YPD media containing 0, 6, and 7.5 mM caffeine, respectively. To determine caffeine sensitivity, optical densities (ODs) of cell population were measured at 2-h intervals over 18 h using a spectrophotometer at 600 nm.

partially, rescued by expression of the plant *DRL1* genes (Fig. 2A). Expression of *DRL1* triggered higher extent of the restoration of yeast growth than expression of *OsDRL1* (Fig. 2A). To confirm yeast growth rate in detail, time-course experiment for quantitative analysis was performed in liquid medium. During growth at 39°C, YKL110C harboring plant *DRL1* genes showed delayed starting points of log phase, compare to WT BY4741 (Fig. 2B). YKL110C harboring plant *DRL1* genes showed earlier starting of log phase than YKL110C (Fig. 2B), indicating that the function of plant *DRL1* genes is partially conserved with that of yeast *KTI12* gene.

In addition, the *kti12Δ* mutant exhibited sensitivity to drugs including caffeine (Fichtner et al., 2002; Frohloff et al., 2001). We performed a complementation assay for caffeine sensitivity in YKL110C, with or without *DRL1* expression. The YKL110C mutant was sensitive to caffeine (Figs. 3A-3C) and *DRL1* expression did not restore growth performance at 30°C or 39°C (Fig. 3A). To further confirm the caffeine sensitivity, we performed a time-course analysis of cell growth on media containing caffeine with different concentration. Expression of *DRL1* restored the growth retardation for 9 h at 0 mM caffeine (Fig. 3C). However, expression of *DRL1* did not restore the caffeine sensitivity when grown on 6 mM or 7.5 mM caffeine (Figs. 3B and 3C), indicating that the function of Arabidopsis DRL1 may not entirely overlap with yeast KTI12 in caffeine sensitivity.

Furthermore, the *kti12Δ* mutant is known to exhibit zymocin toxin resistance (Fichtner et al., 2002; Frohloff et al., 2001). *KTI12* overexpression also causes zymocin resistance, although the degree of zymocin resistance casued by elevated *KTI12* gene expression is lower than that induced by deletion of *KTI12* gene (Frohloff et al., 2001). We also performed an assay for zymocin resistance in WT and *kti12Δ* mutant with or without *DRL1* overexpression. Multicopy *DRL1* in combination with the *GAL1*-driven expression of the γ-toxin tRNase subunit from zymocin from vector pHMS14 in galactose medium failed to elicit zymocin resistance, while in multicopy, the yeast *KTI12*

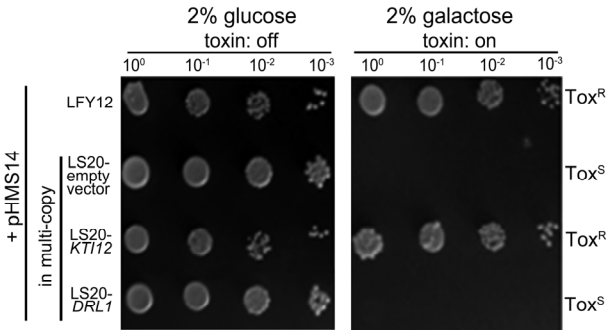

Fig. 4. Effect of *DRL1* overexpression on toxin resistance. *KTI12* deletion mutant, LFY12 was generated by transformation with the pYF6 deletion construct. Yeast strans, WT (LS20) and *kti12Δ* mutant (LFY12) were transformed with GAL1-driven γ-toxin gene expression vector, pHMS14. LS20 transformants were transformed with YEplac181 vector harboring *KTI12* or *DRL1* ORF with *KTI12* promoter, respectively. Empty YEplac181 vector was used as a control. Serial dilution of yeast strains were replica spotted and incubated on YPD media containing 2% glucose (inactivation of γ-toxin synthesis) or 2% galactose (activation of γ-toxin synthesis) for 30 h, respectively. Growth on galactose media means resistance towards toxin (Tox^R) and the inhibition of growth does sensitivity towards toxin (Tox^S).

gene suppressed zymocin and triggered resistance to the tRNase toxin (Fig. 4). This indicates that the function of Arabidopsis DRL1 may not overlap with yeast KTI12 in zymocin mediated growth inhibition.

Based on these results, DRL1 may have a biochemical function that is partially conserved with yeast KTI12, as well as a unique function that is specific to its plant context.

Function of DRL1 during development in Arabidopsis

Previously, we isolated the Arabidopsis *drl1-101* mutant, which contains a Ds transposon in the C-terminal region of the *DRL1* (At1g13870) gene, and performed genetic analysis demonstrating that the *DRL1* gene is involved in leaf patterning and SAM formation (Cho et al., 2007). The *drl1-101* mutant produced narrow leaves with a structure that was trumpet-like and filamentous (Cho et al., 2007). In this study, we found that the leaves of the *drl1-101* mutant were serrated and diminished in size during the early stages of leaf development, compared to the WT (Figs. 5A and 5B). The palisade cells in the WT leaf blades exhibited an oval shape and were regular in size (Fig. 5E). In contrast, the palisade cells in the *drl1-101* mutant leaf blades exhibited irregular sizes and reduced numbers accompanied by an increase in the intracellular spaces (Fig. 5F). The number of cells in the *drl1-101* mutant leaf blades was significantly reduced compare to that in WT leaf blades and resulted in 82% of WT (Fig. 5G). The size of cells in the *drl1-101* mutant leaf blades was increased compare to that in WT leaf blades and resulted in 107% of WT (Fig. 5H). The shapes and sizes of palisade cells in the *drl1-101* mutant leaf blades were very similar to those of sponge cells in WT leaves, indicating that the leaf blades of the *drl1-101* mutant were abaxialized. These results indicated that the processes of cell division and cell differentiation were altered in *drl1-101* mutant leaves.

The establishment of adaxial-abaxial polarity of leaf blades requires communication with the SAM and is necessary for leaf growth. Morphological analysis of longitudinal sections from 10-day-old seedlings in a previous study showed that the SAM structure in the *drl1-101* mutant was defective (Cho et al., 2007). To examine alterations in the *drl1-101* SAM in detail, we observed the external shape and size of the SAM using scanning electron microscopy (SEM). The SEM analysis showed that the *drl1-101* SAM was smaller in size compared to the WT and that the external shape was flatter than that of the WT (Figs. 5C and 5D).

To analyze effects of acquisition and maintenance of meristematic identity on leaf polarity patterning, we examined the expression levels of positive regulators of meristem identity acquisition and maintenance including the *class-I KNOX* genes *KNATTED1* (*KNAT1*), *KNAT2*, and *KNAT6*, and the *SHOOT-MERISTEMLESS* (*STM*) gene in WT and *drl1-101* shoot apexes. The expression levels of the *class-I KNOX* genes in the shoot apex were lower in *drl1-101* than in the WT (Fig. 6A). In particular, *STM* expression was undetected in *drl1-101* (Fig. 6A). Decreased *class-I KNOX* and *STM* expression may result in the smaller size and altered SAM structure in the *drl1-101* mutant.

Because the *drl1-101* mutant exhibited abaxialized leaves with altered cell differentiation, we examined the expression patterns of abaxial-determining genes in WT and mutant leaves. As noted above, the *KAN* and *YAB* gene families promote the abaxialization of leaves by repressing adaxial polarity determinants or integrating abaxial polarity determinants. The *YAB* gene family was expressed at high levels in *drl1-101* mutant leaves relative to WT leaves (Fig. 6B). The *YAB2* gene exhibited the strongest expression of the *YAB* gene family members in *drl1-101* mutant leaves (Fig. 6B). The *KAN1* gene was also expressed at high levels in the *drl1-101* mutant relative to the WT (Fig. 6B). These results indicated that the altered phenotype of the *drl1-101* leaf cells was caused by upregulated *YAB* and *KAN* gene expression, which regulates the abaxialization features of leaves. Taken together, these results indicate that defects in the establishment of adaxial-abaxial polarity might be coupled to genetic alteration within the meristem in the *drl1-101* mutant.

Because the *drl1-101* mutant exhibited altered cell number and size, we examined the expression patterns of cell cycle-related gene *Cyclin D3;1* (*CYCD3;1*) and cell proliferation regulatory genes *AINTEGUMENTA* (*ANT*) and *ARGOS* in WT and *drl1-101* mutant leaves. *CYCD3;1* expression was increased, while *ANT* and *ARGOS* were not changed in the *drl1-101* mutant relative to the WT (Fig. 6C). Despite the reduced cell number in *drl1-101* mutant, the expression of cell cycle gene might be activated by the defects in DNA metabolism. *CycD3;1* directly regulates cell division from various external and internal signals, while *ANT* and *ARGOS* act upstream of cell division regulatory mechanisms (Dewitte et al., 2003; Hu et al., 2003; Mizukami and Fischer, 2000). Therefore, this result indicates that *DRL1* might indirectly regulate cell division in the upstream of *CycD3;1*.

DISCUSSION

Genes homologous to Arabidopsis *DRL1* and yeast *KTI12* are present in the genomes of most organisms including fungi, fruit flies, human, and plants. However, their biological and genetic functions have not been studied in depth. More detailed studies on yeast KTI12 revealed that it acts as a regulator of HAT while associating with the Elongator complex (Otero et al., 1999; Wittschieben et al., 1999). KTI12 is required for the HAT activity of Elongator complex *in vivo*. However, the specific role of

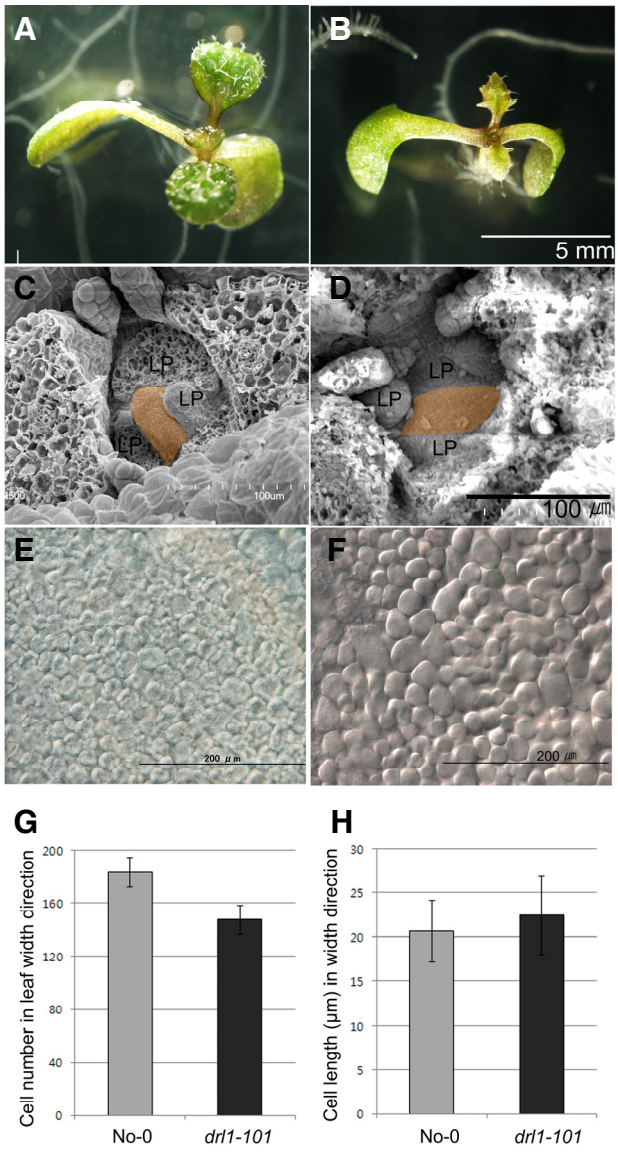

Fig. 5. Phenotypic and morphological alterations in the Arabidopsis *DRL1* mutant, *drl1-101*. (A, B) 7-day old seedlings of the WT (No-0) (A) and *drl1-101* (B). Bar, 5 mm. (C, D) Scanning electron microscopy (SEM) of shoot apical meristem (SAM) in the WT (No-0; C) and the *drl1-101* (D). Orange colored regions in images indicate SAM. LP, leaf primordium Bar, 100 μm. (E, F) Paradermal images of palisade cells in leaves of the WT (No-0; E) and the *drl1-101* (F). Bar, 200 μm. (G, H) Statistical analysis of palisade cell number (G) and size (H) in the 3rd leaf on 21-days old WT (No-0) and *drl1-101*. The 3rd leaves of four independent plants were used for satistical analysis.

KTI12 regulating Elongator HAT activity remains still unclear (Petrakis et al., 2005).

DRL1 homologs exhibited 28-77% amino acid sequence homology with that of Arabidopsis DRL1 (Figs. 1A and 1D). The existence of only a single gene copy of *DRL1* homologs in the genomes of all organisms may suggest that no gene duplication occurred during evolution and that the function of DRL1 homologs may be highly conserved (Cho et al., 2007). In this study, we showed that DRL1 proteins have structurally conserved domains with other species as well as plant-specific sequences (Fig. 1A), which suggest existence of differences in the evolution of conserved biochemical functions specific to plants. Expression of Arabidopsis and rice *DRL1* genes in the yeast *kti12Δ* mutant complemented growth retardation at 39°C, indicating that this function of yeast *KTI12* appears to be conserved in and rescued by plant *DRL1* genes (Fig. 2). In contrast, *KTI* genes conferred resistance to zymocin, which affects RNAPII-dependent gene transcription in yeast and also targets tRNAs for anticodon cleavage (Frohloff et al., 2001; Jablonowski and Schaffrath, 2007; Jablonowski et al., 2006; Lu et al., 2005). Mutations in *KTI* genes including *KTI12* cause caffeine sensitivity as well as zymocin resistance (Ficthner et al., 2002; Frohloff et al., 2001). In this study, *DRL1* expression in the yeast *kti12Δ* mutant did not reveal the suppression of the caffeine sensitive phenotype (Fig. 3). In addition, despite elevation of *DRL1* expression in multicopy in a yeast WT background, *DRL1* failed to suppress the zymocin sensitive phenotype (Fig. 4). This is in contrast to yeast KTI12, which when overexpressed, suppresses zymocin action (Butler et al., 1994; Fichtner et al., 2002; Frohloff et al., 2001). In addition, Arabidopsis *ELP1* encoding a homolog of yeast Elongator subunit ELP1/KTI7 complemented zymocin resistance, but not caffeine sensitivity, in the yeast *elp1* mutant (Chen et al., 2006). Thus, the failure of plant ELP1 and DRL1 to rescue the caffeine sensitivity in yeast mutants strongly suggests that the role of Elongator and DRL1 in response to caffeine may have changed between yeast and plants during the course of evolution.

On the other hand, exchangeable Elongator function in tRNA modification between plant and yeast indicated that remarkable structural conservation remained in animals, fungi and plants (Chen et al., 2009; Huang et al., 2005; Mehlgarten et al., 2010). Despite structural conservation, Elongator function may differ in unicellular and multicellular organisms, based on the functional characterization of Elongator mutants from yeast and plants (Nelissen et al., 2005). Four components of the Elongator complex, ELONGATA1 (ELO1), ELO2, ELO3, and DRL1/ELO4, have been identified as a HAT and have shown to influence plant growth and development (Nelissen et al., 2005). The Arabidopsis *elo* mutants commonly showed aberrant leaf morphology with deficiencies in organ growth due to reduced cell proliferation (Cho et al., 2007; Nelissen et al., 2005). In addition, the Arabidopsis Mediator mutant *med14/struwwelpeter1* showed similar aberrant leaf morphology to *elo* mutants (Nelissen et al., 2005). Arabidopsis *med12/cct* and *med13/gct* showed defect in the specification of central and peripheral identity (Gillmor et al., 2010). Mediator complex subunits are involved in transcription regulation by promoting the interaction of DNA binding transcription factors and RNAPII preinitiation complex in response to developmental and envirionmental cues (Kornberg, 2005; Poss et al., 2013).

A crucial question is why many Elongator and Mediator mutants have defective leaf phenotype and how Elongator and Mediator affects leaf morphology? Perhaps the most controversial answer is the dependent processes on specificity of HAT and/or tRNA modification activity. Actually, Elongator is known to be multifunctional protein involved in biotic and abiotic stresses as well as in leaf morphogenesis (Chen et al., 2006; DeFraia et al., 2010; Nelissen et al., 2003; 2005; 2010; Zhou et al., 2009). How they control specifically to leaf polarity? In this study, we showed that the *DRL1* gene regulates the expression

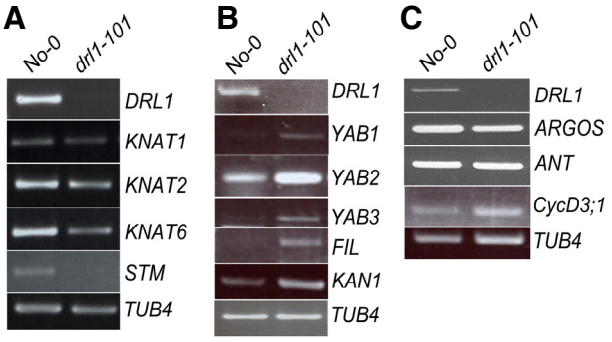

Fig. 6. Semi-quantitative analysis of expression levels of SAM-specific, abaxial-determining, and cell proliferating genes in WT (No-0) and *drl1-101* mutant. Expression level of SAM-specific genes (A), abaxial polarity determinant genes (B), and cell proliferation positive regulator genes (C) in the WT (No-0) and the *drl1-101*, as determined by reverse transcription-PCR (RT-PCR). SAM specific genes include the *class-I KNOX* family [*KNATTED1* (*KNAT1*), *KNAT2* and *KNAT6*] and *SHOOTMERISTEMLESS* (*STM*), abaxial polarity determinant genes include members of the *YABBY* (*YAB*) family [*YAB1*, *YAB2*, *YAB3*, and *FILAMENTOUS LEAVES* (*FIL*)] and *KANADI* (*KAN*) family (*KAN1*), and cell proliferating genes incude *ARGOS*, *AINTEGUMENTA* (*ANT*), and *CyclinD3;1* (*CycD3;1*). Total RNA was isolated from shoot apices of 10-day old plants (A) or rosette leaves of 3-week old plants (B, C). Results from RT-PCR amplification of *β-TUBULIN 4* (*TUB4*) mRNA are shown as control.

of *class-I KNOX* family members and *STM* genes in the SAM, and regulates *YAB* and *KAN* genes in leaves (Figs. 6A and 6B). *Class-I KNOX* genes play an essential role in the identification and maintenance of pluripotent cells in the SAM by preventing cell differentiation. Chromatin remodeling has a critical influence on plant developmental processes including organ formation, leaf patterning, flowering, gametogenesis and embryogenesis, due to its involvement in the maintenance of stable expression patterns of meristem-controlling transcription factors and the control of meristem activity (Shen and Xu, 2009). In addition, histone acetylation is involved in the regulation of leaf initiation and the establishment of leaf polarity (Chua et al., 2005; Ishibashi et al., 2012). It was reported that MINIYO, interacting with RNAPII and Elongator induces cell differentiation through the repression of *STM* (Sanmartín et al., 2011). These results indicate that *DRL1* may be involved in preserving an equilibrium between SAM maintenance and organ differentiation. Genes encoding domains not associated with leaf polarity patterning, such as small RNAs, 26S proteasome, and ribosomal large subunit, function in the identification of leaf polarity (Griffiths-Jones et al., 2006; Huang et al., 2006; Yao et al., 2008). The results of previous studies are consistent with our speculation that *DRL1* might directly or indirectly function in the maintenance of SAM activity and the establishment of leaf polarity by mediating histone acetylation or through other mechanisms. Our future studies will address how chromatin remodeling by *DRL1* and Elongator complex directly regulates the expression of *class-I KNOX* gene family and abaxial-determining families using ChIP assays.

Although comparative analysis of amino acid sequences revealed plant-specific sequences in plant DRL1 homologs, the functions of these domains remain a mystery. Mutation analysis of these plant-specific domains will elucidate the evolutionary differences between Arabidopsis *DRL1* and yeast *KTI12*. In addition, the regulation of cell division and differentiation on the adaxial side of leaves by *DRL1* establishes a functional correlation with yeast *KTI12,* which appears to be involved in cell cycle regulation, implying that the function of *DRL1* may have been modified during the evolution from unicellular to multicellular organisms.

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

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2011-013-C00056 and NRF-2011-0011884 to G.-T.K.) and by the Next-Generation Bio-Green21 Program of the Rural Development Administration (PJ008167 to G.-T.K.). In addition, R.S. acknowledges technical assistance by Daniel Jablonowski and support by the Deutsche Forschungsgemeinschaft (DFG), Germany (SCHA 750/18).

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