Doctoral Dissertation
博士論文

Molecular genetic studies on leaf development in *Oryza sativa*

（イネの葉の形態形成に関する分子遺伝学的研究）

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Contents

Reference

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List of abbreviation

ADL1	*ADAXIALISED LEAF1*
AN	*ANGUSTIFOLIA*
AOS	*ALLENE OXIDE SYNTHASE*
AP2	*APETALA2*
ARF	*AUXIN RESPONSE FACTOR*
AS	*ASYMMETRIC LEAVES*
BB	*BIG BROTHER*
BOP1	*BLADE-ON-PETIOLE1*
Cg1	*Corngrass1*
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats CRISPR-associated proteins 9
cul1	*curled later1*
DEG	differentially expressed genes
DL	*DROOPING LEAF*
elo	*elongate*
ELP	Elongator complex protein
ETT	*ETTIN*
GA	gibberellin
GANT	Gcn5-related N-acetyltransferase
GIF	GRF-INTERACTING FACTOR

GRF	GROWTH REGULATING FACTOR
hal1	*half-pipe-like leaf1*
HAT	histone acetyltransferase
HD-ZIPIII	class III homeodomain-leucine zipper
H3K14	Histone H3 lysine 14
HSP	heat shock protein
JA	jasmonic acid
KNOX	*KNOTTED-LIKE HOMEOBOX*
LSY1	*LATERAL SYMMETRY1*
MNU	N-methyl-N-nitrosourea
NAL	*NARROW LEAF*
nd1/*nrl1*/*sle1*	*narrow leaf and dwarf1*/*narrow and rolled leaf1*/*slender leaf1*
OSH1	*ORYZA SATIVA HOMEOBOX1*
OSK	Oryza sativa SKP1-like protein
PHAN	*PHANTASTICA*
PHB	*PHABULOSA*
PHV	*PHAVOLUTA*
PIN	PIN-FORMED
PPS	*PETER PAN SYNDROME*
PRE	*PRECOCIOUS*
REV	*REVOLUTA*
RL9/SLL1	*ROLLED LEAF9/SHALLOT-LIKE1*

RNA-seq	RNA sequencing
ROT	*ROTUNDIFOLIA*
rSAM	radical S-adenosyl-methionine
SAM	shoot apical meristem
SCF	Skp1-Cullin-F-box
SD	short day
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*
STM	*SHOOT MERISTEMLESS*
SWI/SNF	SWITCH/SUCROSE NONFERMENTING
ta-siRNA	trans-acting small interfering RNA
TOE	*TARGET OF EAT*
TPR	tetratricopeptide repeat
tRNA	transfer RNA
UbL40	ubiquitin fusion ribosomal protein L40
WOX	*WUSCHEL-RELATED HOMEOBOX*

Abstract

Leaves are important for photosynthesis and show diverse morphology in angiosperms. Although molecular genetic mechanism underlying leaf morphogenesis have been understood deeply in eudicots such as *Arabidopsis thaliana*, the knowledge of leaf development in monocots is still insufficient. In this dissertation, I focused on two curling leaf mutants, *curled later1* (*cul1*) and *halfpipe-like leaf1* (*hal1*), in order to get new insights for leaf development in a model monocot, *Oryza sativa* (rice). In chapter 2, I revealed that *CUL1* encoding a large subunit of Elongator complex is required for leaf development at late stage of vegetative phase and for proper maintenance of the shoot apical meristem (SAM). In chapter 3, I characterized the leaf phenotype of *hal1*.

CURLED LATER1 **encoding the largest subunit of the Elongator complex has a unique role in rice development**

In chapter 2, I first characterized the *cul1* mutant with narrow and curled leaves. By histological analysis, I revealed that the development of both the bulliform cells and sclerenchyma cells were impaired in *cul1* leaves and that these defects were a cause of curling leaf phenotype. These leaf phenotypes were not observed in early-middle stages of the vegetative phase and appeared specifically at late stage. It is therefore likely that *cul1* displayed a heteroblastic change. In general, the heteroblastic change is observed in the transition from the juvenile to adult phase in many plants. The heteroblastic change in *cul1* was likely to occur at the late adult phase. In this sense, the *CUL1* seems

to play a unique role in leaf development in rice.

I also revealed that the SAM was smaller in *cul1* than in wild-type, suggesting that *CUL1* function is associated with meristem activity to some extent. Consistent with this, the leaf initiation rate was slow in *cul1*, as compared with wild-type. Unlike leaf phenotype, the partial defect in the SAM appeared independently of growth stage. I also suggested that the appearance of leaf phenotype at the late adult phase seems to be associated with the transition of meristem fate, that is, from the vegetative meristem to the reproductive meristem.

I revealed that *CUL1* encodes a yeast ELP1-like protein, which is the largest subunit of the Elongator complex. Elongator is conserved in eukaryotes such as yeast, animals and plants, whereas its roles are diversified among various organisms. In Arabidopsis, Elongator is known to regulate leaf and root development, hormone action and environmental responses. In other plants, however, the function of Elongator complex is still largely unknown. To get further evidence that Elongator is required for rice development, I disrupted *OsELP3* encoding the catalytic subunit of the Elongator complex by CRISPR-Cas9 technology. Knockout lines of *OsELP3* produced curled narrow leaves with impaired bulliform and sclerenchyma cells, similar to the *cul1* mutant. In addition, the curled narrow leaf phenotype appeared at late stage of the vegetative phase. Therefore, these results confirm the importance of Elongator activity in normal leaf development at later vegetative phase in rice. Furthermore, this result showing that the *cul1* mutant and *OsELP3* knockout line shared mutant phenotypes suggests that the proteins encoded by these two genes act together as components of the

Elongator complex.

To explore the effect of the *cul1* mutation on gene expression, I carried out transcriptome analysis. As a result, the genes involved in protein quality control, such as ubiquitin-associated protein degradation and molecular chaperon, were highly up-regulated specifically in the *cul1* shoot apex at the later vegetative phase (*cul1*-L shoot apex). This result suggests that the genes responsible for protein quality control is induced by the accumulation of abnormal proteins in the *cul1*-L apex probably due to the loss of Elongator activity. Therefore, it seems likely that the Elongator is required for the protein progression of translational processes. The defects in leaf development in *cul1* might be related to the impaired translation of the proteins, which plays essential roles in the differentiation of bulliform and sclerenchyma cells.

Characterization of *half-pipe-like leaf1* mutant that exhibit curled leaf phenotype

In chapter 3, I focused on a semi-dominant mutant, *hal1-d*, which produced the adaxially curled leaves. Histological analysis revealed that the bulliform cells of *hal1-d* leaf were small and abnormal shape. The obseversion of semi-transparent leaves by TOMEI methods showed that the bulliform cell files were often indistinguishable from other cell files in *hal1-d*, suggesting that the bulliform cells were failed to grow. Therefore, the defective development of the bulliform cells were likely to be a main cause of curling leaf phenotype in *hal1-d* mutant. The size of the leaf blade and the spikelet were also influenced by the *hal1-d* mutation.

Conclusion and perspectives

The Elongator complex plays multiple roles in diverse organisms. The loss-of-function of the genes encoding Elongator subunit exhibits various phenotypes depending on diverse species. In addition, the studies on Elongator have not yet been reported in plants, except for Arabidopsis. In this dissertation, I revealed that the Elongator complex is required for leaf development and meristem activity, and associated with heteroblasty in rice. The role of Elongator in rice development seems to be different from that of Elongator in Arabidopsis development to some extent. Therefore, the function of Elongator is likely to have been diversified even within angiosperms. Further studies on Elongator in rice would be expected to provide important information on not only elucidation of leaf and meristem development in rice but also the conservation and diversification of Elongator function in angiosperms.

Chapter 1

General Introduction

Leaf initiation

Leaf is an important organ for photosynthesis; the shape and size of leaves influence light capture and gas exchange. Unlike animals, plants continue to produce new organs after embryogenesis. Leaves develop from the shoot apical meristem (SAM), which consist of a population of pluripotent stem cells. The SAM is divided into three functional domains: the central region, peripheral region, and rib region. The cells in the central region remain undifferentiated, whereas the cells in the peripheral region acquire cell fate to differentiate lateral organs such as leaves. The stem cells in the central region supply cells to this peripheral region, and leaf primordia develop from the peripheral region. The cells in the rib region supply cells to form the stem (reviewed in Leyser and Day, 2003).

The undifferentiated state of the central region in the SAM is maintained by the activity of members of the class I KNOTTED homeobox (*KNOX*) genes. Leaf initiation is associated with down-regulation of the *KNOX* genes. *KNOX* genes are expressed in the central region and the rib region of the SAM, but not expressed in the peripheral region. The absence of *KNOX* gene expression results from the repression by several genes such as *ASYMMETRIC LEAVES1* (*AS1*), *AS2* and *BLADE-ON-PETIOLE1* (*BOP1*) *in Arabidopsis thaliana* (Fig.1-1A) (Byrne et al., 2000; Ori et al., 2000). A phytohormone auxin is also involved in suppressing the expression of *KNOX* genes.

Determination of leaf polarity

After the determination of the cell fate, leaf primordia emerge from the peripheral region of the SAM. Leaf primordia develop along three-dimensional axes: adaxial-abaxial, proximal-distal and central-lateral axes. The molecular mechanisms underlying the establishment of the adaxial-abaxial polarity are well understood and many genes responsible for the adaxial-abaxial polarity have been identified (Fig.1-1B) (reviewed in Husbands et al., 2009).

Surgical experiments using potato (*Solanum tuberosum*) have revealed that the determination of the polarity in the leaf depends on a signal from the SAM (Sussex, 1951). Leaf primordium develops into a radially symmetric structure with abaxial identity by the incision between the primordium and the SAM. This result suggests that the signal from the SAM promotes adaxial cell fate in the leaf primordia.

The *PHANTASTICA* (*PHAN*) gene encoding a MYB-like transcription factor plays a crucial role to determine the adaxial identity in the leaf in *Antirrhinum majus* (Waites and Hudson, 1995; Waites et al., 1998). The *phan* leaf lacks the adaxial-abaxial polarity and shows a needle-like structure in a severe case.

In Arabidopsis, the adaxial cell fate is determined by class III homeodomain-leucine zipper (HD-ZIPIII) genes, such as *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) (McConnell et al., 2001; Emery et al., 2003). The expression of HD-ZIPⅢ genes is negatively regulated by *miRNA165/166* and is restricted to only at the adaxial side. By contrast, *KANADI* and *YABBY* genes are involved in the determination of the abaxial cell fate (Kerstetter et al., 2001; Pekker et al., 2005;

Siegfried et al., 1999; reviewed in Bowman, 2000). In addition, *ETTIN* (*ETT*)/*AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4*, the targets of ta-siRNA, are also required for the establishment of the abaxial cell identity (Sessions et al., 1997; Pekker et al., 2005). Thus, the adaxial-abaxial leaf polarity is regulated by the interaction of several transcription factors and small RNAs. This regulation is basically conserved in angiosperms (reviewed in Chitwood et al., 2007; Husbands et al., 2009).

Leaf shape regulated by cell division and cell elongation

After the establishment of the three axes, cells divide and elongate along each axis. The cell proliferation and elongation play an important role for the determination of the size and shape of the leaf. In Arabidopsis, for example, the cell proliferation and elongation along the proximal-distal or the central-lateral axis are regulated by a large number of genes, such as *ANGUSTIFOLIA*(*AN*), *AN3*, *ROTUNDIFOLIA3* (*ROT3*) and *ROT4* (Tsuge et al., 1996; Horiguchi et al., 2005; Narita et al., 2004).

The *an3* mutant has narrow leaves, which results from reduced cell division along the central-lateral axis. *AN3* is also known as *GRF-INTERACTING FACTOR1* (*GIF1*) (Kim and Kende 2004; Horiguchi et al., 2005). GIFs interact with plant specific GROWTH REGULATING FACTORs (GRFs), which recruit the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complexes to regulate transcription of various genes and promote cell proliferation in leaves (Debernardi et al., 2014; Vercruyssen et al., 2014; reviewed in Kim and Tsukaya, 2015). On the other hands, the *rotundifolia3* (*rot3*) mutant has round-shape leaves, which shows reduced

cell expansion along the proximal-distal axes. *ROT3* encodes a cytochrome P450 (Tsuge et al., 1996; Kim et al., 1998).

Heteroblasty

Plant growth is divided into three distinct developmental stages, embryogenesis, vegetative development and the reproductive development. The vegetative development can be further divided into juvenile and adult phases.

Leaf traits, such as shape and size, change during plant growth in a number of species. This phenomenon is called heteroblasty, which is associated with the transition of developmental stages. For example, *Eucalyptus albida* produces white elliptical leaves during juvenile phase, whereas it produces deep-green lanceolate leaves during adult phase (Kerstetter and Poethig, 1998). Several *Acacia* species, the leaf shape dramatically change between juvenile and adult phases, via transition state (Wang et al., 2011). In Arabidopsis, leaves change in their size, serration pattern and trichome production during juvenile-to-adult phase transition.

SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) genes and *APETALA2* (*AP2*)-like genes, which were targeted by *miR156* and *miR172,* respectively, play an important role to regulate juvenile-to-adult phase change in angiosperms (reviewed in Huijser and Schmid, 2011; Chitwood and Sinha, 2016). The repression of SPLs by miR156 prevents precocious change from juvenile to adult phase. By contract, the negative regulation of AP2-like protein by miR172 promotes this change. In maize, the dominant *Corngrass1* (*Cg1*) mutant, in which *miR156* is overexpressed, has

prolonged juvenile phase (Chuck et al., 2007). By contract, the *glossy15* mutant, the loss-of-function mutant of *AP2*-like gene, generates adult traits in juvenile leaves (Moose and Sisco, 1996; Lauter et al., 2005). In Arabidopsis, *SPL*s and AP2-like genes, such as *SPL9*, *SLP15*, *TARGET OF EAT1* (*TOE1*) and *TOE2,* are involved in heteroblasty as well as in maize (Usami et al., 2009; Wu et al., 2009; reviewed in Huijser and Schmid, 2011). *ETTIN*/*ARF3* and *ARF4*, regulators of the abaxial leaf fate, also play an important role for the juvenile-to-adult phase change in Arabidopsis (Hunter et al., 2006).

Phytohormones, such as gibberellin (GA) and jasmonic acid (JA), are also related to heteroblasty. In Arabidopsis, the mutants defective in GA biosynthesis and its signaling, such as the *ga1-3* and *gai* mutant, exhibit prolonged juvenile phase (Telfer et al., 1997). In rice (*Oryza sativa*), the mutation of *PETER PAN SYNDROME* (*PPS*), which regulates the expression of GA biosynthetic genes, prevents the juvenile-to-adult transition, leading to the prolonged juvenile phase (Tanaka et al., 2011). In addition, *PRECOCIOUS* (*PRE*) also controls juvenile-to-adult transition in rice, via the regulation of *miR156* expression. *PRE* gene encodes *ALLENE OXIDE SYNTHASE* (*AOS*), which is a key enzyme for JA synthesis. These reports suggest that GA and JA play important roles for the regulation of the juvenile-to-adult transition in rice (Hibara et al., 2016).

Leaf structure and heteroblastic traits of rice

Rice generates long and narrow leaves. The rice leaf consists of lead blade and leaf

sheath, which are distinguished along the proximal-distal axis. In the leaf blade, the midrib is formed in the central region and runs together in parallel with small and large vascular bundles (Fig.1-2A). Midrib formation is regulated by the YABBY gene *DROOPING LEAF* (*DL*), which promotes cell division along the adaxial-abaxial axis at the central region of the leaf primordia (Yamaguchi et al., 2004; Ohmori et al., 2011).

Rice leaves change their sizes and shapes with plant growth (reviewed in Itoh et al., 2005). The first leaf is very small, and its leaf blade and leaf sheath are difficult to be distinguished. From the second leaf, the leaf blade is distinguished clearly from the leaf sheath. The first and second leaves have juvenile traits, whereas the third to fifth leaves are regarded as the transition state from the juvenile to adult phase (Hibara et al., 2016). Except for the last leaf, the shape and morphological characteristics are similar among adult leaves. The last leaf is known as the flag leaf, which is shorter and less slender than the other adult leaves.

Different types of cells and tissues are formed between rice leaves and Arabidopsis leaves. In Arabidopsis, for example, mesophyll cells consist of two different tissues, palisade and spongy layers. By contrast, such tissue differentiation is not observed in rice. Unique cells such as bulliform cells and sclerenchyma cells, are differentiated in rice leaves (Fig.1-2B). Sclerenchyma cells, which have rigid cell walls, are formed at both the adaxial and abaxial side of the vascular bundles. Bulliform cells are differentiated in the adaxial epidermal layer and are distinguished from other ordinary epidermal cells by its unique shape and large size.

Genes that regulate leaf development in rice

Molecular genetic mechanism underlying leaf development is not well understood in rice, as compared with Arabidopsis. However, mutants of altered leaf shape, such as narrow leaf and curling leaf mutants, have provided good opportunities to identify various genes that regulate leaf morphology in rice.

Studies using narrow leaf mutants have revealed leaf morphogenesis along the central-lateral axis in rice. The reduction of leaf width is often associated with the reduction of the number of vascular bundles. *NARROW LEAF2* (*NAL2*) and *NAL3*, the orthologs of Arabidopsis *WUSCHEL-RELATED HOMEOBOX3* (*WOX3*), are expressed in leaf margin and regulate leaf width and the number of small veins (Cho et al., 2013; Ishiwata et al., 2013; Kubo et al., 2016). Another duplicated paralog of *WOX3*, *LATERAL SYMMETRY1* (*LSY1*), also regulates leaf development in the central-lateral axis independently of *NAL2/NAL3* (Obara et al., 2004; Honda et al., 2018). *LSY1* is also involved in the establishment of the adaxial-abaxial polarity, because the *lsy1* mutant shows the asymmetric leaf phenotype in addition to the narrow leaf phenotypes. *NAL1* and *NAL7*, which encode trypsin-like serine/cysteine protease and YUCCA protein respectively, also regulate leaf width through modulating the cell number along the central-lateral axis (Qi et al., 2008; Fujino et al., 2008; Cho et al., 2014). YUCCA proteins are required for auxin biosynthesis, suggesting that auxin action is also associated with the regulation of leaf width.

Studies using curling leaf mutants also contribute to understanding of leaf morphogenesis in rice. Rice leaves are flat in normal condition, but the leaf blade is

curled adaxially in dry condition. This curling is thought to be caused by the loss of water from the bulliform cells. *NARROW LEAF AND DWARF1* (*ND1*) / *NARROW AND ROLLED LEAF1* (*NRL1*) / *SLENDER LEAF1* (*SLE1*), encoding cellulose synthase-like protein (OsCSLD4), is required for leaf flattening (Hu et al., 2010; Yoshikawa et al., 2013), because the *nd1/nrl1/sle1* mutant generates adaxially curling leaves due to the reduction of the cell size including bulliform cells. *SHALLOT-LIKE1* (*SLL1*), encoding KANADI transcription factor, promotes abaxial identity of the leaf in rice (Zhang et al., 2009). The *sll1* mutant has the defect in the differentiation of sclerenchyma cells at the abaxial side, resulting in adaxially curling of the leaf. Taken together, bulliform cells and sclerenchyma cells seem to play important roles for leaf flattening.

Composition of this dissertation

In this dissertation, I aimed to elucidate the mechanism of leaf development in monocots using rice. In chapter 2, I revealed that *CURLED LATER1* (*CUL1*), encoding the largest subunit of Elongator complex, is involved in leaf development and meristem function in rice. In chapter 3, I analyzed *half-pipe-like leaf1* (*hal1*) mutant, which shows curling leaf phenotype related to the reduction of the bulliform cell size. Finally, in chapter 5, I summarize the conclusions and discuss some future directions.

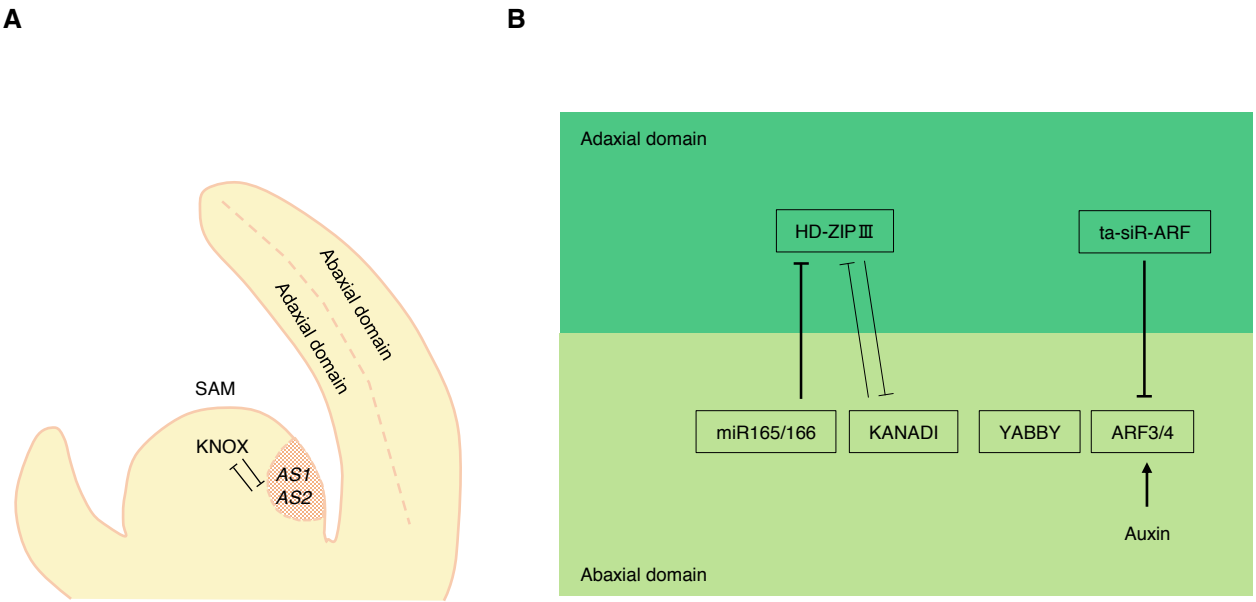

Figure 1-1. Leaf initiation and determination of leaf polarity.
A, The schematic diagram of leaf initiation. B, Genes that determine adaxial-abaxial identity in leaves.

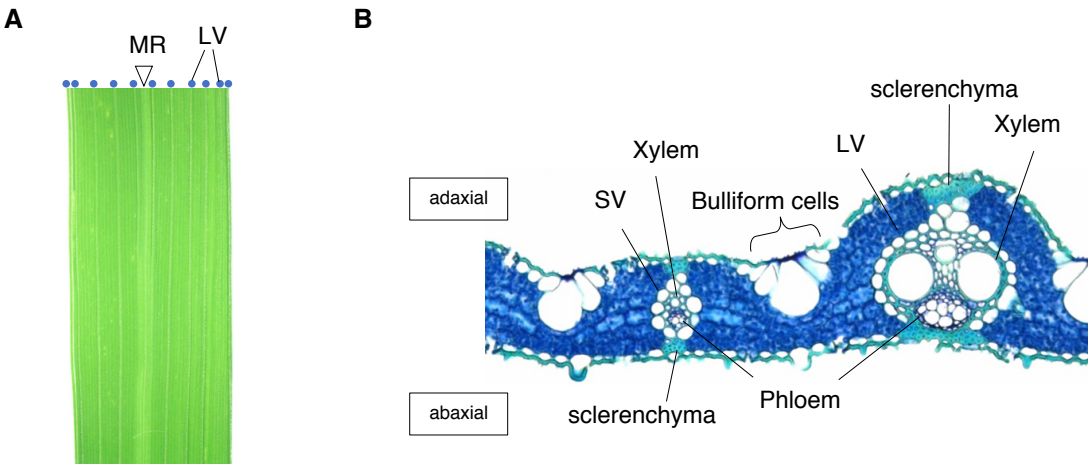

Figure 1-2. Leaf structure of rice.
A, Close-up view showing the leaf blade of rice. Arrowhead indicates midrib and blue dots indicate large veins. B, Transverse section of the leaf blade. MR, midrib. SV, small vascular bundle. LV, large vascular bundle.

Chapter 2

CURLED LATER1 encoding the largest subunit of the Elongator complex has a unique role in rice development

Introduction

Plant development depends on the activity of the shoot apical meristem (SAM). In the vegetative phase, the SAM successively generates leaf primordia. After transition to the reproductive phase, the vegetative SAM converts to the inflorescence meristem, which produces flowers. The vegetative phase is further divided into two distinct phases, the juvenile and adult phases, in which different traits are observed in the leaves (reviewed in Huijser and Schmid, 2011; Chitwood and Sinha, 2016).

The anatomical and morphological changes that occur in leaves depending on plant growth stage are called heteroblasty. The molecular genetic mechanisms underlying the transition from juvenile to adult phase have been elucidated in *Arabidopsis thaliana* based on heteroblastic changes in leaves. MicroRNAs such as miR156 and miR172, which regulate genes encoding SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) and APETALA2 (AP2)-like transcription factors, are key components in the genetic control of these mechanisms in Arabidopsis (reviewed in Huijser and Schmid, 2011; Chitwood and Sinha, 2016). The roles of these microRNAs and transcription factors in the juvenile-to-adult transition are conserved in angiosperms. Mutations in *Corngrass1* and *Glossy15*, which encode miR156 and AP2-

like transcription factors, respectively, strongly affect heteroblasty in maize (*Zea mays*) (Moose and Sisco, 1996; Lauter et al., 2005; Chuck et al., 2007). In addition to these microRNAs, hormones such as jasmonate and gibberellin have been shown to be involved in the juvenile-to-adult transition in rice (*Oryza sativa*) (Tanaka et al., 2011; Hibara et al., 2016).

The rice leaf comprises the leaf blade, leaf sheath, and their boundary region including the ligule and auricle. Rice plants produce 15–20 leaves depending on the strain and growth conditions, and the shape and size of the leaf vary depending on the growth stage. The first to third leaves are formed during embryogenesis (Itoh et al., 2005). The first leaf is very small, and its leaf blade and leaf sheath are indistinguishable. Although the second leaf is also small, the leaf blade is distinguished from the leaf sheath. The first and second leaves are considered to be juvenile, whereas the third to fifth leaves seem to represent the transition from juvenile to adult phase (Hibara et al., 2016). Except for the last leaf, the adult leaves are similar to each other in shape and morphological characteristics. The last leaf is called the flag leaf, and is shorter and less slender than the other adult leaves.

Rice has thin and flat leaves, in which large and small vascular bundles run in parallel together with a midrib (Ohmori et al., 2011; Matsumoto et al., 2017; Kubo et al., 2019). Two kinds of cells, bulliform and sclerenchyma cells, keep the leaf blade flat. The bulliform cells are specialized adaxial epidermal cells, which are distinguished from ordinary epidermal cells by their larger size and unique shape, and they keep the leaf blade flat by absorbing water (Itoh et al., 2005). In dry conditions, the leaf blade is

curled adaxially due to loss of water from the bulliform cells. The *ADAXIALISED LEAF1* (*ADL1*) gene is essential for restricting differentiation of the bulliform cells to the adaxial side (Hibara et al., 2009). The sclerenchyma cells, which are formed on both the adaxial and abaxial sides of the vascular bundle, have thick and rigid cell walls, which provide the leaf blade with physical strength. In a loss-of-function mutant of the *SHALLOR-LIKE1* (*SLL1*) gene, which is similar to Arabidopsis *KANADI*, the leaf blades are curled adaxially due to the failed differentiation of abaxial sclerenchyma cells (Zhang et al., 2009). Leaf development, including shape and size, is regulated by a large number of genes (Fujino et al., 2008; Li et al., 2009; Ohmori et al., 2011; Ishiwata et al., 2013; Yoshikawa et al., 2013; Yasui et al., 2018).

The Elongator complex plays multiple roles in a wide range of eukaryotes, such as plants, animals and fungi (reviewed in Versees et al., 2010; Woloszynska et al., 2016; Dalwadi and Yip, 2018). It was initially identified as a factor that directly associates with RNA polymerase II holoenzyme and was shown to be involved in transcriptional elongation (Otero et al., 1999; Wittschieben et al., 1999). In Arabidopsis, the Elongator complex is involved in leaf and root development; in humans, by contrast, it is involved in neuron development and its mutation is associated with familial dysautonomia (reviewed in Versees et al., 2010; Woloszynska et al., 2016; Dalwadi and Yip, 2018). Elongator comprises two subcomplexes: a large subcomplex consisting of the subunits ELP1, ELP2, and ELP3; and a small subcomplex consisting of ELP4, ELP5, and ELP6. ELP1 is the largest subunit and is thought to be a scaffold protein associated with the stability of the whole complex. ELP3 is the main catalytic subunit and contains two

distinct domains: a radical S-adenosyl-methionine (rSAM) domain and a putative histone acetyltransferase (HAT) domain. The rSAM domain of ELP3 is required to produce the chemically modified wobble uridine of tRNA, whereas ELP1 and ELP6 provide tRNA binding sites (reviewed in Dalwadi and Yip, 2018). The HAT domain shows sequence homology to members of the Gcn5-related N-acetyltransferase (GANT) superfamily. From molecular biological and biochemical studies based on these structural observations, Elongator is involved in multiple molecular functions including transcriptional regulation, translational control and endocytosis (reviewed in Versees et al., 2010; Woloszynska et al., 2016; Dalwadi and Yip, 2018).

In Arabidopsis, Elongator is involved in diverse biological activities, such as leaf and root development, hormone action, and stress/environmental responses (reviewed in Woloszynska et al., 2016). The genes encoding the Elongator subunits are called *ELONGATA* (*ELO*), except for *ELO4* (Nelissen et al., 2005). *elo/elp* mutants exhibit various developmental defects, such as narrow leaf, disordered venation pattern, short primary root, and disturbed hypocotyl elongation (Nelissen et al., 2005; Nelissen et al., 2010; Xu et al., 2012; Woloszynska et al., 2018; Qi et al., 2019). Molecularly, these mutant phenotypes are associated with a partial failure in the regulation of transcription, translation, histone acetylation, tRNA modification and miRNA synthesis (Nelissen et al., 2010; Fang et al., 2015; Leitner et al., 2015). Transcriptome analysis has also shown that the expression of auxin-related genes is affected by *elo/elp* mutation: some of the auxin-related genes have reduced H3K14 acetylation levels, suggesting that Elongator is involved in transcriptional control (Nelissen et al., 2010). Auxin signaling is also

affected at the translational level: the abundance of PIN-FORMED (PIN) proteins such as PIN1 and PIN2, which are responsible for polar auxin transport, is reduced in *elo/elp* mutants, despite no change at the transcript levels (Leitner et al., 2015). This translational control is associated with a defect in modification of the tRNA wobble uridine, similar to observations in yeast and human (Mehlgarten et al., 2010; Leitner et al., 2015). Thus, much progress has been made toward understanding the function of Elongator in Arabidopsis in the past decade. By contrast, the knowledge about the role of Elongator in other plants is limited.

In this chapter, I show that Elongator is required for leaf development and meristem function in rice. Initially, I characterized the *curled later1* (*cul1*) mutant, which produced narrow and adaxially curled leaf blades at a late adult stage of vegetative growth. The curled leaf phenotype was related to impaired differentiation of the bulliform and sclerenchyma cells. As compared with wild type, the SAM was reduced in size in *cul1* independent of growth stage. Gene isolation revealed that *CUL1* encodes a protein similar to yeast ELP1 protein, the largest subunit of the Elongator complex. Disruption of *OsELP3*, an ortholog of yeast *ELP3* and *Arabidopsis ELO3*, led to defects similar to those observed in *cul1*, including the appearance of mutant phenotypes at the same growth stage. Thus, Elongator activity seems to be specifically required for leaf development at later stages of the vegetative phase in rice. Transcriptome analysis showed that genes involved in protein quality control, including ubiquitin-associated protein degradation and molecular chaperoning, were highly up-regulated in the shoot apex at later vegetative stages, suggesting that loss of function of

Elongator results in a partial defect in translational control.

Results

The leaf phenotype appears at later vegetative stages in *cul1*

The *cul1* mutant was isolated as a morphological mutant that generated slender leaves at a later stage of the vegetative phase (before heading) (Fig. 2-1A). By contrast, leaves at younger growth stages looked normal. Therefore, I observed leaf phenotypes at various stages of plant growth. I characterized the phenotype of every leaf at the point when its whole leaf blade emerged from the sheath of the preceding leaf (Fig. 2-2). Thus, the leaf characteristics were analyzed at the same developmental stage of the leaf, but at different growth stages of the plant. The leaves were almost mature at this stage and did not grow much more.

The leaf width was mostly equivalent between *cul1* and wild type until approximately the 12th leaf (Fig. 2-1, B-D). Thereafter, the leaf width of *cul1* became shorter in the 14th leaf, as compared with the wild type (Fig. 2-1, B-D). I found that the *cul1* leaves produced at the later vegetative phase were curled upward (Fig. 2-1C). The curled leaves first appeared after the 14th leaf stage, and the extent of curling became more evident in the 15th leaf onward, and hence the mutant was named a '*cu*rled *l*ater' (Fig. 2-1E). These observations suggest that the phenotype of the *cul1* leaves changes depending on plant growth stage. Hereafter, I refer to the wild-type-like leaves produced during the early and middle growth stages of *cul1* as "N-type" (normal) and the curled slender leaves produced at later growth stages as "C-type" (curled).

The leaf blade consists of the midrib and two laminae formed on the either side of

the midrib. The large and small veins (vascular bundle) run in parallel in each lamina. I found that the number of small veins was reduced in C-type leaves in *cul1* relative to wild type (Fig. 2-1F). I found that the width of the lamina on the two sides often differed from each other in the 13th leaf (Fig. 2-1C), and the number of small veins was reduced in the short lamina of *cul1* as compared with wild-type lamina (Fig. 2-1F; Fig. 2-3). The phenotype of this leaf seemed to represent an intermediate state of the transition from N-type to C-type; therefore, I defined this leaf as "I-type" (intermediate). The timing of the appearance of the I-type and C-type leaves was more or less equal among individual *cul1* plants, indicating that loss of *CUL1* activity leads to a defect in leaves at an exact stage of plant growth (Fig. 2-4).

Histological analysis

To identify the cause of the curled leaf phenotype, I performed a histological analysis. Both the bulliform cells and sclerenchyma, which are required to keep leaves flat, were clearly observed in the N-type leaves of *cul1*, similar to wild-type leaves (Fig. 2-5A). By contrast, the bulliform cells were very small or indistinguishable from ordinary epidermal cells in the *cul1* C-type leaves (Fig. 2-5A). In addition, the differentiation of sclerenchyma adjacent to the small vascular bundle was defective in C-type leaves (Fig. 2-5, A and B). These results suggest that a failure in the formation of bulliform cells and sclerenchyma caused the curling of the C-type leaves in *cul1*. These developmental defects of the bulliform cells and sclerenchyma were also observed in the short lamina of the I-type leaf of *cul1*, but not in the long lamina (Fig. 2-5A). Quantitative analysis

indicated that the defects in the bulliform cells and sclerenchyma occurred at high frequency both in the C-type leaves and in the short lamina of the I-type leaf of *cul1* (Fig. 2-5, C and D).

The size of small vascular bundle seemed to be reduced in the C-type leaves, in addition to showing abnormal cell sizes and arrangement (Fig. 2-3; Fig.2-6A). Measurement of the area clearly indicated a significant reduction in the size of the small vascular bundles as compared with wild type (Fig. 2-6B). Reduced small vascular bundles were also observed in the short lamina of the I-type leaf (Fig. 2-5A).

Taken together, my observations indicate that the defects in tissue differentiation are likely to appear in leaves produced at the later vegetative phase in *cul1* via an intermediate state (I-type leaf).

Meristem activity seems to be related to the appearance of *cul1* leaf phenotype

Wild type and *cul1* produced a similar number of leaves (Table 2-1). By contrast, the period from germination to appearance of the flag leaf (the last vegetative leaf) was longer in *cul1* than in wild type, consistent with the observation that *cul1* displayed a late flowering phenotype. This result indicated that the rate of leaf initiation is slower in *cul1* than in wild type.

Next, I examined meristem activity, which is related to lateral organ differentiation (Tanaka et al., 2012; Suzuki et al., 2019). The size of the SAM was measured after clearing shoot apices obtained at the middle and late stages of the vegetative phase. The 9^{th} to 11^{th} leaves corresponded to P3 to P1 primordia,

respectively, in the shoot apex at the middle stage, whereas the 13th to 15th leaves corresponded to P3 to P1, respectively, at the later stage. The SAM of *cul1* was significantly smaller than that of wild type at the middle stage (Fig. 2-7, A and B). The SAM became larger in both *cul1* and wild type as the plants grew, but the SAM remained significantly smaller in *cul1* at the later stage as compared with wild type. The reduced size of the SAM might be associated with the slower rate of leaf initiation in *cul1*.

Plants enter into the reproductive phase after floral induction: in rice, the SAM that generates leaf primordia is converted into the inflorescence meristem that produces branch meristems. To investigate whether there is a relationship between the formation of C-type leaves and phase transition, I cultivated the *cul1* mutant in a growth chamber in winter (short day [SD] condition). As a result, the timing of the phenotypic appearance was highly accelerated, occurring at the 8th leaf onward (Fig. 2-8A). To confirm this result, I cultivated the *cul1* mutant under the SD condition for 10 days in the spring/summer season (Fig. 2-9). Again, the I- and C-type leaves appeared precociously in the plant cultivated temporally under SD, as compared with normally cultivated plants (Fig. 2-8B). Collectively, these results suggest that the appearance of the mutant leaf phenotype in *cul1* is probably associated with the vegetative-to-reproductive phase transition, which is dependent on SD conditions.

CUL1 encodes a protein similar to yeast ELP1, the largest subunit of the Elongator complex

The gene underlying the *cul1* mutation was isolated by my collaborators, Suzuki et al. The combination of rough mapping and the MutMap method (Abe et al., 2012) identified Os07g0563700 as a candidate gene responsible for the *cul1* mutant (Fig. 2-10). To confirm this, I analyzed the phenotype of transgenic plants, which had the genomic fragment of Os07g0563700 from wild type. T2 plants of transformant #2 with the transgene showed the wild-type leaf phenotype, whereas those without it showed the *cul1*-like leaf phenotype (Fig. 2-11). Histological analysis revealed that the rescued *cul1* mutants generated both bulliform cells and sclerenchyma in the leaves generated at later developmental stages (Fig. 2-12). These observations clearly indicated that the *CUL1* gene is Os07g0563700.

CUL1 encodes a protein similar to yeast ELP1 with two WD40 repeat domains in the N-terminus and a tetratricopeptide repeat (TPR) in the C-terminus (Fig. 2-13A). In yeast, ELP1 is the largest subunit of the Elongator complex, which comprises six subunits. Elongator has been shown to be involved in multiple functions including transcription, tRNA modification and histone acetylation (reviewed in Svejstrup, 2007; Glatt and Muller, 2013; Ding and Mou, 2015). *CUL1* is also orthologous to Arabidopsis *ELO2*, the mutation of which leads to a narrow leaf phenotype (Nelissen et al., 2005) (Fig. 2-13B).

I examined spatial expression pattern of *CUL1* in wild type by in situ hybridization. *CUL1* was expressed in the SAM and leaf primordia (Fig. 2-14A). Furthermore, RT-PCR analysis indicated that *CUL1* was expressed in the shoot apices at both the middle and late vegetative phase of rice development (Fig. 2-14B).

Knockout lines of *OsELP3* show phenotypes similar to *cul1*

The ELP3 subunit has two distinct domains, the rSAM and HAT domains, which are involved in tRNA maturation and histone acetylation. Mutation of the gene encoding ELP3 causes various biological defects in Arabidopsis, yeast and humans, depending on the organism (Nelissen et al., 2005; Nelissen et al., 2010; Leitner et al., 2015; reviewed in Dalwadi and Yip, 2018). Next, therefore, I focused on the function of the ELP3-like gene (*OsELP3*) in rice. A blast search indicated that the rice genome has a single gene similar to *ELP3*. Protein alignment revealed that the rSAM and HAT domains of ELP3-like proteins are highly conserved among distantly related species, suggesting that they have conserved molecular functions (Fig. 2-15).

To explore the developmental role of *OsELP3*, I disrupted the gene by using CRISPR-Cas9 technology and obtained three independent knockout lines with biallelic mutations causing a frameshift in the rSAM domain (Fig. 2-16A). All knockout lines produced curled narrow (C-type) leaves at the later stages of the vegetative phase, although they produced wild-type (N-type) leaves at the early and middle stages (Fig. 2-16B). In addition, I observed the I-type leaf just before the appearance of the C-type leaves in these knockout lines. Histological analysis revealed developmental defects in both the bulliform and sclerenchyma cells in these leaves (Fig. 2-16C). These leaf phenotypes were very similar to those observed for the *cul1* mutant. It seems therefore likely that both CUL1 and OsELP3 act as components of the Elongator complex in rice, and that the catalytic activity of OsELP3 is required for proper leaf development at later stages of the vegetative phase.

Transcriptome analysis

To examine the effect of the *cul1* mutation on gene expression, I performed transcriptome profiling in shoot apices including the meristem and leaf primordia (P1–P3). The shoot apex was sampled for RNA isolation at two different growth stages corresponding to the middle vegetative stage, where the developing 9th to 11th leaves correspond to the P3 to P1 primordia (referred as WT-M and *cul1*-M), and the late vegetative stage, where the developing 14th to 16th leaves correspond to the P3 to P1 primordia (referred as WT-L and *cul1*-L). I expected that the former apices would produce N-type leaves in the *cul1* mutant when matured, whereas the later apices would produce C-type leaves with the narrow and curled phenotype.

From the RNA sequencing (RNA-seq) data, I identified differentially expressed genes (DEGs) between the wild-type and *cul1* shoot apices, and between the middle and late shoot apices (false discovery rate, 0.01; fold change, 2). Notably, DEGs were identified in the comparison not only between *cul1*-M and *cul1*-L but also between WT-M and WT-L (Fig. 2-17). The latter observation suggests the gene expression profile differs in the SAM and leaf primordia (P1–P3) between the middle and late stages of the wild-type vegetative phase. A large number of genes were up-regulated in *cul1*-L as compared with WT-L, whereas fewer genes were down-regulated in *cul1*-L as compared with WT-L.

I looked for DEGs between *cul1*-M and *cul1*-L that were also present between WT-L and *cul1*-L (but not WT-M vs WT-L), as shown by the mulberry-colored area of the Venn diagram in Fig. 2-17. Among the shared DEGs, which are likely to be

associated with the morphological changes in the C-type leaves in *cul1*, the number of up-regulated genes was much larger than the number of down-regulated genes. In particular, genes encoding proteins constituting the SCF complex, such as SKP1-like proteins (OSKs), a cullin, and F-box proteins were highly up-regulated (5- to 50-fold) in *cul1*-L as compared with *cul1*-M, WT-M and WT-L (Fig. 2-18A; Table 2-2). The genes encoding ribosome protein-fused ubiquitin (UbL40) were also up-regulated. These proteins are involved in specific-protein degradation via the 26S proteasome, although the exact function of UbL40 is unclear at present. In addition, genes encoding heat shock proteins (HSPs) and translation-related proteins were also highly up-regulated in the *cul1*-L shoot apex, as compared with the other shoot apices (Fig. 2-18, B and C; Table 2-2). Considering that HSPs are involved in protein refolding, one of the shared features of the genes up-regulated in *cul1*-L seemed to be an association with protein quality control. I am not able to detect any common features among the shared down-regulated genes. I also examined the relative expression levels of genes whose mutation is known to cause a narrow leaf phenotype. None of these genes was down-regulated in *cul1*-L (Fig. 2-18D; Table 2-2).

In yeast, Elongator interacts with the RNA transcript elongation complex (Otero et al., 1999; Wittschieben et al., 1999) and is implicated in transcription elongation. If *CUL1* is also involved in transcriptional elongation in rice, the distribution of mapped-reads over the gene body in RNA-seq would be expected to differ between *cul1* and wild type. Unexpectedly, however, the coverage of mapped-reads in the gene body was indistinguishable between *cul1* and wild type (Fig. 2-19).

Discussion

The *cul1* mutation affects both leaf development and meristem activity

In this study, I first characterized the rice mutant *cul1*, which generates narrow and curled (C-type) leaves. These leaf phenotypes appeared at the late adult phase of vegetative development: in other words, *cul1* displayed heteroblastic changes at a unique growth stage. The narrow leaf phenotype of *cul1* was accompanied by a decreased number of vascular bundles, similar to other narrow leaf mutants described in rice (Fujino et al., 2008; Cho et al., 2013; Ishiwata et al., 2013; Kubo et al., 2019). Histological observation indicated that the C-type leaves contained undeveloped bulliform cells, no sclerenchyma, and small vascular bundles with a disordered cell arrangement. The former two histological defects seem to be related to adaxial curling of the leaf blade, consistent with several previous reports (Zhang et al., 2009; Hu et al., 2010; Zou et al., 2011). In addition, all these histological defects were also observed in the short lamina of the I-type leaf, suggesting that the reduction in leaf width is associated with abnormal cell differentiation in *cul1* leaves.

Longer cultivation periods were required to produce the flag leaf (the last vegetative leaf) in *cul1* as compared with wild type. Because the total number of leaves was approximately equal between *cul1* and wild type, this observation suggests that the rate of leaf generation in *cul1* is slower as compared with wild type. The SAM was significantly smaller in *cul1* than in wild type. The putative lower activity in the smaller SAM might be associated with the slower rate of leaf generation in *cul1*. Thus, *CUL1*

seems to be required to maintain the proper size and activity of the SAM.

In wild type, *CUL1* was expressed uniformly in the SAM and leaf primordia independent of plant growth stage. Consistent with this expression, the reduced SAM was observed in both the middle and late stages of plant growth in *cul1*. Despite the continuous expression of *CUL1*, the leaf phenotype was not observed until the middle growth stage, suggesting that the function of *CUL1* is specifically required for normal leaf development at the later stages of vegetative growth.

A prominent feature of the *cul1* mutant is the timing of the appearance of the mutant leaf phenotypes. The timing of this heteroblastic change in *cul1* is different from that of either the juvenile-to-adult or the vegetative-to-reproductive phase transition. In all *cul1* mutants examined, the C-type phenotype appeared at the 14^{th} or 15^{th} leaf. In addition, an intermediate type (I-type) leaf was frequently seen just before the emergence of the C-type leaves. Thus, the effect of the loss of *CUL1* activity seems to become evident in leaves only at a particular growth stage.

There seem to be two possibilities that might explain this heteroblastic change: the first is that it depends on plant age; the second is that it is associated with a developmental event such as the vegetative-to-reproductive transition. To address this issue, I cultivated *cul1* plants under the continuous SD condition that accelerates flowering in rice. As a result, the *cul1* phenotype was observed in younger plants from the 8^{th} leaf onwards, suggesting that plant age is unrelated to the heteroblastic change. Furthermore, the flag leaf emerged soon after the production of C-type leaves under the SD condition, suggesting that there is a strong link between the heteroblastic change

and the vegetative-to-reproductive phase transition. It is possible that, after perceiving a flowering signal, the vegetative meristem enters a transient state toward its conversion into the inflorescence meristem, and that this "transient meristem" is associated with the heteroblastic change in *cul1*.

CUL1 encodes the largest subunit of the Elongator complex

Gene isolation revealed that *CUL1* encodes a yeast ELP1-like protein in the Elongator complex. ELP1 is the largest subunit of this complex, functioning as a scaffold protein (reviewed in Versees et al., 2010; Woloszynska et al., 2016; Dalwadi and Yip, 2018). By contrast, ELP3 is the catalytic subunit responsible for tRNA maturation and protein acetylation. Notably, disruption of *OsELP3* led to the same defects in the leaf as the *cul1* mutation: bulliform cells and sclerenchyma were not differentiated, resulting in a narrow and curled leaf. In addition, the appearance of this phenotype was also restricted to the leaves that differentiated at the later vegetative phase, as observed in *cul1*. These results suggest that both CUL1 and OsELP3 act together as components of the Elongator complex in rice, and that the developmental alteration in the *cul1* mutant is related to either a defect in tRNA maturation or protein acetylation.

The genes encoding Elongator subunits have been well studied in Arabidopsis: for example, they have been shown to play important roles in leaf and root development, in addition to environmental responses (Nelissen et al., 2005; Nelissen et al., 2010; Kojima et al., 2011; Xu et al., 2012; Fang et al., 2015; Leitner et al., 2015; Qi et al., 2019; reviewed in Ding and Mou, 2015; Woloszynska et al., 2016). Arabidopsis

ELO genes such as *ELO2/AtELP1* and *ELO3/AtELP3* are expressed in the SAM and leaf primordia, similar to rice *CUL1* (Nelissen et al., 2010). However, in contrast to these Arabidopsis *ELO/AtELP* genes, which show localized adaxial expression in the leaf primordia, the rice *CUL1* gene was uniformly expressed in the leaf primordia. The narrow leaf phenotype and altered venation patterns are shared by Arabidopsis *elo/elp* mutants and the rice *cul1* mutant. Unlike the rice *cul1* mutant, however, *elo* mutant phenotypes are observed in leaves independent of the growth stage of Arabidopsis (Nelissen et al., 2005; Nelissen et al., 2010). Auxin-related phenotypes and altered auxin distribution are observed in Arabidopsis *elo* mutants (Nelissen et al., 2005; Malenica et al., 2007; Nelissen et al., 2010). Consistent with those observations, transcriptome analysis shows that the expression of auxin-related genes is affected by *elo* mutation (Nelissen et al., 2010). In the rice *cul1* mutant, however, the observed phenotypes were unrelated to auxin action, and no changes in the expression of auxin-related genes were observed. Taken together, the evidence suggests that loss of elongator activity is likely to have different effects on the development of rice and Arabidopsis. The diverse role of the Elongator complex at both the molecular and phenotypic level in various organisms has previously been suggested (reviewed in Mehlgarten et al., 2010; Dalwadi and Yip, 2018).

Molecular function of CUL1

One of the molecular functions of the Elongator complex is transcriptional elongation, which is associated with histone acetylation (Otero, 1999; Nelissen, 2010;

reviewed in Van Lijsebettens and Grasser, 2014). To address whether CUL1 is involved in transcriptional regulation in rice, I performed RNA-seq analysis. As a result, I was not able to find any differences in the distribution of sequence reads in the 5' to 3' direction in the gene body between *cul1* and wild type, suggesting that overall transcription elongation is not probably affected in the *cul1* mutant. However, transcriptome analysis revealed that a large number of genes are differentially expressed between the middle and late shoot apices not only in *cul1* but also in wild type. This result suggests that the properties of the SAM and leaf primordia change depending on plant growth in the vegetative phase even in wild type, and that the change in SAM size is associated with some of the changes in the gene expression profile.

A prominent feature of the transcriptome data is that the genes encoding components of the SCF complex and HSPs were markedly up-regulated specifically in the *cul1*-L shoot apex, which is responsible for the differentiation of C-type leaves. These proteins operate specifically in protein degradation and protein refolding, in other words, protein quality control. The highly elevated levels of the genes encoding these proteins suggest that abnormal proteins may accumulate in *cul1*-L shoot apices due to disturbed translation. By transcriptome analysis, I was not able to identify putative genes that might be related to the defects in leaf development in *cul1*. In addition, transcripts of genes known to be associated with a narrow leaf phenotype were not reduced in the *cul1*-L apices relative to other apices. Taking these observations altogether, it seems likely that the defects in the leaf development of *cul1* might result from a disturbance in translational control.

Similar to yeast and humans, Elongator has been shown to be involved in producing the wobble uridine modification of tRNA, which is essential for efficient translation, in Arabidopsis (Mehlgarten et al., 2010). In fact, the abundance of PIN proteins is regulated at the translational level via the maturation of tRNAs by Elongator (Leitner et al., 2015). Accordingly, it is plausible that CUL1 might play a similar role in translational control in rice. In future studies, proteome analysis might provide clues to identify factors associated with the partially lowered SAM activity and altered leaf morphology in *cul1* and to elucidate the mechanism underlying the heteroblastic change associated with meristem function.

Table 2-1 The total number of leaves and the period from the germination to the emergenece of the flag leaf

	2016		2017	
	WT	*cul1*	WT	*cul1*
Number of leaves	18.5 ± 0.33	18.7 ± 0.17	17.2 ± 0.19	18.1 ± 0.21
Period (days)	100.8 ± 1.32	122.7 ± 1.11	92.5 ± 1.19	121.5 ± 1.08
n	8	9	13	11

Table 2-2 Gene ID of up-regulated genes specifically in *cul1*-L in the transcriptome analysis.

Name	*	Gene ID (RAP)	Gene function
UbL40-1		OS09G0452700	ubiquitin fusion ribosomal protein L40
UbL40-2		OS03G0259500	ubiquitin fusion ribosomal protein L40
OSK17		OS07G0624900	S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN 17
OSK19		OS07G0625600	S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN 19
OSK22		OS07G0625500	S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN 22
OSK28		OS07G0625400	S-PHASE KINASE-ASSOCIATED PROTEIN1-LIKE PROTEIN 28
Cullin		OS01G0369200	Component of cullin-RING E3 ubiquitin ligase (CRL) complex
E3 ligase		OS06G0687200	Putative RING E3 ubiquitin (Ub) ligase
F-box 231		OS04G0591900	F-box protein 231
F-box 479		OS09G0342000	F-box protein 479
F-box	1	OS07G0459500	F-box domain, Skp2-like domain containing protein.
HSP	2	OS01G0136100	16.9 KDA CLASS I HEAT SHOCK PROTEIN 1
HSP	3	OS03G0266900	17.3 KDA CLASS I HEAT SHOCK PROTEIN
HSP	4	OS02G0782500	18.0-KD CLASS III HEAT SHOCK PROTEIN
HSP70-1		OS05G0460000	CYTOSOLIC HEAT SHOCK PROTEIN 70-1
HSP90.1		OS04G0107900	Heat shock protein 90.1
HSP101		OS05G0519700	HEAT SHOCK PROTEIN 101
EF-1		OS02G0134300	Transcription Elongation Factor 1
EF-2		OS01G0723000	Similar to Elongation factor EF-2
eIF-6		OS01G0280500	Similar to Eukaryotic translation initiation factor 6 (eIF-6)
eIF1A-1		OS02G0300700	EUKARYOTIC INITIATION FACTOR 1A-1
OS04G0627900	5	OS04G0627900	Translation initiation factor SUI1 domain containing protein.
Ribosomal P0		OS07G0251301	Ribosomal phosphoprotein P0
VAL-tRNA SYNTHETASE		OS10G0506200	VAL-TRNA SYNTHETASE 3
NAL1		OS04G0615000	NARROW LEAF1
NAL7		OS03G0162000	NARROW LEAF7
LSY1		OS05G0118700	LEAF LATERAL SYMMETRY1
SRL1		OS07G0102300	SEMI-ROLLED LEAF 1
SLE1/ND1		OS12G0555600	NARROW LEAF AND DWARF 1

* Shown in Fig. 2-18.

Table 2-3 Primers used in this study.

Amplification of the partial *CUL1* and *ACTIN1* cDNA for RT-PCR	
CUL1-RT-d5	5'-CTTGCAGCCAAGCTCCAGTC-3'
CUL1-RT-u6	5'-ATTCGCCAACAGGTGGTTTC-3'
ACT1 d1	5'-TCTGCGATAATGGAACTGGT-3'
ACT1 u2	5'-CATAGTCCAGGGCGATGTAGG-3'
Amplification of the CRISPR-Cas9 target site of *OsELP3*	
ELP3-geneseq-d3	5'-GCAGGCCAGAAGCAGGATAG-3'
ELP3-geneseq-u4	5'-GCCCCAAGCAATAATCAGGT-3'

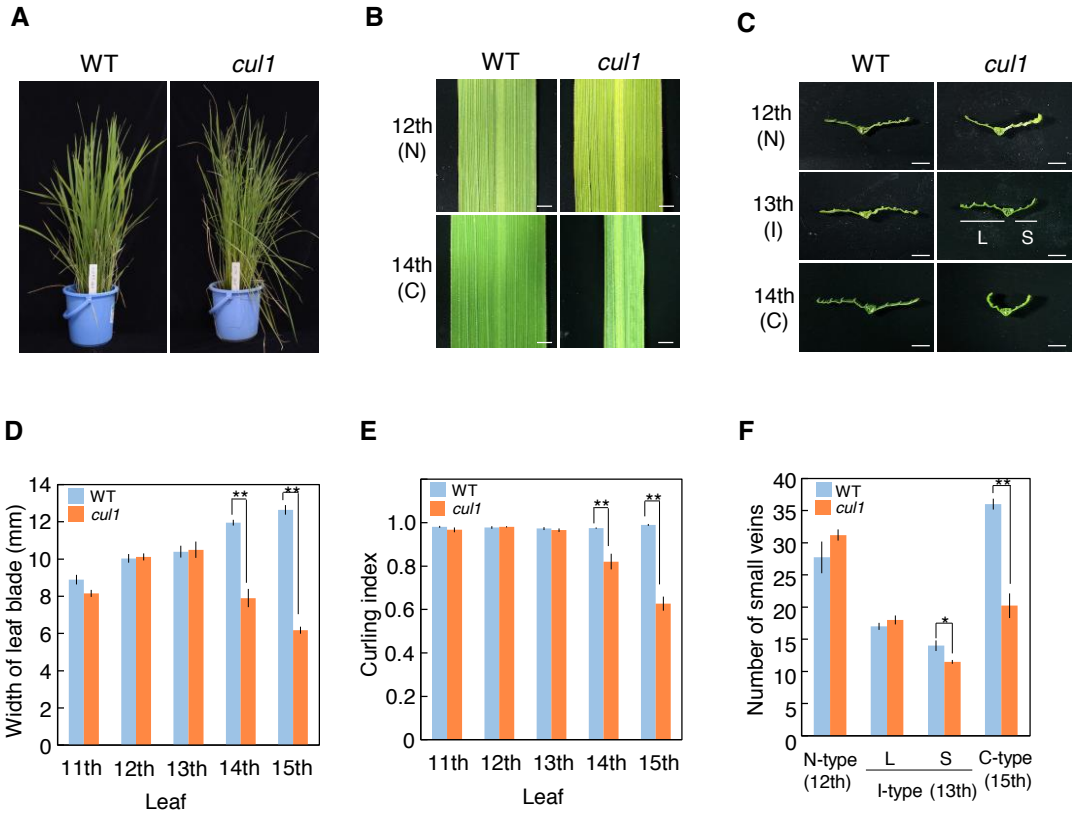

Figure 2-1. Leaf phenotypes of *cul1* and wild type.
A, Adult rice plants at the growth stage when the flag leaves are emerging, but the panicles are not. Wild type, 97 days after germination (dag); *cul1*, 128 dag. Note that the wild type and *cul1* plants are at similar growth stages, despite the different periods since germination. B, Middle part of the leaf blade. C, Free hand section of the leaf blade. D, Width of the leaf blade. E, Curling index showing the extent of curling of the leaf blade. The curling index is the distance between the margins of the curling leaf and the width of the leaf blade after flattening (Matsumoto, 2017). F, Number of small veins in the normal (N-), intermediate (I-), and curling (C-) type leaf. L, long lamina; S, short lamina. Asterisks (D-F) indicate significant differences (Student's t-test: **P<0.01, *P<0.05). Error bars represent SE. Scale bars, 2 mm in B, C.

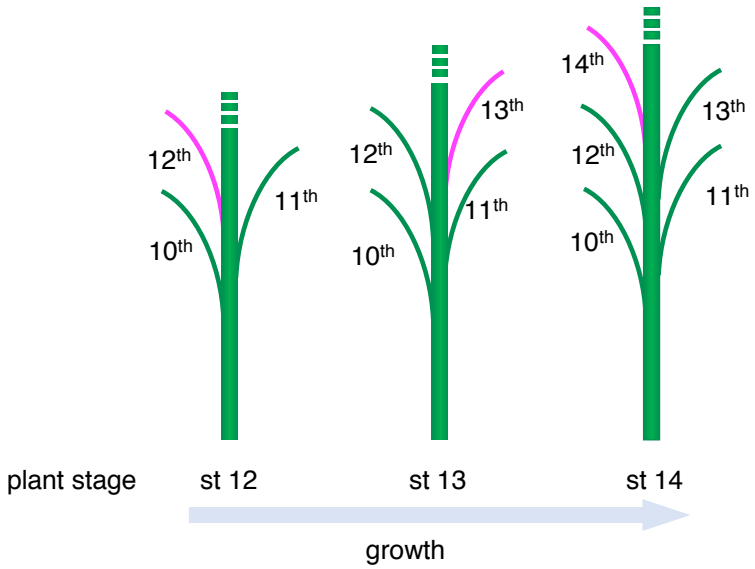

Figure 2-2. Schematic representation of the order of leaf emergence. The leaf blade with magenta was characterized in different stages of plant growth.

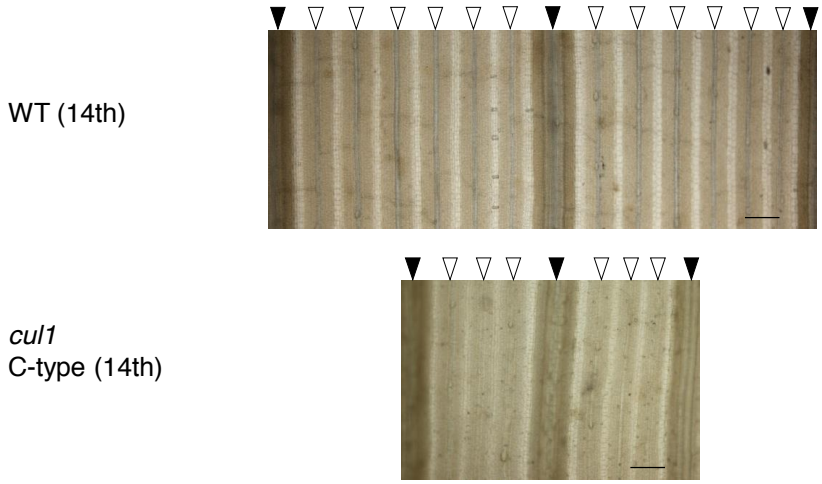

Figure 2-3. Reduction in the number of small vascular bundles in *cul1*. The leaf was treated with a clearing agent. The lines of the small vascular bundles in *cul1* are not clear, suggesting their small size. Closed triangles, large vascular bundles; open triangles, small vascular bundles.

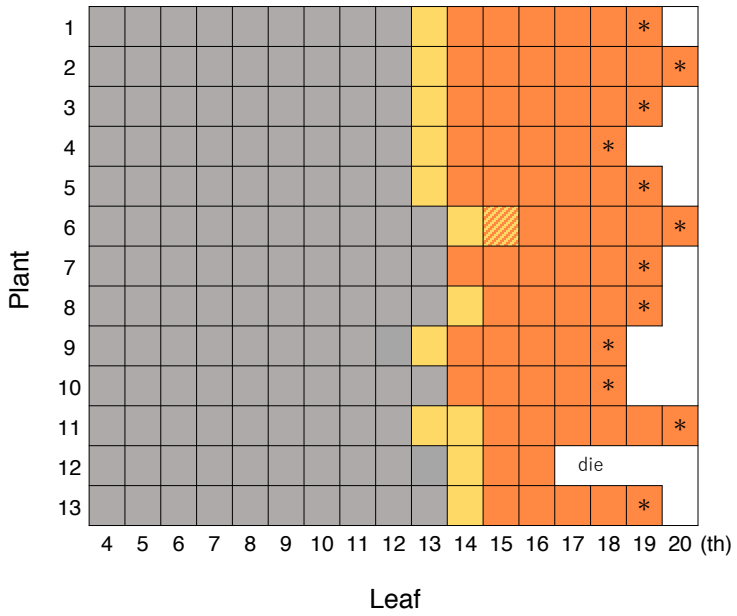

Figure 2-4. Schematic representation of the timing of the appearance of the *cul1* leaf phenotype.
gray, N-type leaf; orange, C-type leaf; yellow, I-type leaf; star, flag leaf.

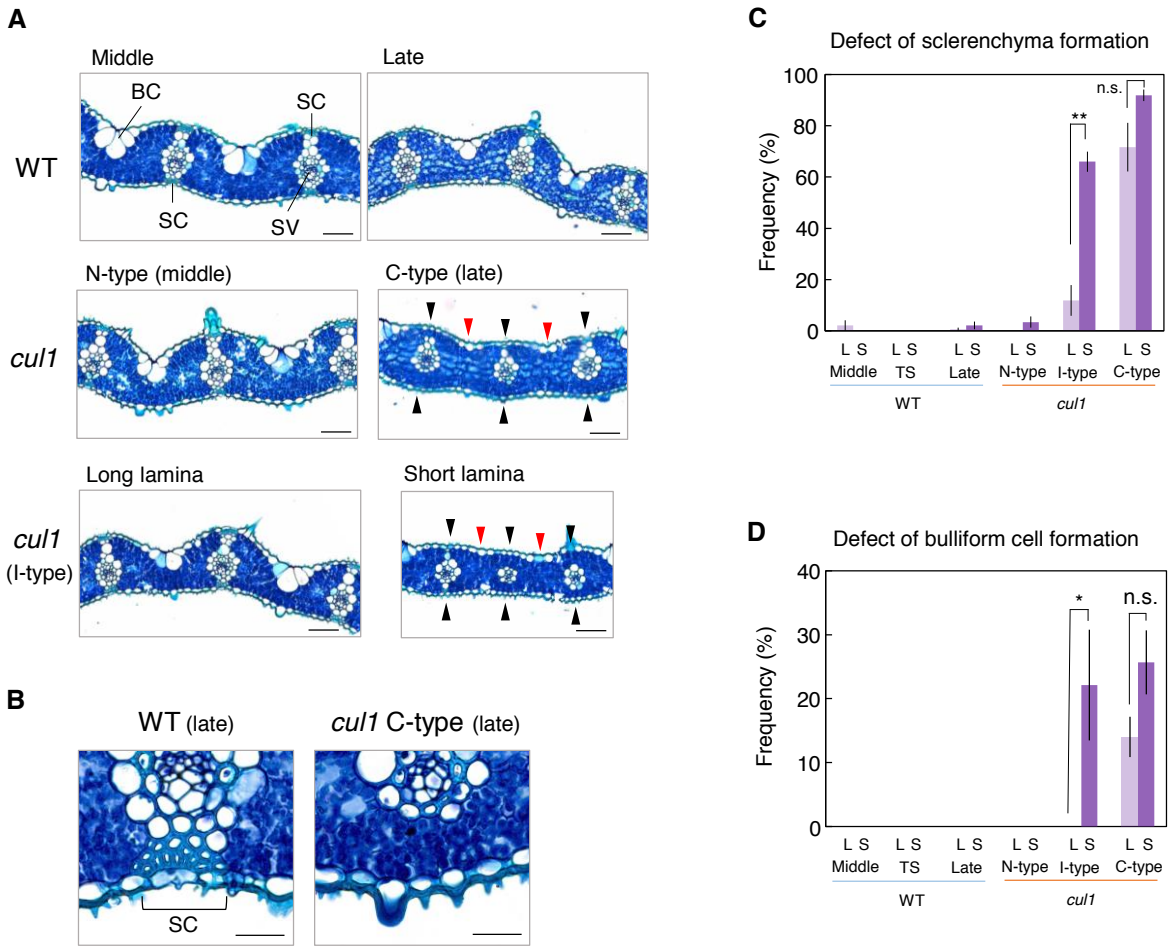

Figure 2-5. Histological observation of the leaf blade of *cul1* and wild type. A, Transverse sections of the leaf blade. Red and black arrowheads indicate developmental defects of bulliform cells and the absence of sclerenchyma, respectively. B, Close-up view showing the sclerenchyma abaxial to the small vascular bundle in wild type and the absence of it in *cul1*. C, Frequency of the absence of the sclerenchyma in *cul1* leaves. D, Frequency of incomplete differentiation of bulliform cells in *cul1* leaves. BC, bulliform cell; SC, sclerenchyma; SV, small vascular bundle. Asterisks (C, D) indicate significant differences (Student's t-test: **P<0.01, *P<0.05). Error bars represent SE. Scale bars, 50 μm in A; 20 μm in B.

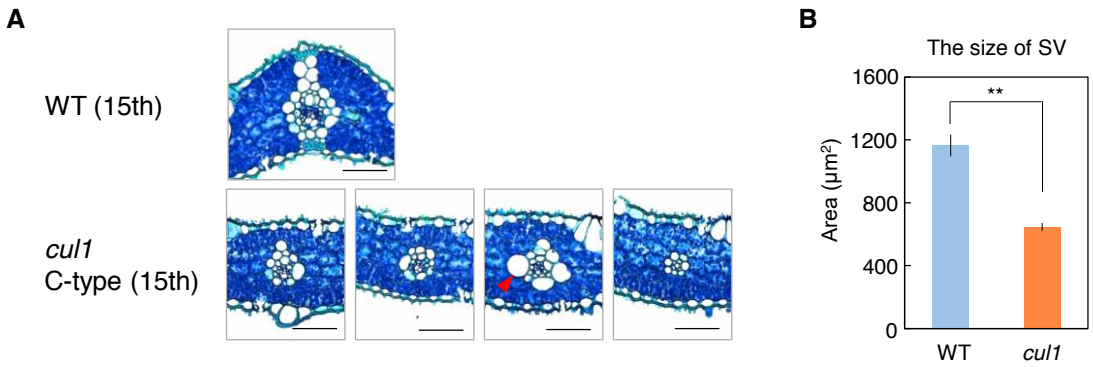

Figure 2-6. The small vascular bundles in the C-type leaf of *cul1* mutant. A, Reduction in size and appearance of abnormal cells in the small vascular bundle in the C-type leaf of *cul1*, as compared with the wild-type leaf. Red triangle indicates an abnormally enlarged bundle sheath cell. B, Size of the small vascular bundle in the 15th leaf in wild type and *cul1*. Asterisks indicate significant differences (Student's t-test: **P<0.01). Error bars represent SE. Scale bars, 50 μm in A.

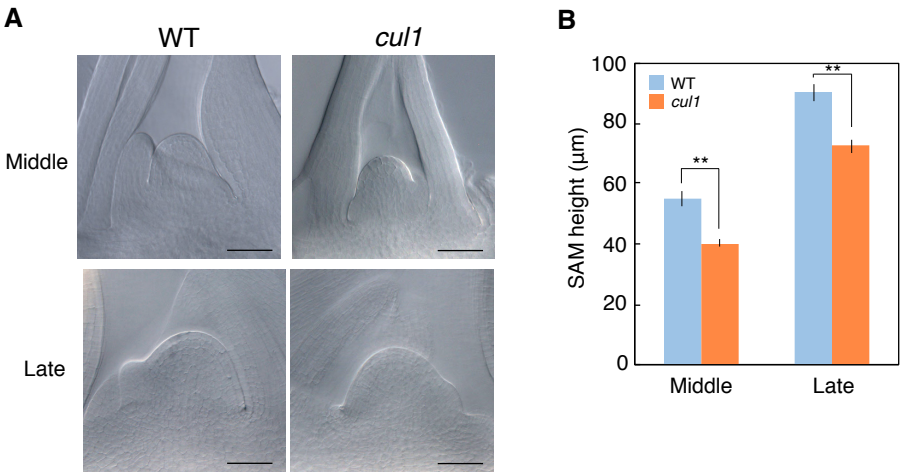

Figure 2-7. Comparison of the SAM size.
A, Wild-type and *cul1* SAM after treatment with a clearing agent.
B, Height of the SAM.Asterisks indicate significant differences (Student's t-test: **P<0.01). Error bars represent SE. Scale bars, 50 μm in A.

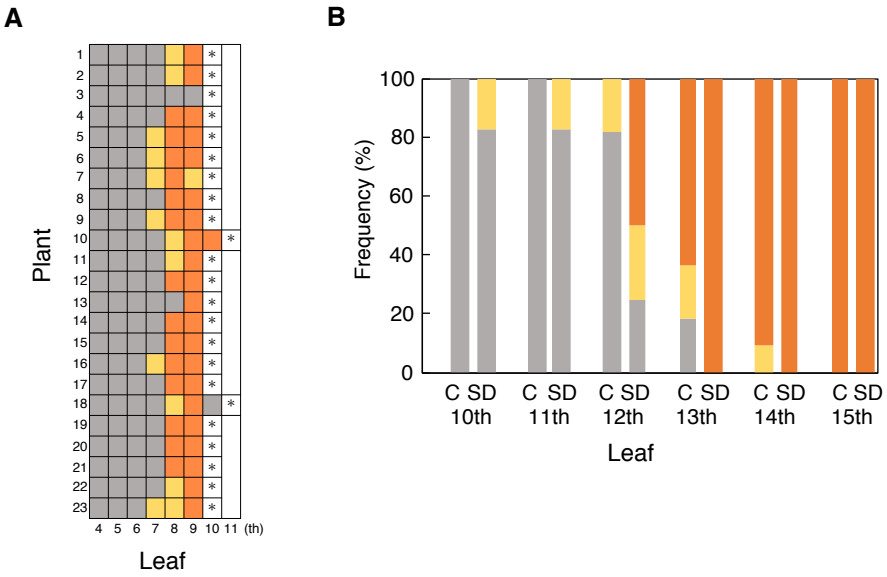

Figure 2-8. Effect of short daylight treatment on the leaf phenotype of *cul1*.

A, Schematic representations of the timing of the appearance of the *cul1* leaf phenotype in plants grown under the SD condition (winter season). Day length, 9.75–10 hours. Gray, N-type leaf; orange, C-type leaf; yellow, I-type leaf; star, flag leaf. C, Effect of SD treatment on the appearance of the *cul1* leaf phonotype (see Fig. 2-9 for the treatment). C, control (normal growth); SD, short daylight treatment.

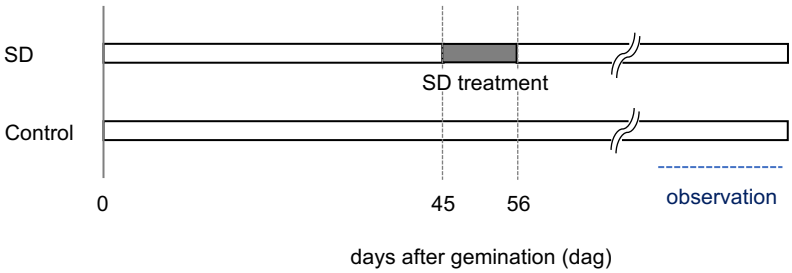

Figure 2-9. Time schedule of short daylight treatment. Plants were grown outdoors in summer (day length: 13.5–14.5 hours). For SD treatment, plants were placed in the dark for 14 hours (before sunset and after sunrise).

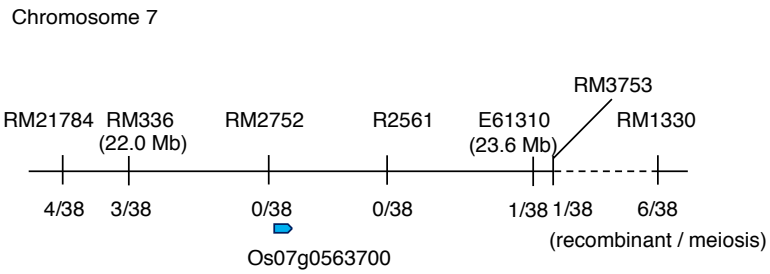

Figure 2-10. Rough mapping of the *CUL1* locus.
Rough mapping and gene isolation were performed by my collaborators, Suzuki et al (see acknowledgment).

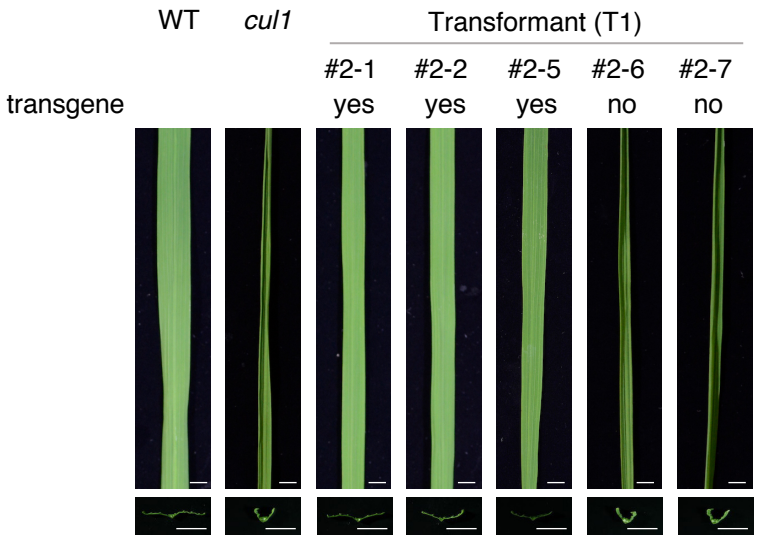

Figure. 2-11. Complementation of the *cul1* mutant by introducing the wild-type genomic DNA of Os07g0563700.
Leaf phenotypes of the T1 generation of a transformant (#2), and presence or absence of the transgene. Scale bars, 5 mm.

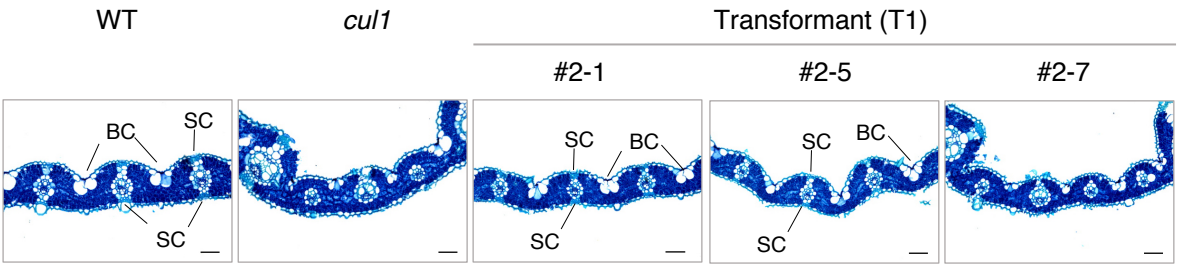

Figure 2-12. Histological analysis of transgenic plants carrying the wild-type genomic DNA of Os07g0563700.
Normal development of bulliform cells and sclerenchyma in the leaf of transgene-rescued plants. Scale bars, 50 μm.

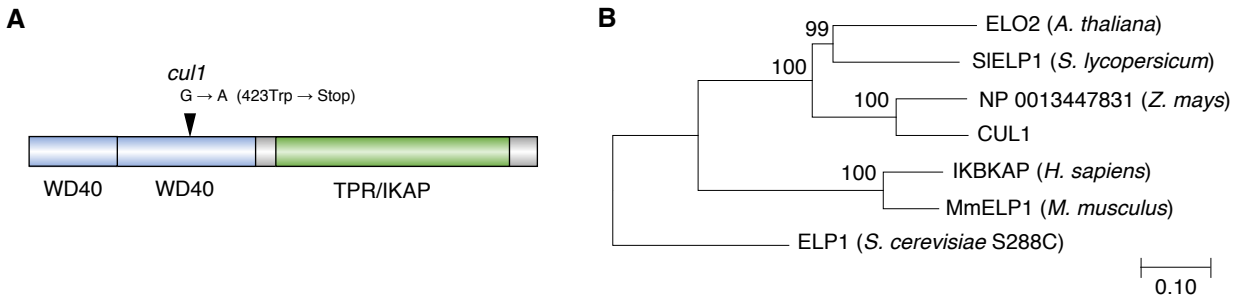

Figure 2-13. Protein structure of CUL1 and phylogenetic tree of CUL1-related proteins (ELP1 homologs).

A, Schematic representation of the *CUL1* protein. Domains rich in WD40 repeats are indicated in light blue, and the TPR/IKA domain is indicated in green. B, Phylogenetic tree of ELP1 proteins. The tree was constructed by a neighbor-joining method using MEGA7 (Kumar et al., 2016). Numbers above the branches indicate the percentage of bootstrap values calculated from 1000 replicates.

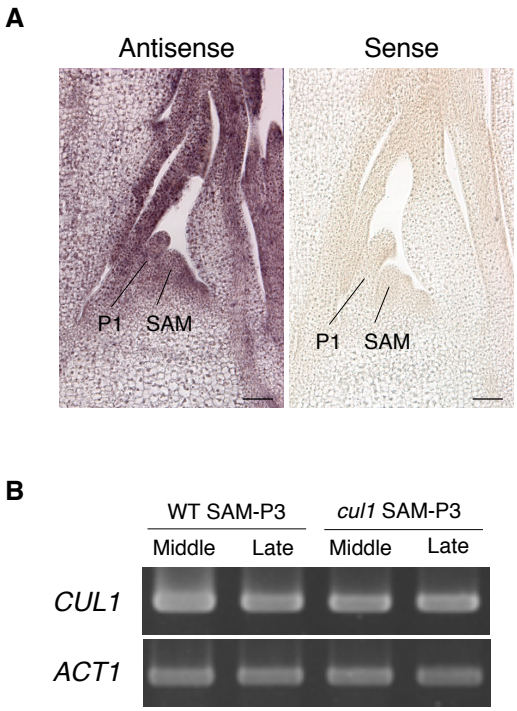

Figure 2-14. Expression of *CUL1* transcripts. A, In situ localization of *CUL1* transcripts in the shoot apex of a wild-type seedling 2 weeks after germination. B, RT-PCR analysis. Scale bars, 50 μm in A.

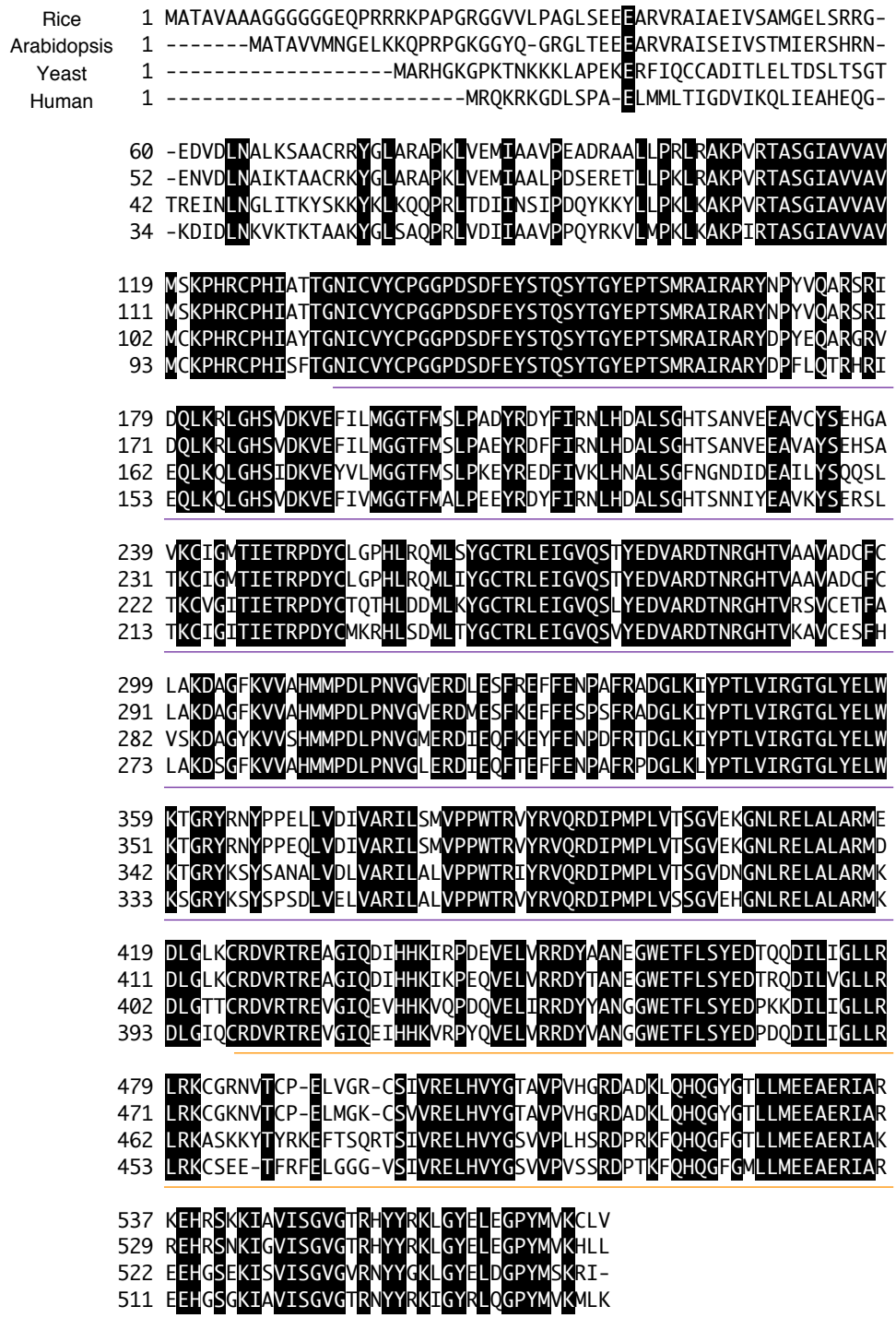

Figure 2-15. Alignment of the amino acid sequence of OsELP3. Purple and orange lines indicate the radical SAM and HAT domains, respectively.

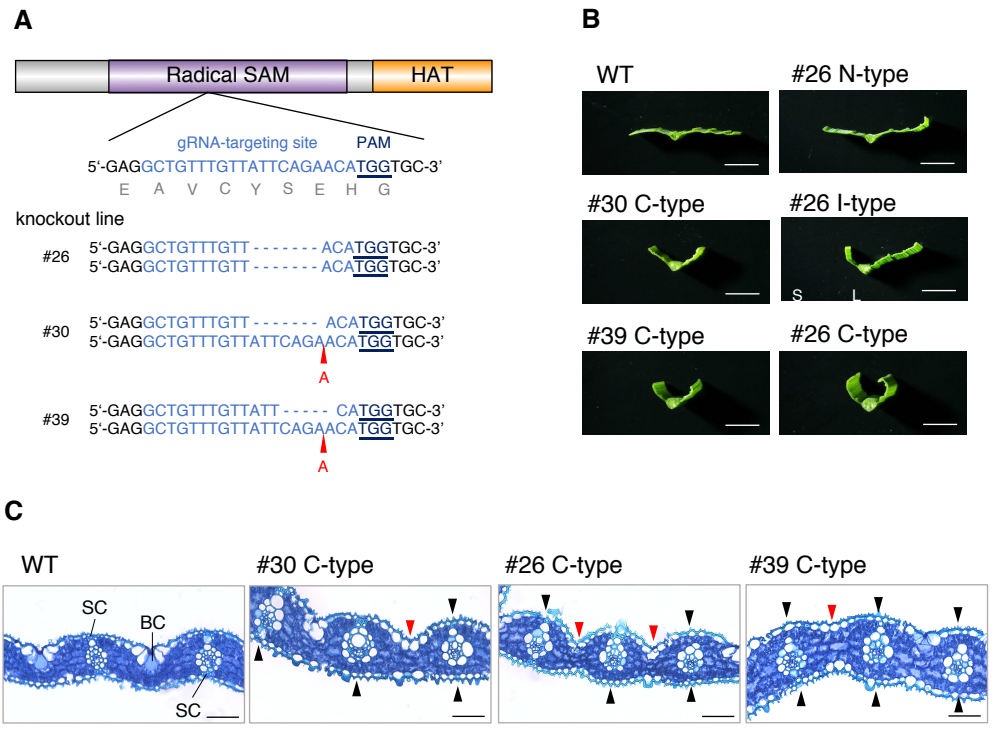

Figure 2-16. *OsELP3* knockout plants generated by CRISPR-Cas9 technology.

A, The target sequence in *OsELP3* in the wild-type genome and the sequences mutated by the CRISPR-Cas9 method. B, Free hand sections of the leaf blade. C, Cross-sections of the leaf blade tissue. Red and black arrowheads indicate the developmental defects of bulliform cells and the absence of sclerenchyma in *OsELP3* knockout lines, respectively. BC, bulliform cell; SC, sclerenchyma. Bars, 2 mm in B; 50 μm in C.

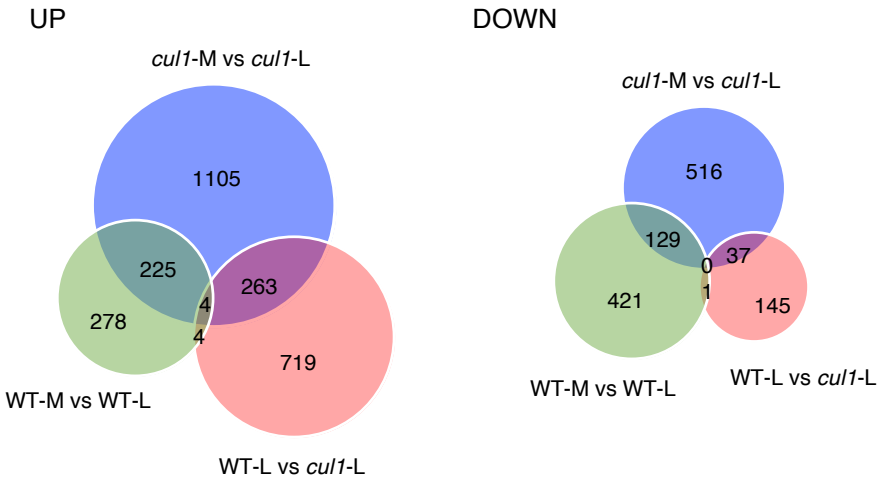

Figure 2-17. Transcriptome analysis in wild-type and *cul1* shoot apices at the middle and late vegetative phase.
Venn diagrams of genes that were up-regulated and down-regulated in each comparison.

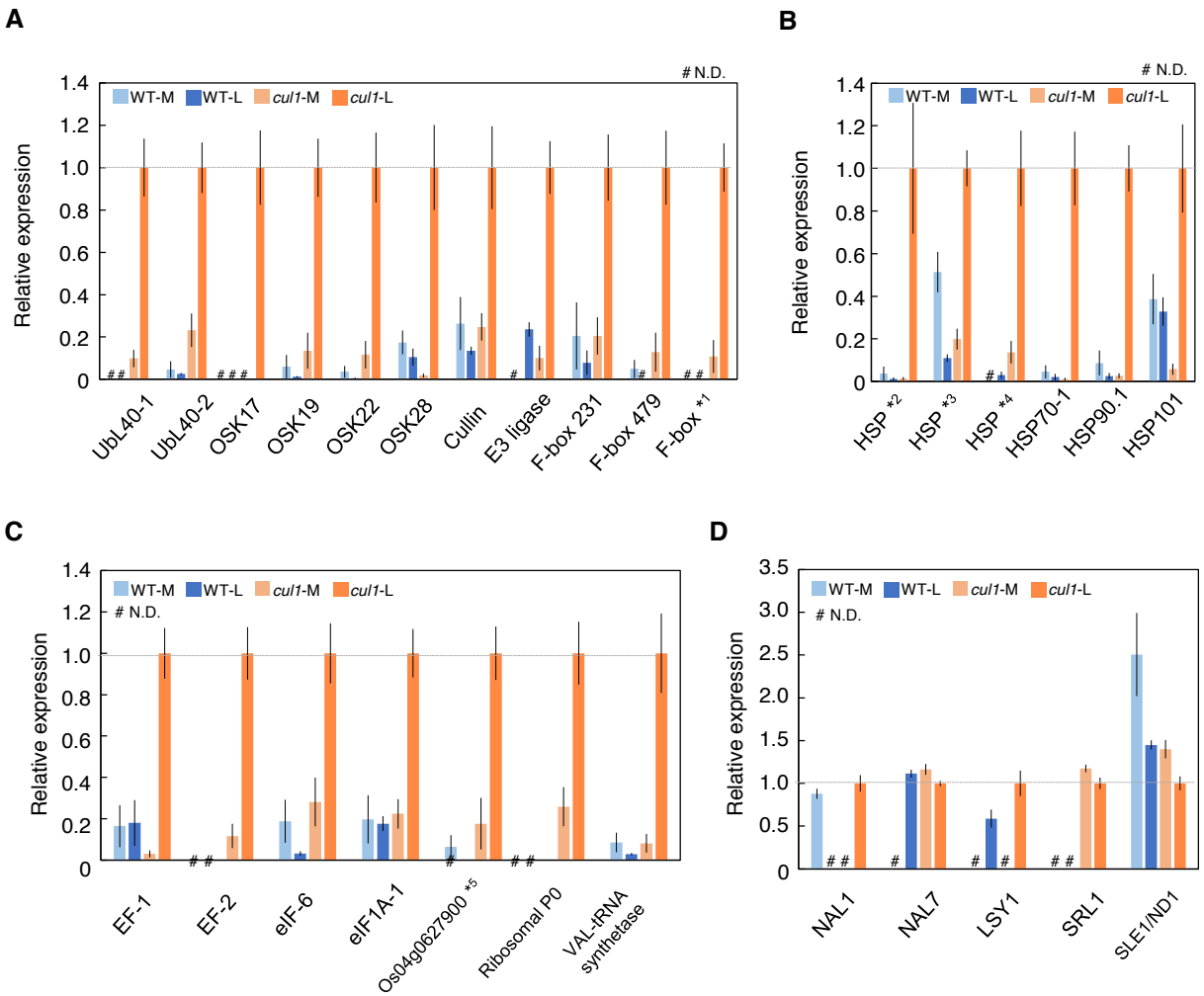

Figure 2-18. Relative expression levels of genes from RNA-seq data.
A, Relative expression levels of genes related to the SCF complex. B, Relative expression levels of HSP genes. C, Relative expression levels of genes related to translation. D, Relative expression levels of genes associated with a narrow leaf phenotype. OSK, *O. sativa* SKP1-like gene; UbL40, ubiquitin-fused ribosomal large subunit 40; HSP, heat shock protein.

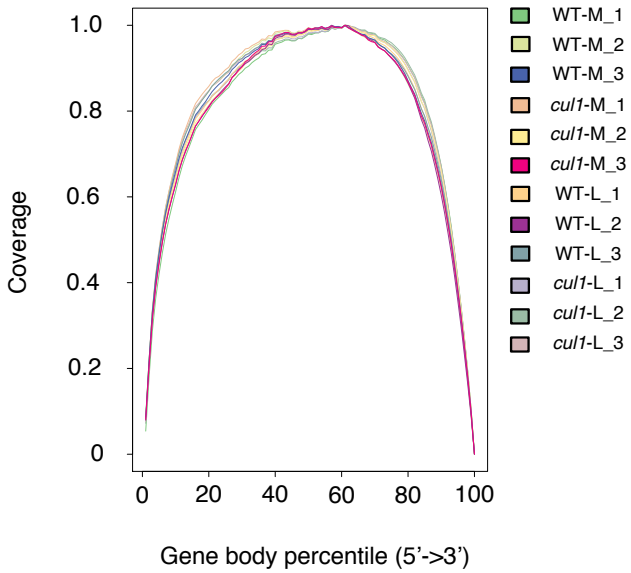

Figure 2-19. Read coverage in the gene body from RNA-seq data.

Chapter 3

Characterization of *half-pipe-like leaf1* mutant that exhibit curled leaf phenotype

Introduction

Plants successively generate leaves that perceive sunlight for photosynthesis. Leaf primordia initiate at the peripheral region of the meristem, where the leaf founder cells are recruited from undifferentiated cells supplied from the stem cells (Aichinger et al. 2012). In the leaf primordia, three developmental axes (apical-basal, adaxial-abaxial, and centrolateral axis) are established and then specific cell types are differentiated along these axes (Kuhlemeier and Timmermans 2016). At the peripheral region of the meristem, *KNOX* genes, such as *SHOOT MERISTEMLESS* (*STM*) of *Arabidopsis thaliana* and *OSH1* of *Oryza sativa* (rice), are down regulated to acquire the fate of the leaf founder cells (Long et al. 1996; Sentoku et al. 1999). The HD-ZIPIII and *ETTIN* genes and small RNAs targeting these genes are involved in the establishment in the adaxial-abaxial cell fate (Chitwood et al. 2007; Husbands et al. 2009). These initial developmental processes and genes regulating these processes in leaf development are likely to be conserved in eudicots and monocots.

In contrast to this conservation, the morphology and size of the leaves are highly diverse in angiospems. Venation pattern also differs between eudicots and monocots.

Therefore, developmental mechanism after leaf primordium formation seems to be diversified in angiosperms. However, understanding of gene function involved in the formation of diverse leaf form or cell differentiation specific to each species is still poor at present.

In rice, like other grasses such as *Zea mays* (maize) and *Brachypodium distachyon*, the leaf is composed of mainly two parts, leaf blade and leaf sheath. Between the two distinct structures, an auricle and a ligule are formed. Two types of vascular bundles, large and small ones, are formed in parallel in both leaf blade and leaf sheath. The two types of vascular bundles are composed of partially different cell types. In the central region of the leaf blade, a strong structure, midrib, is formed. The midrib is developed from enough amounts of cells, which are proliferated in the central region of early leaf primordia (Ohmori et al. 2011; Yamaguchi et al. 2004). The *DROOPING LEAF* (*DL*) gene plays a central role in midrib formation by promoting the cell proliferation (Ohmori et al. 2008; Yamaguchi et al. 2004). The midrib is indispensable for erection of the leaf blade, because it is thin and very long in rice. In *dl* mutant, the leaves fail to erect due to the lack of the midrib.

A number of genes are involved in the formation and elaboration of the leaf in rice. Failures in the establishment of the adaxial-abaxial axis generate abnormal leaves in the mutants such as *shoot organization2* and *wavy leaf1* (Abe et al. 2010; Itoh et al. 2008). These abnormalities are often closely associated with the partial defects in the shoot apical meristem. On the contrary, slight defects in abaxial identity produce rolled leaves, in which other characteristic are largely normal (Yan et al. 2008; Zhang et al.

2009). Mutations in the genes for auxin synthesis and for some transcription factors cause narrow leaf phenotype, together with a reduction in the number of vascular bundles (Cho et al. 2013; Fujino et al. 2008; Ishiwata et al. 2013; Kubo et al. 2016). To understand the genetic mechanism underlying rice leaf morphogenesis in more detail, I focused on the *half-pipe-like leaf1* (*hal1*) mutant, which has curled leaves, in this chapter.

Results

I searched for a rice mutant population, in which mutation had been induced by the chemical mutagen, N-methyl-N-nitrosourea (MNU) (Satoh et al. 2010). The *hal1-d* mutant was found as a mutant showing an erect and curled leaf phenotype (Fig. 3-1, A and B). Leaves were flat in wild type, whereas they were curled adaxially in *hal1-d* (Fig. 3-1, B and C). The cross section of the *hal1-d* leaf looked like a half-pipe (Fig. 3-1C). Genetic analysis revealed that *hal1-d* is a semi-dominant trait: F1 plants between *hal1-d* and wild type (Kinmaze, a genetic background of *hal1-d*) showed mildly curling leaf phenotype (Fig. 3-1C). F2 plants showing curled, mildly curled and flat leaves segregated 52:101:32, respectively (1:2:1; P=0.053, χ^2=5.89). In this article, I used the mutant homozygous for *hal1-d.*

To describe the extent of curling, I measured the width of the leaf blade in natural state (a) and in forcedly flattened state (b) in the leaf, when the whole leaf blade came out from the older leaf sheath. The curling index (a/b) in wild type was about 1.0 (Fig. 3-1D). By contrast, it was approximately 0.5 in *hal1-d*, and this value was constant in the all examined leaves (Fig. 3-1D). This result suggests that the extent of leaf curling is independent of the leaf position, that is, it was not affected by the developmental stage of rice plants.

To reveal the cause of leaf curling, I performed histological analysis. Bulliform cells are differentiated in the adaxial epidermis between the vascular bundles.

In wild type, a large teardrop-shaped bulliform cells were observed, and these cells are clearly distinguished from normal epidermal cells (Fig. 3-2, A, C and E). By contrast, the bulliform cells were small and round in *hal1-d* mutant (Fig. 3-2, B, D and G). In some cases, peripheral bulliform cells were indistinguishable from normal epidermal cells in *hal1-d* (Fig. 3-2G). Although the bulliform cells between the large and small vascular bundles and those between small vascular bundles were reduced in size in *hal1-d*, the bulliform cells of the latter case seemed to be more profoundly affected (Fig. 3-2, D and G). I measured the area of the bulliform cell clusters between the small veins using the cross section of the leaf (Fig. 3-2, E and G). The area of the bulliform cell cluster was reduced to about 50% in *hal1-d*, as compared to wild type (Fig. 3-2K). Next I examined bulliform cell files by making the leaf transparent by TOMEI method (Hasegawa et al. 2016). The bulliform cell files were relatively wide and were clearly distinguished from other tissues in wild type (Fig. 3-2I). By contrast, the cell files were narrower in *hal1-d* than in wild type, and, in some cases, they were indistinguishable from other parts in *hal1-d* leaves (Fig. 3-2J). Size measurement showed that the width of the bulliform cell file was significantly smaller in *hal1-d* leaves than in wild type leaves (Fig. 3-2L). Taking together, the size of the bulliform cells was reduced in *hal1-d*. Other tissues such as sclerenchyma were not obviously affected by the *hal1-d* mutation (Fig. 3-2, F and H). Therefore, the reduction in the bulliform cell size seems to be a major cause of leaf curling in *hal1-d*.

To examine the *hal1-d* effect, I measured the size of the leaf blade. Each size was measured just after the whole leaf blade came out from the older leaf sheath. The

leaf size did not change substantially thereafter. In wild type, the last leaf (just before the flag leaf) was shorter than the 14th leaf (Fig. 3-3A); the length of the leaf blade varied depending on the leaf position. By contrast, the last leaf was longer than the 14th leaf in *hal1-d*. Thus, the changing pattern of the leaf blade length during plant growth differed between wild type and *hal1-d*. The width of the leaf blade also exhibited a similar result (Fig. 3-3B). These observations suggest that the reduction in the sizes of the last leaf in wild type was partially alleviated in the *hal1-d* mutant. The last two leaves of *hal1-d* had more veins than those of wild type, consistent with the fact that *hal1-d* had broader last leaves than wild type (Fig. 3-3C).

The effect of *hal1-d* appeared weakly in the reproductive phase. The spikelet (lemma and palea) appeared to be seemingly slender in *hal1-d* just before anthesis, as compared to wild type (Fig. 3-3D). The measurement of spikelet size showed a significant reduction in the spikelet width (Fig. 3-3F). I often observed the seeds, in which the lemma and palea failed to close, probably due to the reduced width of these organs and an increase in the endosperm volume (Fig. 3-3E). The hulled seeds of *hal1-d* were slightly narrower than those of wild type (Fig. 3-3E).

Discussion

In this chapter, I characterized the phenotype of rice *hal1-d* mutant. Leaves in *hal1-d* mutant were curled adaxially and were slightly altered in their size. Histological observation revealed that bulliform cells were small in size and abnormal in shape in *hal1-d* leaves, as compared with wild-type leaves. In grasses, the bulliform cells are involved in leaf rolling by their reduced volumes under dry condition, suggesting that these cells are responsible for leaf flattening under normal condition. Therefore, undeveloped small bulliform cells are likely to be a major cause of curled leaves in *hal1-d* mutant. Because incomplete bulliform cells were formed in *hal1-d*, the *HAL1* gene seems to be required for growth of bulliform cells, but not for differentiation.

A defect in a gene encoding a cellulose synthase-like proteins (OsCSLD4) affects bulliform cell formation in rice, resulting in the generation of rolled and narrow leaf blade. This gene is identified independently and named *SLENDER LEAF 1* (*SLE1*) (Yoshikawa et al. 2013), *NARROW AND ROLLED LEAF1* (*NRL1*) (Hu et al. 2010), or *NARROW LEAF AND DWARF1* (*ND1*) (Li et al. 2009). Unlike *hal1-d*, the mutant of this gene produces narrow leaves and shows dwarf phenotype. Rolled leaf is also associated with a partial defect in the establishment of adaxial-abaxial polarity in the leaf. For example, a mutation in the *ROLLED LEAF9* (*RL9*) */SHALLOT-LIKE1* (*SLL1*) gene, a member of KANADI gene encoding GRAS transcription factor, also bring about rolled leaf phenotype (Yan et al. 2008; Zhang et al. 2009). In this case, rolled leaf is related to incomplete development of sclerenchyma cells at the abaxial side. It will be

interesting to know what protein is encoded by *HAL1* and how the protein act in the growth of bulliform cells.

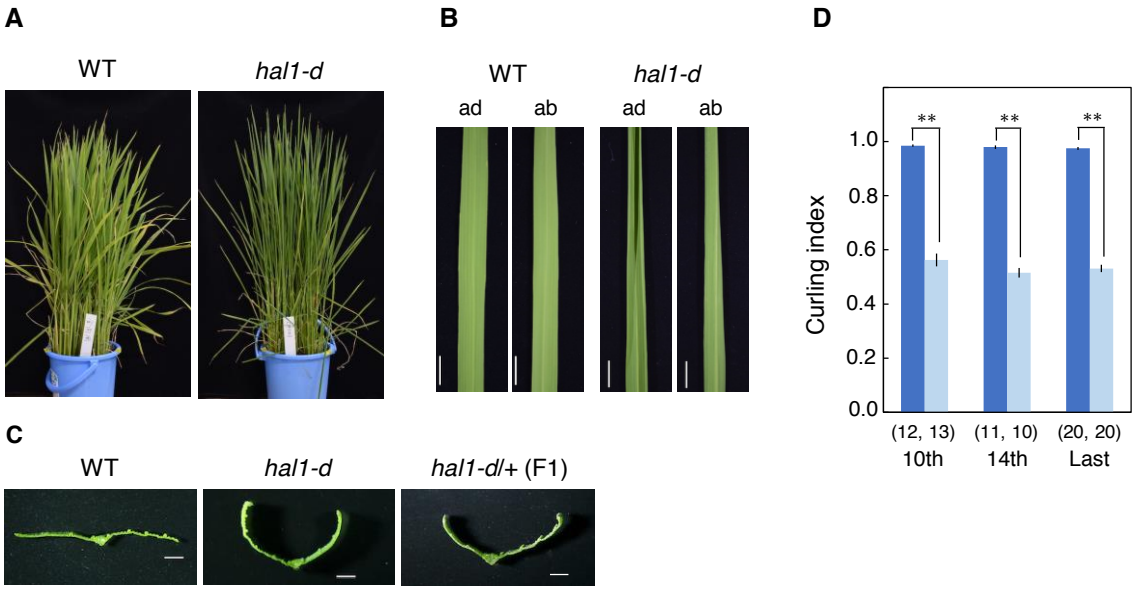

Figure 3-1. Morphological characteristics of *hal1-d* mutant.

A, 108-days-old plants of wild type and *hal1-d*. B, The leaf blade. ab, abaxial side; ad, adaxial side. C, Cross section of the leaf blade. D, Curling index. Blue, wild type; light blue, *hal1-d*. '10th', '14th' and 'last' denote leaf position, the last leaf means the one formed just before the flag leaf. The number in parentheses indicates the number of leaves examined. Asterisks indicate significant differences (Student's t-test: **P < 0.01). Error bars represent SE. Scale bars, 1 cm in B; 1 mm in C.

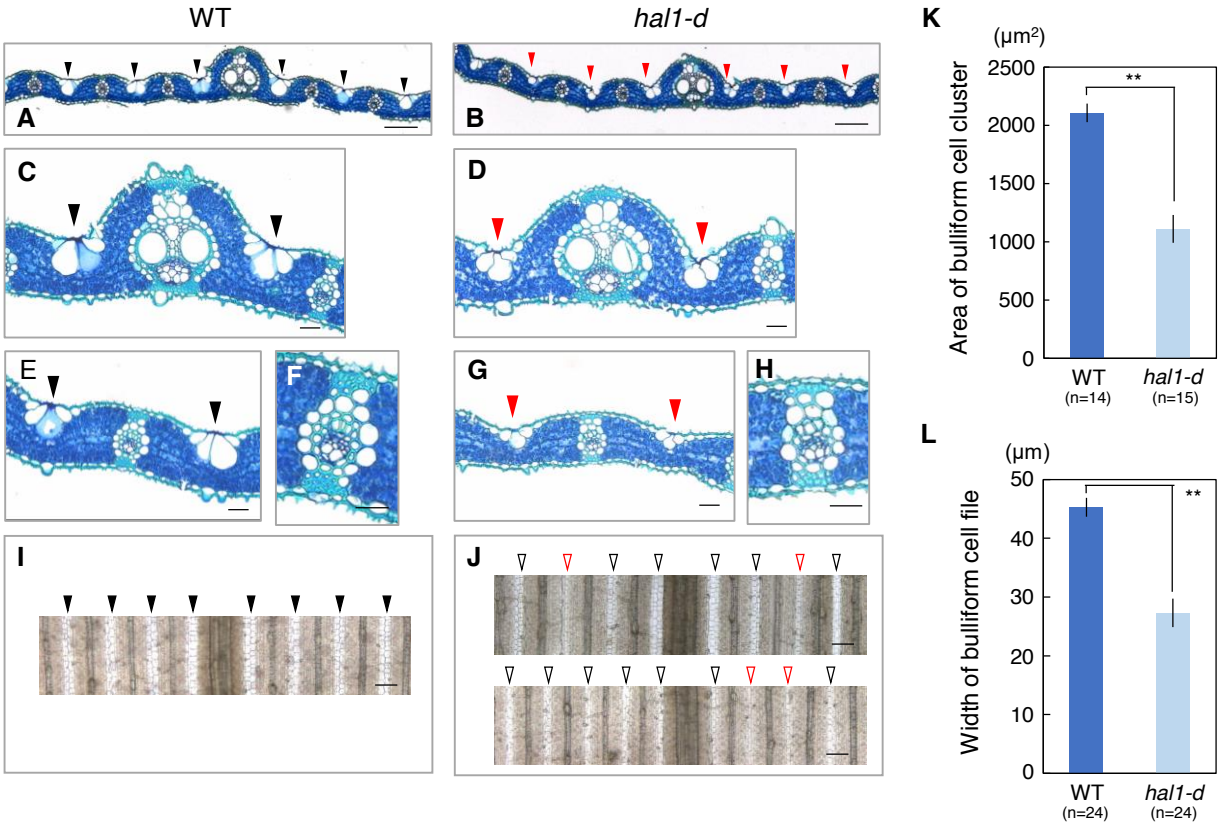

Figure 3-2. Histological analysis of the leaf blade.

A and B, Transverse sections of the leaf blade. C and D, The bulliform cells between large and small veins. E and G, The bulliform cells between small veins. Black arrowheads indicate normal bulliform cells, and red arrowheads indicate small and abnormal-shaped bulliform cells. F and H, Close-up view of sclerenchyma next to the small vascular bundle. I and J, Segments of the leaf blade cleared by TOMEI method. Arrowhead indicates the position of the bulliform cell file, and red closed arrowhead indicates narrower or unclear bulliform cell file. K, The area of the bulliform cell cluster. L, The width of the bulliform cell file. Asterisks indicate significant differences (Student's t-test: **P < 0.01). Error bars represent SE. Scale bars, 100 μm in A, B, I, J; 20 μm in C to H.

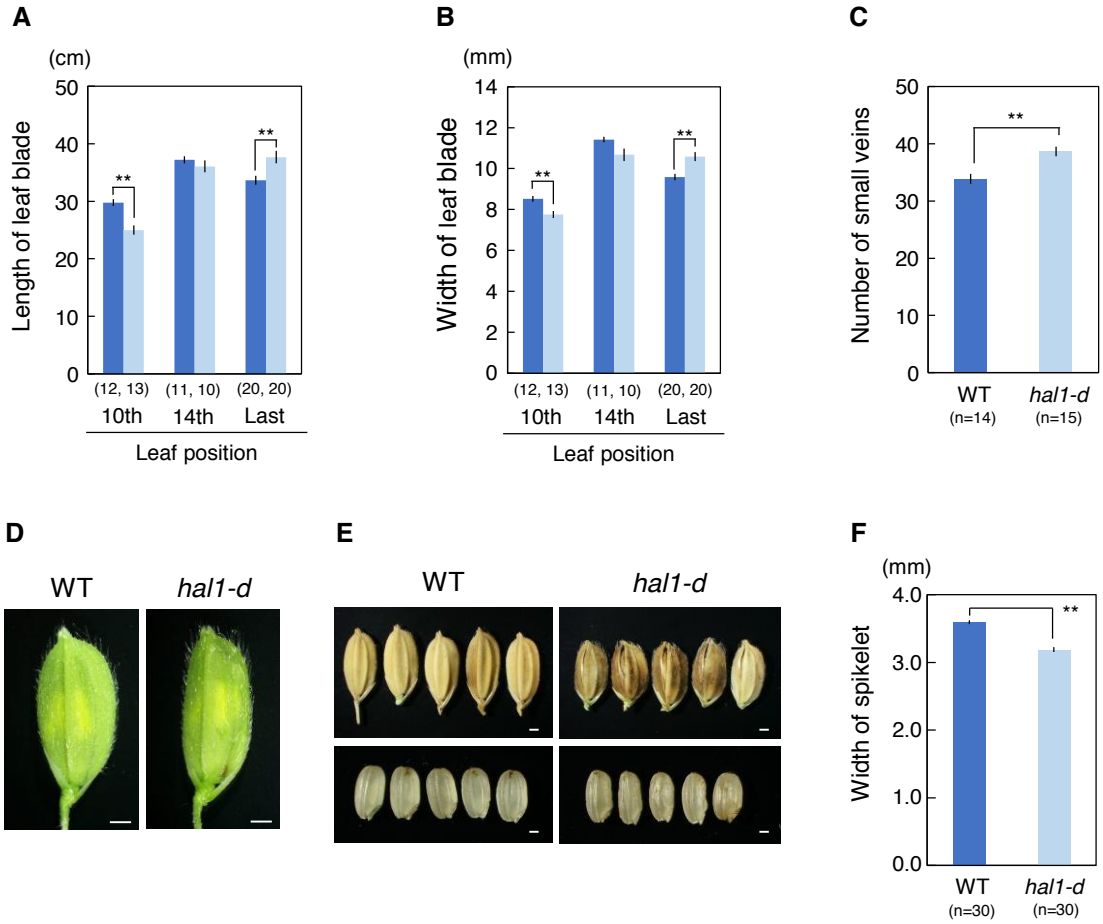

Figure 3-3. Size of the leaf blade and the spikelet.

A, The length of the leaf blade. B, The width of the leaf blade. Blue, wild type; light blue, *hal1-d*. C, The number of small veins in the last two leaves. D, The spikelet of wild type and *hal1-d*. E, Mature seeds of wild type and *hal1-d*. Typical unclosed sees of *hal1-d* were shown. F, The width of the spikelet. The number in parentheses indicate the number of leaves examined. Asterisks indicate significant differences (Student's t-test: **P < 0.01). Error bars represent SE. Scale bars, 1 mm in D, E.

Chapter 4

Materials and methods

Plant materials and growth conditions

Rice (*O. sativa* ssp. *japonica*) variety Koshihikari was used as the wild type. The *cul1* mutant was identified as a recessive mutant showing narrow leaves among M2 plants of Koshihikari that had been mutagenized with sodium azide. Rice plants were usually grown in pots containing soil (outdoor). Transgenic plants were grown in an NK System Biotron (model LH-350S, LH- 220S; Nippon Medical & Chemical Instruments, Osaka, Japan). *hal1* (CM1103) was identified as narrow leaf mutant among M2 plants of Kinmaze that had been mutagenized by N-methyl-N-nitrosourea and provided by Kyushu University as part of the National BioResource Project.

Histological analysis and in situ hybridization

Leaf segments were dissected, fixed, and dehydrated by the methods of Toriba and Hirano (2018). After replacement of the solution with xylene, the leaf tissue samples were embedded in Paraplast Plus (McCormick Scientific, St Louis, MO, USA). For histological analysis, microtome sections (7 μm) were stained with 1% toluidine blue-O and observed by a BX50 optical microscope (Olympus, Tokyo, Japan). To measure the size of small vascular bundles, I obtained the cross-sections of the 15th leaf blade of wild-type and *cul1* (C-type) and measured the area of the small vascular bundles using ImageJ software (https://imagej.nih.gov/ij/). To measure the size of the SAM, shoot

apices were dissected and fixed in acetic alcohol (1:3). After treatment with a clearing agent (16 g of chloral hydrate dissolved in 8 mL of 25% [v/v] glycerol), the samples were observed under DIC optics. The height of the SAM was measured by using ImageJ software. To observe cell files in the leaf, I cleared leaves by the transparency agent TOMEI-I (Hasegawa et al., 2016).

To generate the *CUL1* probe, partial cDNA fragments were amplified with the primer pair 5'-CACCCCGTAGCGAACCTCCTCTGT -3' and 5'-CCTCTCCAATCCCCACAATC-3' (located in the third exon). The PCR products were then cloned into a pENTR/D-TOPO vector (Thermo Fisher Scientific). Labeling of probes and tissue preparation were carried out as described previously (Toriba and Hirano, 2018). In situ hybridization and immunological detection were performed on 10 μm thick sections using the method of Toriba and Hirano (2018).

Isolation of the *CUL1* gene and complementation test

(The experiments below is performed by my collaborators Suzuki et al.)

The *CUL1* locus was mapped to a region between molecular markers RM336 (22.0 Mb) and E61310 (23.6 Mb) by using the F2 population from a cross between *cul1* and Nipponbare. To identify *CUL1* gene candidates, the whole-genome sequence of the *cul1* mutant was determinated by the MutMap method using 38 *cul1* homozygotes (Abe et al., 2012). For complementation analysis, a 7-kb fragment encompassing the putative *CUL1* gene (Os07g0563700) was cloned into a pZH2Bi-KXB vector. The recombinant

plasmid was introduced into *Agrobacterium tumefaciens*, and transformation of the *cul1* mutant was performed as described by Hiei et al. (1994).

RT-PCR analyses

RNA was isolated as described above. After DNase I treatment, first-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and the oligo(dT)15 primer. Amplification (30 cycles) was then performed with the primers listed in Table 2-2.

Generation of knockout mutants of *OsELP3* by CRISPR–Cas9 technology

To disrupt OsELP3, 20-bp sequences corresponding to the SAM domain were selected as the target of guide RNA (gRNA) (Fig. 2-16A). Synthetic oligonucleotides containing the target sequence and adopter sequence were annealed, and subcloned into the gRNA cloning vector, pU6gRNA-oligo (Mikami et al., 2015). The DNA fragment containing the OsU6 promoter and the gRNA region was then cloned into pZH_OsU3gYSA_MMCas9, as described previously (Tanaka et al., 2017; Yasui et al., 2017). The resulting construct was introduced into scutellum-derived calli via *A. tumefaciens* (Hiei et al., 1994). To identify knockout lines of *OsELP3*, the target genomic region of each regenerating plant was sequenced by using the primers in Table 2-2 (Fig. 2-16A).

RNA-seq experiments

Shoot apices including SAMs and P1–P3 leaf primordia were pooled (>5 apices per sample) and used for RNA isolation. Each sample (WT-M, WT-L, *cul1*-M, *cul1*-L; see Results) was prepared in biological triplicate. Total RNA was extracted by using TRIsure (BIOLINE) and treated with RNase-free DNase I. mRNA was isolated from total RNA (>2 μg) using the Ribo-Zero™ rRNA removal kit (illumina) to remove rRNA. For RNA-seq library preparation, the cDNA was synthesized using NEB Next Random Primers. The resulting libraries were sequenced by using the Illumina HiSeq instrument at GENEWIZ (South Plainfield, NJ, America), which generated 2x150 bp paired-end reads. The data ranged between 35.8 and 65.8 million reads per library (average 48.3 million). Reads were aligned to the rice reference sequence (Nipponbare) from RAP-DB (https://rapdb.dna.affrc.go.jp), and an average of 94.9% reads was mapped to the reference genome. Short-read alignment was performed by using Hisat2 (v2.0.1) (Kim et al., 2015) with default parameters. To obtain expression data, mapped-reads were counted for each gene by featureCounts (Liao et al., 2014) using Oryza_sativa.IRGSP-1.0.43.gtf from EnsemblPlants (http://plants.ensembl.org/info/website/ftp/index.html) as a gene annotation file. Normalization and differential expression analysis of the expression data were performed with the R package RUVSeq (Risso et al., 2014). The uniformity of mapped-read coverage over the gene body was examined using the geneBody coverage.py program in the RSeQC package (v2.6.6) (Wang et al., 2012). The required reference gene model file (BED12 format) was generated from the same gtf file using the

gtf2bed.pl program in the GenomicsTools package on Github (https://github.com/timothyjlaurent).

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL/DDBJ databases under the following accession numbers: LC511180 (*CUL1*), LC511181 (*OsELP3*). The raw RNA-Seq reads have been deposited into the DDBJ Sequence Read Archive (DRA) under accession number DRA009274.

Chapter 5

Concluding remarks

My purpose of this dissertation is to understand the molecular genetic mechanism underlying leaf development in *Oryza sativa* (rice). In this study, I performed molecular developmental genetic analysis of two mutants showing curling leaf phenotype, *curled later1* (*cul1*) *and half-pipe-like leaf1* (*hal1*). In chapter 2, I analyzed *cul1* mutant and revealed that CUL1 encoding the largest subunit of Elongator complex is required for proper morphogenesis of the leaf and meristem activity in rice. The disruption of the *OsELP3* gene encoding another Elongator subunit showed phenotypes similar to those observed in *cul1*. Therefore, I suggest that Elongator complex plays an important role for leaf development in rice. In chapter 3, I focused on *hal1* mutant and suggested that *HAL1* is necessary to the growth of bulliform cells and to keep the leaves flat.

In this dissertation, I revealed that both *cul1* and *hal1* mutants, which showed curling leaf phenotype, were defective in the development of bulliform cells. Therefore, bulliform cells are likely to play an important role for leaf flattening in rice. In *cul1*, differentiation of sclerenchyma cells was also compromised, suggesting that sclerenchyma cells are also responsible for keeping leaves flat in rice. The gene responsible for *hal1* mutant remains unknown in this study. Isolation of the *HAL1* gene would help to understand the development of bulliform cells in future.

In chapter 2, I revealed the importance of Elongator complex in rice development. The most unique feature of *cul1* is the timing of appearance of leaf

phenotype. Unlike other rice leaf mutants (Fujino et al., 2008; Cho et al., 2013; Yoshikawa et al., 2013; Zhang et al., 2009; Honda et al., 2018), the leaf development was normal during early-middle stages of the vegetative phase. However, curled narrow leaf phenotype appeared at late stage of the vegetative phase, that is, the late adult phase of vegetative development. To my knowledge, mutants that exhibit a heteroblastic change in such a unique developmental stage have not yet reported so far. This unique heteroblastic change in *cul1* also suggests that the adult phase is subdivided by the difference in the requirement of activities of several genes. *CUL1* was expressed in the SAM and leaf primordia from an early stage of the vegetative phase. Therefore, the heteroblastic change in *cul1* suggests that the activity of Elongator complex is strongly required for the leaf development at later stage of the vegetative phase, as compared to early-middle stages. Unlike the leaf development, the SAM activity of *cul1* is defective from early-middle stages. Therefore, the maintenance of the SAM activity is likely to require to some extent the Elongator function in all stages of vegetative development.

By transcriptome analysis, the expression of genes encoding components of SCF complex and HSPs, which were involved in protein quality control, were up-regulated specifically in the *cul1* shoot apex at the late vegetative stage (*cul1*-L shoot apex). This result suggests that the genes for protein quality control might be induced to remove the abnormal proteins accumulating specifically in *cul1*-L shoot apex, probably due to loss of Elongator activity. My analysis showed that the transcriptional level of genes regulating leaf width, such as *NAL2*, *NAL3* and *NAL7*, were not changed in *cul1*-L shoot apex. However, it would not be denied that these gene are affected at the protein

level.

Although the regulation of leaf development by Elongator complex is unclear at present, two possibilities would be raised to explain the cause of *cul1* leaf phenotype. One possibility is that the defect in translation of the genes responsible for leaf development leads curled narrow leaf phenotype in *cul1*. In Arabidopsis, the mutation of Elongator subunits leads to the reduction of PIN protein levels, leading to auxin-related phenotype (Leitner et al., 2015). Similarly, it is possible that the level of protein responsible for the differentiation of bulliform and sclerenchyma cells reduced in *cul1*-L shoot apex. The other possibility is that the up-regulation of the genes involved in protein quality control, such as SCF complex and HSPs, affects leaf morphogenesis in *cul1*. In Arabidopsis, overexpression of *BIG BROTHER* (*BB*), encoding E3 ubiquitin ligase, causes organ size reduction (Disch et al., 2006). However, the reason why overexpression of *BB* affects leaf development remains unknown.

To elucidate the relationship between the function of Elongator and leaf development, proteome analysis would be required in future. This analysis would provide a wealth of information concerning the function of Elongator in rice and factors regulating leaf development and meristem activity associated with *cul1* phenotypes.

References

Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H, Cano L, Kamoun S, Terauchi R (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat. Biotechnol. **30:** 174-178

Abe M, Yoshikawa T, Nosaka M, Sakakibara H, Sato Y, Nagato Y, Itoh J-I (2010) *WAVY LEAF1*, an ortholog of Arabidopsis *HEN1*, regulates shoot development by maintaining MicroRNA and trans-acting small interfering RNA accumulation in rice. Plant Physiol. **154:** 1335-1346

Aichinger E, Kornet N, Friedrich T, Laux T (2012) Plant stem cell niches. Annu. Rev. Plant Biol. **63:** 615-636

Bowman, JL (2000) The YABBY gene family and abaxial cell fate. Curr. Opin. in Plant Biol. **3:** 17-22

Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham, M, Hudson A, Martienssen RA (2000) *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. Nature **408:** 967-971

Chitwood DH, Sinha NR (2016) Evolutionary and environmental forces sculpting leaf development. Curr. Biol. **26:** R297-R306

Chitwood DH, Guo M, Nogueira FT, Timmermans MC (2007) Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. Development **134:** 813-823

Cho SH, Yoo SC, Zhang H, Lim JH, Paek NC (2014) Rice *NARROW LEAF1* Regulates Leaf and Adventitious Root Development. Plant Mol. Biol. Rep. **32:** 270-281

Cho SH, Yoo SC, Zhang H, Pandeya D, Koh HJ, Hwang JY, Kim GT, Paek NC (2013) The rice *narrow leaf2* and *narrow leaf3* loci encode WUSCHEL-related homeobox 3A (OsWOX3A) and function in leaf, spikelet, tiller and lateral root development. New Phytol. **198:** 1071-1084

Chuck G, Cigan AM, Saeteurn K, Hake S (2007) The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nature Genet. **39:** 544-549

Dalwadi U, Yip CK (2018) Structural insights into the function of Elongator. Cell Mol.

Life Sci. **75:** 1613-1622

Debernardi JM, Mecchia MA, Vercruyssen L, Smaczniak C, Kaufmann K, Inze D, Rodriguez RE, Palatnik JF (2014) Post-transcriptional control of *GRF* transcription factors by microRNA miR396 and GIF co-activator affects leaf size and longevity. Plant J. **79:** 413-426

Ding Y, Mou Z (2015) Elongator and its epigenetic role in plant development and responses to abiotic and biotic stresses. Front. Plant Sci. **6:** 296

Disch S, Anastasiou E, Sharma VK, Laux T, Fletcher JC, Lenhard M (2006) The E3 ubiquitin ligase BIG BROTHER controls *Arabidopsis* organ size in a dosage-dependent manner. Curr. Biol. **16:** 272-279

Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL (2003) Radial Patterning of *Arabidopsis* Shoots by Class III HD-ZIP and KANADI Genes. Curr. Biol. **13:** 1768-1774

Fang X, Cui Y, Li Y, Qi Y (2015) Transcription and processing of primary microRNAs are coupled by Elongator complex in *Arabidopsis*. Nat. Plants **1:** 15075

Fujino K, Matsuda Y, Ozawa K, Nishimura T, Koshiba T, Fraaije MW, Sekiguchi H (2008) *NARROW LEAF 7* controls leaf shape mediated by auxin in rice. Mol. Genet. Genomics **279:** 499-507

Glatt S, Muller CW (2013) Structural insights into Elongator function. Curr. Opin. Struct. Biol. **23:** 235-242

Hasegawa J, Sakamoto Y, Nakagami S, Aida M, Sawa S, Matsunaga S (2016) Three-dimensional imaging of plant organs using a simple and rapid transparency technique. Plant Cell Physiol. **57:** 462-472

Hibara K-I, Isono M, Mimura M, Sentoku N, Kojima M, Sakakibara H, Kitomi Y, Yoshikawa T, Itoh J-I, Nagato Y (2016) Jasmonate regulates juvenile-to-adult phase transition in rice. Development **143:** 3407-3416

Hibara K-I, Obara M, Hayashida E, Abe M, Ishimaru T, Satoh H, Itoh J-I, Nagato Y (2009) The *ADAXIALIZED LEAF1* gene functions in leaf and embryonic pattern formation in rice. Dev. Biol. **334:** 345-354

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. **6:** 271-282

Honda E, Yew CL, Yoshikawa T, Sato Y, Hibara K-I, Itoh J-I (2018) *LEAF*

LATERAL SYMMETRY1, a Member of the *WUSCHEL-RELATED HOMEOBOX3* Gene Family, Regulates Lateral Organ Development Differentially from Other Paralogs, *NARROW LEAF2* and *NARROW LEAF3* in Rice. Plant Cell Physiol. **59:** 376-391

Horiguchi G, Kim GT, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant J. **43:** 68-78

Hu J, Zhu L, Zeng D, Gao Z, Guo L, Fang Y, Zhang G, Dong G, Yan M, Liu J, Qian Q (2010) Identification and characterization of *NARROW AND ROLLED LEAF 1*, a novel gene regulating leaf morphology and plant architecture in rice. Plant Mol. Biol. **73:** 283-292

Huijser P, Schmid M (2011) The control of developmental phase transitions in plants. Development **138:** 4117-4129

Hunter C, Willmann MR, Wu G, Yoshikawa M, de la Luz Gutierrez-Nava M, Poethig SR (2006) Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in *Arabidopsis*. Development **133:** 2973-2981

Husbands AY, Chitwood DH, Plavskin Y, Timmermans MC (2009) Signals and prepatterns: new insights into organ polarity in plants. Genes Dev. **23:** 1986-1997

Ishiwata A, Ozawa M, Nagasaki H, Kato M, Noda Y, Yamaguchi T, Nosaka M, Shimizu-Sato S, Nagasaki A, Maekawa M, Hirano H-Y, Sato Y (2013) Two *WUSCHEL-related homeobox* genes, *narrow leaf2* and *narrow leaf3*, control leaf width in rice. Plant Cell Physiol. **54:** 779-792

Itoh J-I, Nonomura K-I, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol. **46:** 23-47

Itoh J-I, Sato Y, Nagato Y (2008) The *SHOOT ORGANIZATION2* gene coordinates leaf domain development along the central-marginal axis in rice. Plant Cell Physiol. **49:** 1226-1236

Kerstetter RA, Bollman K, Taylor RA, Bomblies K, Poethig RS (2001) *KANADI* regulates organ polarity in *Arabidopsis*. Nature **411:** 706-709

Kerstetter RA, Poethig RS (1998) The specification of leaf identity during shoot development. Annu. Rev. Plant Biol. **14:** 373-398

Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low

memory requirements. Nat. Methods **12:** 357-360

Kim GT, Tsukaya H, Uchimiya H (1998) The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. Genes Dev. **12:** 2381-2391

Kim JH, Tsukaya H (2015) Regulation of plant growth and development by the GROWTH-REGULATING FACTOR and GRF-INTERACTING FACTOR duo. J. Exp. Bot. **66:** 6093-6107

Kim, JH, Kende H (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **101:** 13374-13379

Kojima S, Iwasaki M, Takahashi H, Imai T, Matsumura Y, Fleury D, Van Lijsebettens M, Machida Y, Machida C (2011) Asymmetric leaves2 and Elongator, a histone acetyltransferase complex, mediate the establishment of polarity in leaves of *Arabidopsis thaliana*. Plant Cell Physiol. **52:** 1259-1273

Kubo FC, Yasui Y, Kumamaru T, Sato Y, Hirano H-Y (2016) Genetic analysis of rice mutants responsible for narrow leaf phenotype and reduced vein number. Genes Genet. Syst. **91:** 235-240

Kubo FC, Yasui Y, Ohmori Y, Kumamaru T, Tanaka W, Hirano H-Y (2019) DWARF WITH SLENDER LEAF1 encoding a histone deacetylase plays diverse roles in rice developmen. Plant Cell Physiol. **(in press)**

Kuhlemeier C, Timmermans MC (2016) The Sussex signal: insights into leaf dorsiventrality. Development **143:** 3230-3237

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33:** 1870-1874

Lauter N, Kampani A, Carlson S, Goebel M, Moose SP (2005) *microRNA172* down-regulates *glossy15* to promote vegetative phase change in maize. Proc. Natl. Acad. Sci. USA **102:** 9412-9417

Leitner J, Retzer K, Malenica N, Bartkeviciute R, Lucyshyn D, Jager G, Korbei B, Bystrom A, Luschnig C (2015) Meta-regulation of *Arabidopsis* auxin responses depends on tRNA maturation. Cell Rep. **11:** 516-526

Leyser O, Day S (2003) Mechanisms in plant development. John Wiley & Sons

Li M, Xiong G, Li R, Cui J, Tang D, Zhang B, Pauly M, Cheng Z, Zhou Y (2009) Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and

plant growth. Plant J. **60:** 1055-1069

Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **30:** 923-930

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. Nature **379:** 66-69

Malenica N, Abas L, Benjamins R, Kitakura S, Sigmund HF, Jun KS, Hauser MT, Friml J, Luschnig C (2007) *MODULATOR OF PIN* genes control steady-state levels of Arabidopsis PIN proteins. Plant J. **51:** 537-550

Matsumoto H, Yasui Y, Kumamaru T, Hirano H-Y (2017) Characterization of a *half-pipe-like leaf1* mutant that exhibits a curled leaf phenotype. Genes Genet. Syst. **92:** 287-291

McConnell JR., Emery J, Eshed Y, Bao N, Bowman J, Barton MK (2001) Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. Nature **411:** 709-713

Mehlgarten C, Jablonowski D, Wrackmeyer U, Tschitschmann S, Sondermann D, Jager G, Gong Z, Bystrom AS, Schaffrath R, Breunig KD (2010) Elongator function in tRNA wobble uridine modification is conserved between yeast and plants. Mol. Microbiol. **76:** 1082-1094

Mikami M, Toki S, Endo M (2015) Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. Plant Mol. Biol. **88:** 561-572

Moose SP, Sisco PH (1996) *Glossy15*, an *APETALA2*-like gene from maize that regulates leaf epidermal cell identity. Genes Dev. **10:** 3018-3027

Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, Goodrich J, Tsukaya H (2004) Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in *Arabidopsis thaliana*. Plant J. **38:** 699-713

Nelissen H, De Groeve S, Fleury D, Neyt P, Bruno L, Bitonti MB, Vandenbussche F, Van der Straeten D, Yamaguchi T, Tsukaya H, Witters E, De Jaeger G, Houben A, Van Lijsebettens M (2010) Plant Elongator regulates auxin-related genes during RNA polymerase II transcription elongation. Proc. Natl. Acad. Sci. USA **107:** 1678-1683

Nelissen H, Fleury D, Bruno L, Robles P, De Veylder L, Traas J, Micol JL, Van

Montagu M, Inze D, Van Lijsebettens M (2005) The elongata mutants identify a functional Elongator complex in plants with a role in cell proliferation during organ growth. Proc. Natl. Acad. Sci. USA **102:** 7754-7759

Obara M, Ikeda K, Itoh J-I, Nagato Y (2004) Characterization of leaf lateral symmetry 1 Mutant in Rice. Breed. Sci. **54:** 157-163

Ohmori Y, Abiko M, Horibata A, Hirano H-Y (2008) A transposon, *Ping*, is integrated into intron 4 of the *DROOPING LEAF* gene of rice, weakly reducing its expression, and causing a mild drooping leaf phenotype. Plant Cell Physiol. **49:** 1176-1184

Ohmori Y, Toriba T, Nakamura H, Ichikawa H, Hirano H-Y (2011) Temporal and spatial regulation of *DROOPING LEAF* gene expression that promotes midrib formation in rice. Plant J. **65:** 77-86

Ori N, Eshed Y, Chuck G, Bowman JL, Hake S (2000) Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. Development **127:** 5523-5532

Otero G, Fellows J, Li Y, de Bizemont T, Dirac AM, Gustafsson CM, Erdjument-Bromage H, Tempst P, Svejstrup JQ (1999) Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. Mol. Cell **3:** 109-118

Pekker I, Alvarez JP, Eshed Y (2005) Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. Plant Cell **17:** 2899-2910

Qi J, Qian Q, Bu Q, Li S, Chen Q, Sun J, Liang W, Zhou Y, Chu C, Li X, Ren F, Palme K, Zhao B, Chen J, Chen M, Li C (2008) Mutation of the rice *Narrow leaf1* gene, which encodes a novel protein, affects vein patterning and polar auxin transport. Plant Physiol. **147:** 1947-1959

Qi L, Zhang X, Zhai H, Liu J, Wu F, Li C, Chen Q (2019) Elongator is required for root stem cell maintenance by regulating *SHORTROOT* transcription. Plant Physiol. **179:** 220-232

Risso D, Ngai J, Speed TP, Dudoit S (2014) Normalization of RNA-seq data using factor analysis of control genes or samples. Nat. Biotechnol. **32:** 896-902

Satoh H, Matsusaka H, Kumamaru T (2010) Use of N-methyl-N-nitrosourea treatment of fertilized egg cells for saturation mutagenesis of rice. Breed. Sci. **60:** 475-485

Sentoku N, Sato Y, Kurata N, Ito Y, Kitano H, Matsuoka M (1999) Regional

expression of the rice *KN1*-type homeobox gene family during embryo, shoot, and flower development. Plant Cell **11:** 1651-1664

Sessions A, Nemhauser JL, McColl A, Roe JL, Feldmann KA, Zambryski PC (1997) *ETTIN* patterns the *Arabidopsis* floral meristem and reproductive organs. Development **124:** 4481-4491

Siegfried KR, Eshed Y, Baum SF, Otsuga D, Drews GN, Bowman JL (1999) Members of the *YABBY* gene family specify abaxial cell fate in *Arabidopsis*. Development **126:** 4117-4128

Sussex, IM (1951) Experiments on the cause of dorsiventrality in leaves. Nature **167:** 651-652

Suzuki C, Tanaka W, Hirano H-Y (2019) Transcriptional Corepressor ASP1 and CLV-Like Signaling Regulate Meristem Maintenance in Rice. Plant Physiol. **180:** 1520-1534

Svejstrup JQ (2007) Elongator complex: how many roles does it play? Curr. Opin. Cell Biol. **19:** 331-336

Tanaka N, Itoh H, Sentoku N, Kojima M, Sakakibara H, Izawa T, Itoh J-I, Nagato Y (2011) The *COP1* ortholog *PPS* regulates the juvenile-adult and vegetative-reproductive phase changes in rice. Plant Cell **23:** 2143-2154

Tanaka W, Toriba T, Hirano H-Y (2017) Three *TOB1*-related *YABBY* genes are required to maintain proper function of the spikelet and branch meristems in rice. New Phytol. **215:** 825-839

Tanaka W, Toriba T, Ohmori Y, Yoshida A, Kawai A, Mayama-Tsuchida T, Ichikawa H, Mitsuda N, Ohme-Takagi M, Hirano H-Y (2012) The *YABBY* gene *TONGARI-BOUSHI1* is involved in lateral organ development and maintenance of meristem organization in the rice spikelet. Plant Cell **24:** 80-95

Telfer A, Bollman KM, Poethig RS (1997) Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. Development **124:** 645-654

Toriba T, Hirano H-Y (2018) Two-color in situ hybridization: a technique for simultaneous detection of transcripts from different loci. *In* N Yamaguchi, ed, Plant Transcription Factors: Methods and Protocols. Springer New York, New York, NY, pp 269-287

Tsuge T, Tsukaya H, Uchimiya H (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L.) Heynh.

Development **122:** 1589-1600

Usami T, Horiguchi G, Yano S, Tsukaya H (2009) The more and smaller cells mutants of *Arabidopsis thaliana* identify novel roles for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* genes in the control of heteroblasty. Development **136:** 955-964

Van Lijsebettens M, Grasser KD (2014) Transcript elongation factors: shaping transcriptomes after transcript initiation. Trends Plant Sci. **19:** 717-726

Vercruyssen L, Verkest A, Gonzalez N, Heyndrickx KS, Eeckhout D, Han SK, Jegu T, Archacki R, Van Leene J, Andriankaja M, De Bodt S, Abeel T, Coppens F, Dhondt S, De Milde L, Vermeersch M, Maleux K, Gevaert K, Jerzmanowski A, Benhamed M, Wagner D, Vandepoele K, De Jaeger G, Inze D (2014) ANGUSTIFOLIA3 binds to SWI/SNF chromatin remodeling complexes to regulate transcription during *Arabidopsis* leaf development. Plant Cell **26:** 210-229

Versees W, De Groeve S, Van Lijsebettens M (2010) Elongator, a conserved multitasking complex? Mol. Microbiol. **76:** 1065-1069

Waites R, Hudson A (1995) *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. Development **121:** 2143-2154

Waites R, Selvadurai HRN, Oliver, IR, Hudson A (1998) The *PHANTASTICA* Gene Encodes a MYB Transcription Factor Involved in Growth and Dorsoventrality of Lateral Organs in *Antirrhinum.* Cell **93:** 779-789

Wang JW, Park MY, Wang LJ, Koo Y, Chen XY, Weigel D, Poethig RS (2011) MiRNA control of vegetative phase change in trees. PLoS Genet. **7:** e1002012

Wang L, Wang S, Li W (2012) RSeQC: quality control of RNA-seq experiments. Bioinformatics **28:** 2184-2185

Wittschieben BO, Otero G, de Bizemont T, Fellows J, Erdjument-Bromage H, Ohba R, Li Y, Allis CD, Tempst P, Svejstrup JQ (1999) A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol. Cell **4:** 123-128

Woloszynska M, Gagliardi O, Vandenbussche F, De Groeve S, Alonso Baez L, Neyt P, Le Gall S, Fung J, Mas P, Van Der Straeten D, Van Lijsebettens M (2018) The Elongator complex regulates hypocotyl growth in darkness and during photomorphogenesis. J. Cell Sci. **131:** jcs203927

Woloszynska M, Le Gall S, Van Lijsebettens M (2016) Plant Elongator-mediated

transcriptional control in a chromatin and epigenetic context. Biochim. Biophys. Acta **1859:** 1025-1033

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. Cell **138:** 750-759

Xu D, Huang W, Li Y, Wang H, Huang H, Cui X (2012) Elongator complex is critical for cell cycle progression and leaf patterning in *Arabidopsis*. Plant J. **69:** 792-808

Yamaguchi T, Nagasawa N, Kawasaki S, Matsuoka M, Nagato Y, Hirano H-Y (2004) The *YABBY* gene *DROOPING LEAF* regulates carpel specification and midrib development in *Oryza sativa*. Plant Cell **16:** 500-509

Yan S, Yan CJ, Zeng XH, Yang YC, Fang YW, Tian CY, Sun YW, Cheng ZK, Gu MH (2008) *ROLLED LEAF 9*, encoding a GARP protein, regulates the leaf abaxial cell fate in rice. Plant Mol. Biol. **68:** 239-250

Yasui Y, Ohmori Y, Takebayashi Y, Sakakibara H, Hirano H-Y (2018) *WUSCHEL-RELATED HOMEOBOX4* acts as a key regulator in early leaf development in rice. PLOS Genet. **14:** e1007365

Yasui Y, Tanaka W, Sakamoto T, Kurata T, Hirano H-Y (2017) Genetic enhancer analysis reveals that *FLORAL ORGAN NUMBER2* and *OsMADS3* co-operatively regulate maintenance and determinacy of the flower meristem in rice. Plant Cell Physiol. **58:** 893-903

Yoshikawa T, Eiguchi M, Hibara K-I, Ito J-I, Nagato Y (2013) Rice *SLENDER LEAF 1* gene encodes cellulose synthase-like D4 and is specifically expressed in M-phase cells to regulate cell proliferation. J. Exp. Bot. **64:** 2049-2061

Zhang GH, Xu Q, Zhu XD, Qian Q, Xue HW (2009) SHALLOT-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. Plant Cell **21:** 719-735

Zou LP, Sun XH, Zhang ZG, Liu P, Wu JX, Tian CJ, Qiu JL, Lu TG (2011) Leaf rolling controlled by the homeodomain leucine zipper class IV gene *Roc5* in rice. Plant Physiol. **156:** 1589-1602