DOCTORATE DISSERTATION

博士論文

Molecular genetic studies on stem cell maintenance in *Oryza sativa*

（イネにおける幹細胞維持に関する分子遺伝学的研究）

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List of abbreviation

AHP	authentic histidine-containing phosphotransfer
APO	ABERRANT PANICLE ORGANIZATION
ARR	ARABIDOPSIS RESPONSE REGULATOR
ASP1	ABERRANT SPIKELET AND PANICLE1
BAM	BARELY ANY MERISTEM
Cas9	CRISPR-associated protein 9
CIK	CLAVATA3 INSENSITIVE RECEPTOR KINASE
CKX	cytokinin oxidase/dehydrogenase
CLE	CLAVATA3/EMBRYO SURROUNDING REGION
CLV	CLAVATA
CRISPR	clustered regularly interspaced short palindromic repeat
CRN	CORYNE
CT2	COMPACT PLANT2
CZ	central zone
DEG	differentially expressed gene
DEP1	DENSE AND ERECT PANICLE 1
DP1	DEPRESSED PALEA1
EG1	EXTRA GLUME1
F-type	Increased Floral Organ-type
FCP	FON2-LIKE CLE PROTEIN
FEA	FASCIATED EAR
FM	flower meristem
FON	FLORAL ORGAN NUMBER
FOS1	FON2 SPARE1
fsp1	fickle spikelet1
G-type	Extra Glume-type
GO	Gene Ontology

gRNA	guideRNA
HK	histidine kinase
IM	inflorescence meristem
L-type	Double Lemma-type
LAX	LAX PANICLE
LOG	LONELY GUY
LOGL	LOG LIKE
LRR-RLK	leucine-rich repeat receptor-like kinase
MAPK	mitogen-activated protein kinase
MNU	*N*-methyl-*N*-nitrosourea
NS	NARROW SHEATH
OC	organizing center
OSH1	ORYZA SATIVA HOMEOBOX1
P-type	Degenerated Palea-type
pBM	primary branch meristem
PHP	pseudo-histidine-containing phosphotransfer
PZ	peripheral zone
RA1	RAMOSA1
REL2	RAMOSA1 ENHANCER LOCUS2
RPK2	RECEPTOR-LIKE PROTEIN KINASE 2
RR	RESPONSE REGULATOR
RZ	rib zone
SAM	shoot apical meristem
SCM2	STRONG CULM2
SEM	scanning electron microscopy
SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
TAB1	TILLERS ABSENT1
TAW1	TAWAWA1
TD1	THICK TASSEL DWARF1
TDIF	tracheary element differentiation inhibitory factor

TDR	TDIF RECEPTOR
TH1	TRIANGULAR HULL1
TPL	TOPLESS
TPR	TOPLESS-RELATED
WOX	WUSCHEL-RELATED HOMEOBOX
WSIP	WUS-interacting protein
WUS	WUSCHEL

Abstract

The shoot apical meristem (SAM) plays crucial roles in producing the above-ground part of the plant body. The SAM harbors pluripotent stem cells, which provide daughter cells for organ differentiation. Therefore, it is essential for proper plant development to appropriately maintain stem cell population in the SAM. In *Arabidopsis thaliana* (Arabidopsis), the *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV*) genes constitute a negative feedback loop to regulate stem cell homeostasis in the SAM: *WUS* promotes stem cell proliferation, and *CLV3* and its downstream genes including *CLV1* negatively regulate the expression of *WUS*. However, our knowledge of the molecular details of this system remains limited. In *Oryza sativa* (rice), *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2*, respective orthologs of *CLV1* and *CLV3*, function in the negative regulation of stem cell proliferation, indicating that the WUS–CLV-like signaling is conserved (FON signaling). In this thesis, I aim to explore molecular mechanisms underlying stem cell maintenance by focusing on FON signaling.

***ASP1* encoding the TOPLESS-related transcriptional corepressor regulates meristem maintenance in concert with CLV-like signaling in rice**

In Chapter II, to gain new insights into FON signaling, I used a genetic enhancer approach. Loss of either *FON1* or *FON2* results in increased number of floral organs such as stamens and pistils. Among a mutagenized population of *fon2* mutants, I focused on strain 1B-280, which exhibited an enhanced floral phenotype of *fon2*.

1B-280 showed an increase in pistil number compared with *fon2*, indicating that 1B-280 is a genetic enhancer mutant of *fon2*. In addition to the flower phenotype, 1B-280 exhibited a dramatic inflorescence phenotype, a bifurcated rachis, which was not observed not only in the *fon2* single mutant but also in inflorescence mutants so far reported. I found that 1B-280 has a mutation in the *ABERRANT SPIKELET AND PANICLE1* (*ASP1*) gene, that is, 1B-280 is a *fon2 asp1* double mutant. *ASP1* encodes an Arabidopsis TOPLESS (TPL)-like transcriptional corepressor. Complementation and genetic analysis confirmed that the *asp1* mutation was responsible for enhancing the flower phenotype of *fon2* and for disrupting inflorescence architecture in 1B-280. The *asp1* mutation synergistically enhanced the flower phenotype in not only *fon2* but also *fon1*, suggesting that ASP1 function is associated with FON signaling.

I found that the inflorescence meristem of *fon2 asp1* was extremely larger than that of each single mutant, which could be a cause of a bifurcated rachis. The *FON2* expression domain, corresponding to putative stem cells, was markedly expanded in the inflorescence meristem of *fon2 asp1* compared with *fon2*, indicating that stem cells showed marked overproliferation leading to extreme enlargement of the meristem in *fon2 asp1*, and that *ASP1* negatively regulates stem cell proliferation.

In Arabidopsis, stem cell overproliferation in the *clv* mutants is caused by the enhanced *WUS* action. Unexpectedly, however, genetic analysis indicated that *TILLERS ABSENT1* (*TAB1*), the *WUS* ortholog in rice, was not involved in promoting stem cell proliferation in *fon2 asp1*; rather, the loss-of-function *tab1* mutant showed slight enlargement of the meristem, a feature related to an increase in stem cells.

To evaluate the effects of ASP1 and FON signaling on stem cell maintenance in terms of gene expression, I carried out transcriptome analysis. As a result, a large number of genes upregulated in *asp1* were also elevated in *fon2* and *fon2 asp1*. This suggests that a considerable proportion of the genes repressed by the ASP1 corepressor are also repressed by FON signaling. In addition, GO analysis indicated that *ASP1* and FON signaling negatively regulate a set of genes with similar functions. Considering that WUS–CLV signaling acts in the fine-tuning of stem cell numbers in Arabidopsis, FON signaling probably fine-tune the expression of genes responsible for stem cell proliferation in rice. It is possible that moderate expression levels of those genes facilitate this fine-tuning. ASP1 might function in partial suppression of various genes to ensure the fine-tuning by FON signaling.

Taken altogether, I showed that TPL-like corepressor activity plays a crucial role in meristem maintenance, and suggested that stem cell proliferation is properly maintained via the cooperation of *ASP1* and FON signaling.

Analysis of a rice *fickle spikelet1* mutant that displays an increase in flower and spikelet organ number

In Chapter III, I analyzed a novel mutant, *fickle spikelet1* (*fsp1*), showing an increase in organ number, a phenotype related to a defect in stem cell maintenance. In rice, floral organs develop in the spikelet, an inflorescence unit unique to grass species. The floral organs, such as carpels, stamens and lodicules, are enclosed by two spikelet organs, the palea and lemma. Mutations in the *FON* genes cause an increase in the number of floral

organs due to overproliferation of stem cells; however, the spikelet organs are less affected in the *fon* mutants. I found that *fsp1* displayed an increased number not only of floral organs but also of spikelet organs. *fsp1* also showed a defect in pollen development, often leading to sterility. The expressivity of the *fsp1* phenotype varied from plant to plant, and also from panicle to panicle within a single plant. Furthermore, the phenotype of *fsp1* seems to be strongly influenced by environmental conditions. I revealed that *fsp1* has a mutation in the essential amino acid in FON1. However, the *fsp1* phenotypes cannot be fully explained by a loss of FON1, suggesting the possibility that *fsp1* might have another mutation.

Conclusion and perspectives

In this thesis, I revealed the importance of FON signaling, which functions in stem cell regulation in the all above-ground meristems, by focusing on the ASP1 corepressor and the *fsp1* mutant. The findings of this study will provide a key to further understanding the molecular mechanisms underlying stem cell maintenance.

In rice, there is another CLV-like signaling pathway other than FON signaling: FON2-LIKE CLE PROTEIN1 (FCP1) and FCP2 are known to regulate stem cell maintenance in the SAM, similarly to FON2. It will be of great interest to determine the genetic interaction between FCP signaling and FON, ASP1 or both.

Chapter I

General introduction

Function and architecture of the shoot apical meristem

One of the striking features of plants is that they can differentiate organs even after embryogenesis and continuously develop throughout their lives. This feature, namely post-embryonic development, largely depends on the activity of the meristem, a group of undifferentiated tissues where stem cells are maintained and lateral organs differentiate (reviewed in Aichinger et al., 2012). At the apex of the shoot, the shoot apical meristem (SAM) functions in producing the aerial part of the plant body. The SAM is established during embryogenesis and successively differentiates leaf primordia in the vegetative phase. When perceiving a flowering signal, the SAM enters the reproductive phase and turns into the inflorescence meristem (IM). The IM initiates either the flower meristems (FMs) which differentiates floral organs directly, or intermediate types of meristems which initiates the FMs.

The SAM is composed of an outer region, tunica, and an inner region, corpus (Figure I-1; reviewed in Steeves and Sussex, 1989; Leyser and Day, 2003). The tunica consists of a few distinct cell layers (two in most eudicots, one in monocots). In general, the outermost layer of tunica, called L1, differentiates into the epidermis, and the second-outermost layer L2 differentiates into subepidermal tissues and germ cells. The corpus is the cell mass underlying tunica and differentiates into remaining internal tissues. In terms of function, the SAM can also be classified into three zones, the central

zone (CZ), the peripheral zone (PZ), and the rib zone (RZ) (Figure I-1; reviewed in Steeves and Sussex, 1989; Leyser and Day, 2003). The CZ is situated at the top of the SAM. The PZ surrounds the CZ, and the RZ is located underneath the CZ. The CZ functions as a reservoir of pluripotent stem cells. Stem cells, residing in the tip of the CZ, proliferate to self-renew and to supply daughter cells to the PZ and RZ. The descendants of cells supplied to the PZ and RZ differentiates into lateral organs and the stem pith, respectively. Thus, it is essential to maintain stem cells in the CZ for continuous organ differentiation, that is, post-embryonic development.

Stem cell maintenance in the shoot apical meristem

The molecular mechanisms underlying stem cell maintenance in the SAM have been revealed through studies of a model eudicot, *Arabidopsis thaliana* (Arabidopsis). The WUSCHEL (WUS)–CLAVATA (CLV) signaling plays a critical role in stem cell maintenance in the Arabidopsis SAM (Figure I-2). In brief, a stem cell promoting factor and a signaling peptide secreted from stem cells constitute a negative feedback loop to maintain stem cell population appropriately: the former is encoded by the *WUS* gene, and the latter is derived from the *CLV3* gene. This mechanism is mostly conserved also in monocots such as *Oryza sativa* (rice) and *Zea mays* (maize) (Figure I-3). In this section, I summarize stem cell maintenance by the WUS–CLV feedback signaling in Arabidopsis, rice and maize.

WUS–CLV feedback signaling in Arabidopsis thaliana

WUS encodes a homeobox transcription factor belonging to the WUSCHEL-RELATED HOMEOBOX (WOX) class (Mayer et al., 1998; Haecker et al., 2004). The loss-of-function *wus* mutant exhibits a defect in stem cell maintenance and a precocious termination of the SAM in the vegetative phase, clearly indicating that *WUS* positively regulates stem cell proliferation (Mayer et al., 1998). *WUS* is expressed in the small region underlying stem cells, called the organizing center (OC), and acts non-cell-autonomously to regulate stem cell fate (Mayer et al., 1998). WUS has a dual function, acting as a transcriptional activator and also as a repressor (Ikeda et al., 2009), and controls transcription of a large number of genes (Busch et al., 2010). WUS moves from the OC to stem cells through plasmodesmata to directly promote the expression of *CLV3* (Yadav et al., 2011; Daum et al., 2014). To prevent *CLV3* activation in the OC, the HAIRY MERISTEM transcription factor family members confine WUS function to stem cell region, probably by physical interaction in the corpus of the SAM (Zhou et al., 2015, 2018). The TOPLESS (TPL) and TOPLESS-RELATED4 (TPR4) transcriptional co-repressors are also known to physically interact with WUS (Kieffer et al., 2006). Although this interaction is suggested to be necessary for the WUS function as a repressor (Kieffer et al., 2006), the specific function of the TPL family in stem cell maintenance remains to be defined. As a repressor, WUS directly controls the transcription of several A-type *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes (Leibfried et al., 2005). The A-type ARRs are negative regulators of cytokinin signaling which functions in promoting cell proliferation (reviewed in Kieber and Schaller, 2018),

indicating that positive regulation of stem cell fate by WUS is executed at least in part via repression of the A-type ARRs.

CLV3 is a founder member of the *CLAVATA3/EMBRYO SURROUNDING REGION* (*CLE*) genes, which function in diverse signaling pathways (reviewed in Yamaguchi et al., 2016). CLV3 is initially expressed as prepropeptide in stem cells, and preproCLV3 is processed and modified into a mature 13-amino acid arabinosylated glycopeptide (Kondo et al., 2006; Ohyama et al., 2009). In contrast to the *wus* phenotype, the loss-of-function *clv3* mutants exhibit overproliferation of stem cells and an enlargement of the SAM, indicating that *CLV3* is a negative regulator of stem cell proliferation in the SAM (Clark et al., 1995; Fletcher et al., 1999). In the *clv3* mutants, the *WUS* expression domain is markedly expanded, therefore *CLV3* is considered to regulate stem cell fate via repression of *WUS* (Brand et al., 2000; Schoof et al., 2000). Consistent with this, *wus* is epistatic to *clv3*, and *WUS* overexpression driven by the CLV1 promoter, which is active in the CZ including the OC (see below), mimics the *clv3* phenotype (Schoof et al., 2000). Furthermore, strong induction of *CLV3* leads to rapid downregulation of the *WUS* expression and termination of the SAM (Brand et al., 2000; Müller et al., 2006).

The mature CLV3 peptide is secreted from stem cells and perceived by multiple receptor complexes, each of which consists of CLV1, CLV2/CORYNE (CRN), and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) (Clark et al., 1993, 1995, 1997; Kayes and Clark, 1998; Fletcher et al., 1999; Jeong et al., 1999; Miwa et al., 2008; Müller et al., 2008; Ogawa et al., 2008; Kinoshita et al., 2010). Loss of function of these

results in an enlargement of the SAM, which is similar to but milder than that of *clv3*. *CLV1* encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) and is expressed in the CZ region including the OC (Clark et al., 1997). CLV2 is an LRR receptor-like protein which lacks intracellular signaling domain, and is considered to function as a receptor complex together with CRN (Jeong et al., 1999; Bleckmann et al., 2010). Both CLV2 and CRN are expressed throughout the SAM. RPK2 belongs to an LRR-RLK subfamily different from that of CLV1, and is expressed in the entire SAM, especially in the PZ (Kinoshita et al., 2010). The direct binding of CLV1 to CLV3 peptide is confirmed by biochemical assays, although no direct binding of CLV2 and RPK2 to CLV3 peptide is detected (Shinohara and Matsubayashi, 2015). Nevertheless, CLV2/CRN and RPK2 are proposed to function as CLV3 receptors because *clv2*, *crn*, and *rpk2* are insensitive to overexpression or exogenous application of CLV3 peptide (Brand et al., 2000; Müller et al., 2008; Kinoshita et al., 2010). Recently, novel LRR-RLKs, CLAVATA3 INSENSITIVE RECEPTOR KINASES (CIKs), are reported to function as co-receptors of CLV1, CLV2/CRN, and RPK2 (Hu et al., 2018). It is therefore possible that the interaction with CIKs is required for CLV2/CRN and RPK2 to perceive CLV3 peptide.

It is still largely unknown how the CLV signaling downstream of the receptors leads to the downregulation of WUS. CLV1 is autophosphorylated probably after perceiving the CLV3 peptide (Williams et al., 1997), indicating the involvement of the phosphorelay. Actually, several protein phosphatases, including POLTERGEIST, POLTERGEIST-LIKE1, and KINASE-ASSOCIATED PROTEIN PHOSPHATASE

(KAPP), function downstream of the CLV3 receptors (Williams et al., 1997; Yu et al., 2000; Song et al., 2006). Mitogen-activated protein kinases (MAPKs) and heterotrimeric G proteins are also proposed to be involved in CLV signaling (Betsuyaku et al., 2011; Ishida et al., 2014).

CLV-like signaling in rice and maize

Rice *FLORAL ORGAN NUMBER2* (*FON2*) is the first *CLV3* ortholog characterized outside of Arabidopsis. *FON2* is expressed in a small number of cells residing at the apical region of the CZ, probably corresponding to stem cells (Suzaki et al., 2006; Figures I-3A and I-3B). The loss-of-function *fon2* mutants exhibit an enlargement of the FM and increased numbers of floral organs. Overexpression of *FON2* results in a precocious termination of the IM or FM, indicating that *FON2* is a negative regulator of stem cell maintenance, similar to *CLV3* in Arabidopsis. *FON1* is the gene encoding an LRR-RLK orthologous to CLV1, and its loss-of-function leads to almost the same phenotype as *fon2* (Suzaki et al., 2004). Unlike *CLV1*, *FON1* is expressed throughout the entire SAM. *fon1* is insensitive to overexpression of *FON2*, and the *fon1 fon2* double mutant displays a similar phenotype to that of *fon2*, indicating that FON1 acts as a receptor of FON2.

In rice, there are some *CLV3*-related *CLE* genes other than *FON2*. *FON2-LIKE CLE PROTEIN1* (*FCP1*) and *FCP2* encode CLE peptides highly similar to FON2 peptide, and are expressed in the entire SAM (Suzaki et al., 2008). Overexpression of *FCP1* leads to a termination of the vegetative SAM (Suzaki et al., 2008; Ohmori et al.,

2013). Furthermore, induction of co-silensing of *FCP1* and *FCP2* causes an enlargement of the *FON2* expression domain (Ohmori et al., 2013). These findings show that *FCP1* and *FCP2* negatively regulate stem cell proliferation in the vegetative SAM. In *indica* varieties of rice, moreover, *FON2 SPARE1* (*FOS1*) functions redundantly with *FON2* (Suzaki et al., 2009). Although *FOS1* is expressed in the entire SAM of both *indica* and *japonica* varieties, it is not functional in *japonica* varieties. Overexpression of *FOS1* results in a termination of the SAM, similar to *FCP1*. *fon1* is sensitive to overexpression of both *FCP1* and *FOS1*, suggesting the possibility that FCP1 and FOS1 peptides act through receptors other than FON1 (Suzaki et al., 2009).

There is little knowledge about positive regulators of stem cell maintenance in rice. *OsWOX4* is the only gene suggested to be involved in promoting stem cell fate in the SAM (Ohmori et al., 2013). Downregulation of *OsWOX4* causes a termination of the SAM. Overexpression of *FCP1* results in downregulation of *OsWOX4*, raising the possibility that *FCP1* and *OsWOX4* constitute a negative feedback loop to maintain stem cells, similar to *CLV3* and *WUS* in Arabidopsis (Ohmori et al., 2013). Interestingly, however, *OsWOX4* is expressed in the entire SAM, unlike *WUS* in the OC. The *WUS* ortholog of rice, *TILLERS ABSENT1* (*TAB1*), is not expressed in the vegetative SAM but is in the premeristem zone, a transient state during axillary meristem establishment (Tanaka et al., 2015). Although loss-of-function *tab1* mutant shows a defect in axillary meristem formation, its SAM is maintained properly, indicating that *TAB1* is not involved in stem cell maintenance, unlike *WUS* in Arabidopsis (Tanaka et al., 2015).

In maize, *THICK TASSEL DWARF1* (*TD1*) and *FASCIATED EAR2* (*FEA2*),

respective orthologs of Arabidopsis *CLV1* and *CLV2*, negatively regulate stem cell maintenance (Taguchi-Shiobara et al., 2001; Bommert et al., 2005; Figures I-3A and I-3C). FEA2 is suggested to act as a receptor complex together with ZmCRN, the maize CRN ortholog, similar to CLV2/CRN (Je et al., 2018). FEA2 has another interacting partner, COMPACT PLANT2 (CT2), a predicted α-subunit of heterotrimeric G protein to regulate stem cell maintenance (Bommert et al., 2013; Je et al., 2018). FEA3 is a novel LRR receptor-like protein, which was first characterized in maize (Je et al., 2016). FEA3 is expressed specifically in the OC-like region in the SAM. Loss of *FEA3* leads to an enlargement of the SAM and an expansion of the expression domain of *ZmWUS1*, one of two maize orthologs of *WUS* (Je et al., 2016). FEA3 is suggested to function as a receptor of ZmFCP1, the maize FCP1 ortholog, expressed in leaf primordia (Je et al., 2016). Exogenous application of ZmFCP1 peptide leads to a reduction in SAM size in wild type, but not in *fea3*. These findings suggest that ZmFCP1 peptide, acting as a signaling molecule from organ primordia, is transmitted to the OC via FEA3 to regulate stem cell maintenance. Je et al. (2016) showed that the putative orthologs of FCP1 and FEA3 also function in stem cell regulation in Arabidopsis, raising the possibility that this FCP1/FEA3-like pathway is conserved in diverse plant species.

In summary, the studies in rice and maize have been contributed to the elucidation of the mechanisms underlying stem cell maintenance, not only by confirming that the WUS–CLV signaling is basically conserved in monocots, but also by discovering new systems involved in stem cell regulation, such as FCP1/FEA3 pathway.

Composition of this thesis

In this thesis, I aimed to uncover the molecular mechanisms of stem cell control in the SAM using rice. In Chapter II, I revealed that the *ABERRANT SPIKELET AND PANICLE1* (*ASP1*) gene, encoding a transcriptional corepressor, negatively regulates stem cell maintenance in concert with FON signaling. In Chapter III, I analyzed a new spikelet mutant, *fickle spikelet1* (*fsp1*), which displays an increase in organ number, a feature related to a defect in stem cell maintenance. Finally, in Chapter V, I briefly describe the conclusion of this study and some future directions.

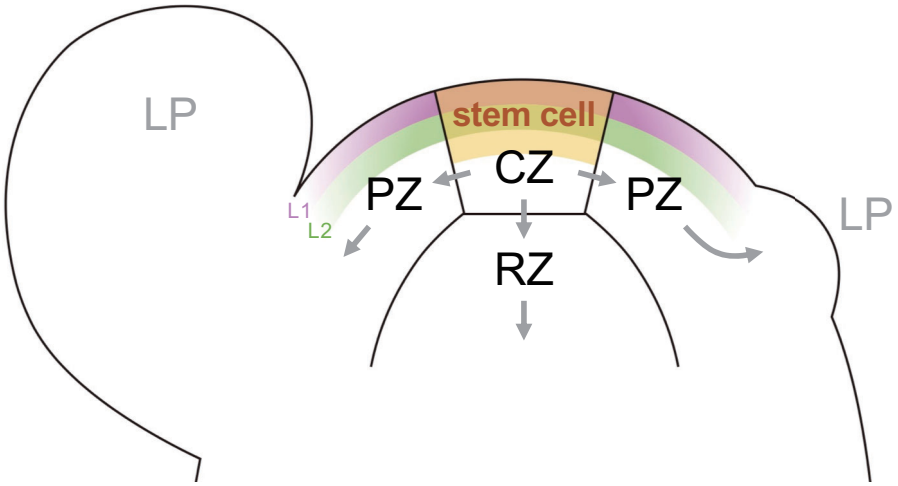


Figure I-1. Schematic diagram of the structure of the shoot apical meristem. CZ, central zone; LP, leaf primordium; PZ, peripheral zone; RZ, rib zone.

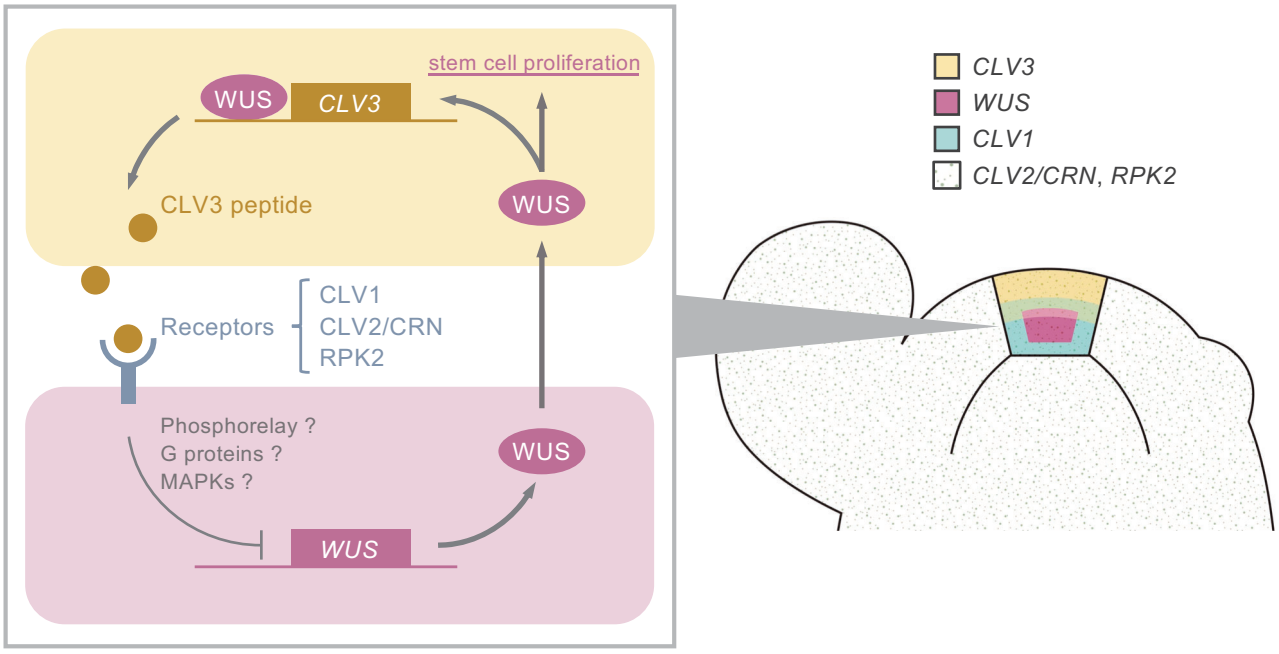


Figure I-2. WUS–CLV signaling in Arabidopsis.
Schematic expression patterns of genes involved in WUS–CLV signaling are indicated in the right figure. *CLV3* is expressed in stem cells, and *WUS* is expressed in the OC. *CLV1* is expressed in the region surrounding and including the OC. *CLV2*/*CRN* and *RPK2* are expressed throughout the SAM, including lateral primordia. In the left panel, schematic representation of WUS–CLV signaling in stem cells (pale yellow) and in the OC cells (pale pink) is indicated. CLV3 peptide is secreted from stem cells and perceived by multiple receptors, including CLV1, CLV2/CRN, and RPK2. Signaling via these receptors finally represses transcription of *WUS*. WUS moves from the OC to stem cells, and directly promote transcription of *CLV3*.

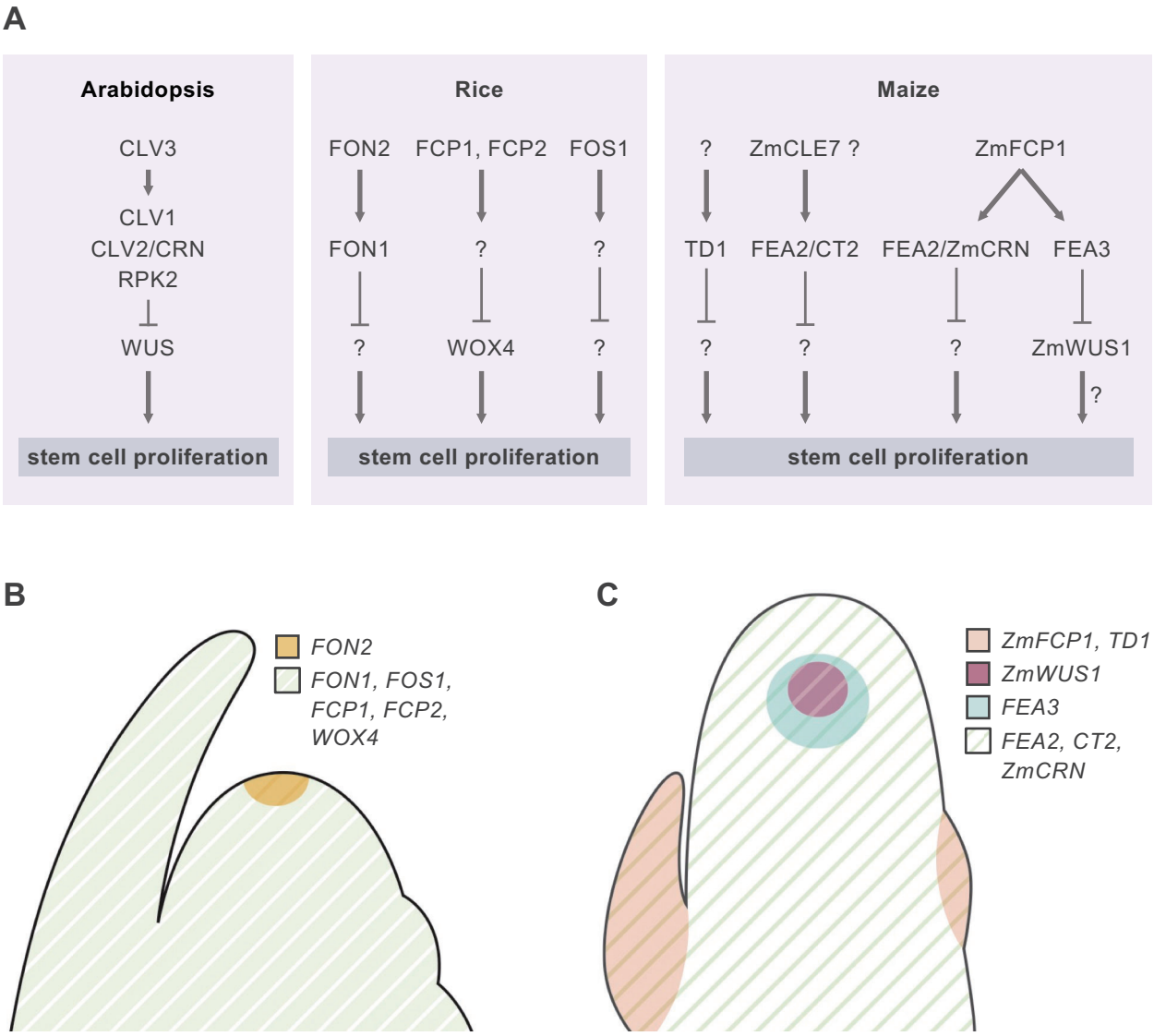


Figure I-3. CLV-like signaling in rice and maize.
(A) Models of CLV-like signaling in Arabidopsis, rice, and maize.
(B) Schematic expression patterns of genes involved in CLV-like signaling in rice. *FON2* is expressed in the apical region of the SAM, putative stem cells. *FON1*, *FOS1*, *FCP1*, *FCP2*, and *WOX4* are expressed throughout the SAM, including lateral primordia.
(C) Schematic expression patterns of genes involved in CLV-like signaling in maize. The expression patterns of maize *CLV3* orthologs, *ZmCLE7* and *ZmCLE14* (Je et al, 2016), have not been characterized. *ZmFCP1* is expressed in leaf primordia. *TD1* is also expressed in leaf primordia, but in the reproductive phase, it is expressed throughout the SAM. *ZmWUS1* is expressed in the predicted OC region. *FEA3* is expressed in and below the *ZmWUS1* expression domain. *FEA2*, *CT2*, and *ZmCRN* are expressed throughout the SAM.

Chapter II

ASP1 encoding the TOPLESS-related transcriptional corepressor regulates meristem maintenance in concert with CLV-like signaling in rice

Introduction

Plant development depends on the activity of the shoot apical meristem (SAM), which harbors a group of stem cells at the tip. These stem cells proliferate to maintain self-renewal and to supply cells for organ differentiation (reviewed in Ha et al., 2010; Aichinger et al., 2012; Somssich et al., 2016). The vegetative SAM differentiates leaf primordia and turns into the inflorescence meristem when it enters the reproductive phase after perceiving a flowering signal. The inflorescence meristem directly initiates either flower meristems differentiating floral organs or intermediate types of meristems such as the branch and spikelet meristems (see below; reviewed in Tanaka et al., 2013; Hirano et al., 2014). In each meristem, the appropriate balance between stem cell self-renewal and organ/meristem differentiation is essential for proper plant development.

In *Arabidopsis thaliana* (Arabidopsis), the WUSCHEL–CLAVATA (WUS–CLV) feedback loop is a fundamental mechanism underlying stem cell maintenance in the SAM (Brand et al., 2000; Schoof et al., 2000; reviewed in Ha et al., 2010; Aichinger et al., 2012; Somssich et al., 2016). The CLAVATA3 (CLV3) peptide is secreted from stem cells and perceived by multiple receptors, including CLV1, CLV2/CORYNE, and

RECEPTOR-LIKE PROTEIN KINASE 2 (Clark et al., 1997; Fletcher et al., 1999; Müller et al., 2008; Kinoshita et al., 2010), which transmit the signal to negatively regulate expression of the *WUS* gene, encoding a homeodomain transcription factor (Mayer et al., 1998). *WUS* is expressed in the organizing center located under the stem cell region, and acts non-cell autonomously in stem cells through movement of the WUS protein itself (Mayer et al., 1998; Yadav et al., 2011). WUS positively regulates stem cell proliferation partly in association with cytokinin action, and activates *CLV3* expression (Leibfried et al., 2005; Gordon et al., 2009; Yadav et al., 2011; Chickarmane et al., 2012). This negative feedback loop between CLV signaling and WUS action regulates stem cell homeostasis in the meristem.

Genetic regulation similar to Arabidopsis WUS–CLV is conserved in other plants, such as *Oryza sativa* (rice) and *Zea mays* (maize) (reviewed in Tanaka et al., 2014; Somssich et al., 2016). In rice, *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2*, respective orthologs of Arabidopsis *CLV1* and *CLV3*, negatively regulate meristem maintenance in the reproductive phase (Suzaki et al., 2004, 2006). The loss of function of either *FON1* or *FON2* results in enlargement of the floral meristem, resulting in increased numbers of floral organs such as pistils and stamens. *FON2* is expressed in the apical region of the floral meristem, putative stem cells, and its expression domain is expanded in the enlarged floral meristem in the *fon1* mutant. Overexpression of *FON2* causes a failure in maintenance of the floral meristem in wild type; by contrast, *fon1* is insensitive to this overexpression (Suzaki et al., 2006). Thus, FON2 acts as a negative regulator of stem cell proliferation through FON1, similar to CLV3 action in

Arabidopsis. *FON2-LIKE CLE PROTEIN1* (*FCP1*) and *FCP2*, close paralogs of *FON2*, play a role in maintenance of the SAM in the vegetative phase by repressing the expression of *WOX4*, a member of *WUSCHEL-RELATED HOMEOBOX* (*WOX*) gene family (Ohmori et al., 2013). By contrast, *TILLERS ABSENT1* (*TAB1*; also known as *OsWUS*), an ortholog of *WUS*, plays an important role in initiation of the axillary meristem in rice, but is not involved in maintenance of the established meristem (Tanaka et al., 2015).

In maize, *THICK TASSEL DWARF1* and *FASCIATED EAR2* (*FEA2*), respective orthologs of *CLV1* and *CLV2*, negatively regulate meristem maintenance (Taguchi-Shiobara et al., 2001; Bommert et al., 2005; reviewed in Somssich et al., 2016). Recent studies revealed that *ZmFCP1*, a maize *FCP1* ortholog, also functions in meristem maintenance (Je et al., 2016, 2018). ZmFCP1 is expressed in organ primordia and is thought to be perceived by FEA3, a novel CLV-related LRR receptor-like protein. Je et al. (2016) also showed that this FCP1/FEA3-like pathway is conserved in Arabidopsis.

Rice produces an inflorescence, known as a panicle, consisting of a main axis (rachis), primary and secondary branches, and spikelets. During inflorescence development, the inflorescence meristem initiates the primary branch meristems, which in turn initiate the secondary branch and spikelet meristems. Floral organs including lodicules, stamens, and a pistil are differentiated from the flower meristem, whose identity is changed from the spikelet meristem. Loss- or gain-of-function mutations in genes such as *ABERRANT SPIKELET AND PANICLE1* (*ASP1*), *ABERRANT PANICLE ORGANIZATION 1* (*APO1*)/*STRONG CULM2* (*SCM2*), *RFL*/*APO2*, *DENSE AND*

ERECT PANICLE 1 (*DEP1*)/*DENSE PANICLE 1*, *TONGARI-BOUSH1-3*, *LAX PANICLE1* (*LAX1*), *LAX2*, *OsCKX2*, *OsSPL14*, and *TAWAWA1* (*TAW1*) lead to small or large inflorescences (Komatsu et al., 2003; Ashikari et al., 2005; Ikeda et al., 2007; Rao et al., 2008; Huang et al., 2009; Jiao et al., 2010; Miura et al., 2010; Ookawa et al., 2010; Tabuchi et al., 2011; Ikeda-Kawakatsu et al., 2012; Yoshida et al., 2012, 2013; Tanaka et al., 2017). The functions of most of these genes have been shown to be associated with meristem activity and fate. Unlike these genes, loss-of-function mutations in *FON1* or *FON2* do not cause obvious defects in inflorescence architecture (Suzaki et al., 2004, 2006).

TOPLESS (*TPL*) and four *TOPLESS*-RELATED genes (*TPR1–TPR4*) encode transcriptional corepressors that play a pivotal role in development, such as embryo and flower patterning in Arabidopsis; for example, RNA silencing of *TPR2* in the *tpl tpr1 tpr3 tpr4* quadruple mutant results in transformation of the shoot pole into a root pole (Long et al., 2002, 2006; Krogan et al., 2012). These genes are also required for signaling of hormones such as auxin, jasmonate, and brassinosteroid (Szemenyei et al., 2008; Pauwels et al., 2010; Oh et al., 2014; Ryu et al., 2014). The TPL and TPRs corepressors, which have no DNA binding motif, physically interact with transcriptional factors that recognize a specific DNA sequence, and silence a large number of target genes by recruiting histone deacetylases (Long et al., 2006; Causier et al., 2012). TPL (reported as WSIP1) and TPR4 (WSIP2) physically interact with WUS, and this interaction is necessary for meristem maintenance (Kieffer et al., 2006). Furthermore, *WUS* regulates the expression of *TPL* and some *TPRs* in the meristem (Busch et al.,

2010). Despite these observations, it is not clear how individual *TPL/TPR* genes function in stem cell maintenance, probably because of their high genetic redundancy in Arabidopsis.

Rice has three *TPL*-like genes including *ASP1*, suggesting that this type of transcriptional corepressor has relatively low functional redundancy in rice (Yoshida et al., 2012). Indeed, using an *asp1* single mutant that displays pleiotropic defects in inflorescence architecture and flower development, it is previously revealed that *ASP1* plays a critical role in the regulation of meristem fate in the reproductive phase (Yoshida et al., 2012). In maize, a mutation in *RAMOSA1 ENHANCER LOCUS2* (*REL2*), a *TPL*-like gene, enhances the highly branching phenotype of *ramosa1* (*ra1*) inflorescence (Gallavotti et al., 2010). REL2 has been shown to regulate axillary meristem fate by physically interacting with RA1, a transcription factor with a C2H2-type zinc-finger motif.

Although several genes involved in the WUS–CLV-like pathway in rice have been identified (Suzaki et al., 2004, 2006, 2008, 2009; Ohmori et al., 2013), our knowledge of the genetic mechanisms underlying meristem maintenance in this plant remains limited. To explore these mechanisms further, in this study I used a genetic approach to identify a gene whose mutation enhances the *fon2* flower phenotype. As a result, I found that a loss-of-function mutation in *ASP1* led to an enhanced floral phenotype in *fon2*. In addition, *fon2 asp1* double mutants showed a marked inflorescence phenotype, bifurcation of the rachis, due to massive enlargement of the inflorescence meristem, suggesting that *FON2* and *ASP1* are required for the negative regulation of stem cell

proliferation in both flower and inflorescence meristems. Transcriptome analysis indicated that FON2 signaling and ASP1 action are likely to negatively regulate a common set of genes, such as those related to transcriptional regulation. Lastly, genetic analysis indicated that, unexpectedly, *TAB1* is not associated with the overproliferation of stem cells in the *fon2 asp1* double mutant.

Results

Phenotypes of strain 1B-280, in which the *fon2* mutation is enhanced

To gain new insight into the FON signaling pathway, I used a genetic approach. Among mutagenized population of *fon2-3* mutants (Yasui et al., 2017), I focused on strain 1B-280 that showed an enhanced floral phenotype of *fon2-3*.

Whereas wild-type rice flowers had one pistil, about 60% of the *fon2-3* flowers had two or three pistils (Figures II-1A, II-1B, and II-1G), and the pistil number in 1B-280 was further increased (Figures II-1C and II-1G). In particular, about 20% of 1B-280 flowers produced more than four pistils, a feature that was rarely observed in the *fon2-3* single mutant (Figure II-1G).

In addition to the flower, 1B-280 showed an abnormality in the inflorescence. Wild-type rice inflorescences consist of a rachis, primary and secondary branches, and spikelets (Figures II-2A and II-2B). The inflorescence in the *fon2-3* single mutant was more or less normal (Figure II-2C). By contrast, 1B-280 exhibited a marked inflorescence phenotype in which the rachis was bifurcated (Figure II-2D), and each bifurcated rachis produced shorter primary branches.

I observed that the flower and inflorescence phenotypes in 1B-280 plants were heritable through several generations. These results suggest that a second site mutation was responsible for enhancing the flower phenotype of *fon2* and for disturbing the inflorescence architecture in 1B-280.

Identification of the gene responsible for enhancement of the *fon2* floral phenotype in 1B-280

Next, I tried to identify the gene affected by the second site mutation in 1B-280. I noted several other characteristic phenotypes of 1B-280, including acute curvature of branches and elongation of sterile lemmas, which were not observed in the *fon2-3* single mutant (Figure II-3). These characteristics are similar to those caused by an *asp1* mutation (Figures II-3D and II-3H; Yoshida et al., 2012), raising the possibility that 1B-280 has a mutation in the *ASP1* gene.

To test this possibility, I sequenced *ASP1* in 1B-280 and found that an essential nucleotide for RNA splicing was mutated at the donor splice site of intron 14 (Figure II-4A). I therefore examined expression of the *ASP1* transcript in 1B-280 by RT-PCR analysis. The experiment using two primer sets clearly indicated that mis-spliced *ASP1* mRNA containing intron 14 was expressed in 1B-280 (Figure II-4B). It is therefore likely that this mutation in *ASP1*, which results in the production of a truncated protein, is the second mutation responsible for enhancement of the *fon2* phenotype in 1B-280. I designated this new *asp1* allele as *asp1-fe* (*fe*; *fon2 enhancer*).

Next, I introduced *gASP1-GFP*, containing a 12.6-kb genomic fragment of the *ASP1* gene, into *fon2-3 asp1-fe* (1B-280). The resulting transformants showed rescue of the enhanced flower phenotypes in 1B-280 (Figure II-4C); the frequency of flowers with four or more pistils was greatly reduced in the rescued plants (Figure II-4F). The inflorescence phenotype of 1B-280 was also rescued by the introduction of *gASP1-GFP* (Figure II-4E). I then disrupted the *ASP1* gene in *fon2-3* by using the CRISPR-Cas9

system and obtained a biallelic mutant of *ASP1* (*asp1-ko*) (Figure II-5). The resulting *fon2-3 asp1-ko* double mutant showed a marked increase in pistil number, that is, it was a phenocopy of 1B-280 (Figures II-4D and II-4F). Collectively, these results strongly indicated that the mutation in *ASP1* was responsible for the enhanced *fon2* phenotype of 1B-280.

Genetic analysis of the enhancing effect of *asp1*

To confirm the phenotype-enhancing effect of *asp1*, I combined *fon2-3* with *asp1-1*, a previously isolated loss-of-function allele (Yoshida et al., 2012) that produces one pistil in its flower (Figures II-1E and II-1G). The resulting *fon2-3 asp1-1* mutant, similar to 1B-280 (*fon2-3 asp1-fe*), showed an increased number of pistils relative to the *fon2-3* single mutant (Figures II-1F and II-1G). *asp1-1* produced an inflorescence with short primary branches (Figure II-2F), as described previously (Yoshida et al., 2012). The *fon2-3 asp1-1* mutant exhibited a bifurcated rachis (Figure II-2G), similar to 1B-280 (*fon2-3 asp1-fe*), but this rachis bifurcation was not observed in either the *fon2-3* or *asp1-1* single mutant (Figures II-2C and II-2F). Therefore, the *fon2* and *asp1* mutations seemed to have a synergistic effect on inflorescence architecture.

FON1 encodes an LRR receptor-like kinase, a putative receptor of FON2, and the *fon1* mutant exhibits phenotypes similar to those of *fon2* (Suzaki et al., 2004, 2006). I therefore checked whether the *asp1* mutation also enhances the *fon1* phenotype. I crossed the *fon1-5* mutant, harboring a loss-of-function null allele (Suzaki et al., 2008), with the *asp1-10* mutant, harboring an allele bearing the same mutation as *asp1-fe*. The

resulting *fon1-5 asp1-10* double mutant showed an enhanced phenotype of *fon1-5* (Figure II-6), suggesting that ASP1 function synergistically interacts with FON signaling.

ASP1 is expressed in various types of above-ground meristem

I examined the spatial expression patterns of *ASP1* in wild type by *in situ* hybridization. In the vegetative phase, *ASP1* mRNA was expressed in the SAM and leaf primordia (Figures II-7A and II-7E), and in the premeristem (Figure II-7F), a transient state during axillary meristem formation (Tanaka et al., 2015). In the reproductive phase, *ASP1* mRNA was also detected in the inflorescence meristems, primary branch meristems, and floral meristems (Figures II-7B to II-7D), consistent with previous results (Yoshida et al., 2012).

Next, I introduced *gASP1-GFP* into *asp1-10* and analyzed the expression pattern of ASP1-GFP in the resulting transformants. ASP1-GFP protein was detected uniformly throughout the vegetative SAM and leaf primordia (Figures II-7G to II-7I). ASP1-GFP was also highly expressed in the premeristem (Figures II-7J to II-7L). Thus, *ASP1* is expressed in various types of the above-ground meristems, suggesting that it has functional roles in these meristems.

The inflorescence meristem is markedly enlarged in *fon2 asp1*

I examined the early stages of inflorescence development by using scanning electron microscopy (SEM). I first observed the inflorescence meristem at the stage when some

primary branch meristems had just started to initiate. The inflorescence meristem of *fon2-3* was a little larger than that of wild type (Figures II-8A, II-8B, II-8E, and II-8F), whereas the *asp1* meristem was indistinguishable from the wild-type meristem (Figures II-8C and II-8G). In *fon2-3 asp1*, however, the inflorescence meristem was extremely enlarged and fasciated (Figures II-8D and II-8H); furthermore, it was often split into two or three independent meristems (Figure II-9). A little later, when the primary branch meristems continued to initiate, no obvious differences in the inflorescence meristem were observed among wild type, *fon2-3*, and *asp1* (Figures II-8I to II-8K, II-8M to II-8O). In *fon2-3 asp1*, by contrast, two inflorescence meristems enclosed by a bract were formed, and each meristem initiated primary branch meristems (Figures II-8D and II-8H). These results indicate that the bifurcated inflorescences observed in *fon2-3 asp1* were caused by extreme enlargement of the inflorescence meristem, followed by a subsequent split of this meristem.

fon2 asp1 exhibited phenotypes not observed in either single mutant, such as a fasciated inflorescence meristem, indicating that the *fon2* and *asp1* mutations act synergistically, and that *ASP1* is functionally associated with *FON2* in the regulation of meristem maintenance.

To characterize the extremely enlarged inflorescence meristems of *fon2 asp1*, I analyzed the expression patterns of *ORYZA SATIVA HOMEOBOX1* (*OSH1*), a molecular marker of undifferentiated meristematic cells (Matsuoka et al., 1993; Sato et al., 1996), and *FON2*, which probably marks stem cells similar to Arabidopsis *CLV3* (Fletcher et al., 1999; Suzaki et al., 2006). Similar expression patterns of *OSH1* were

observed in the inflorescence meristem of wild type, *fon2-3*, and *asp1-1* (Figures II-10A to II-10C). In *fon2-3 asp1-fe* (1B-280), by contrast, the expression domain of *OSH1* was markedly expanded in the enlarged meristem (Figure II-10D). *FON2* expression was detected in the apical region of the inflorescence meristem in wild type and *asp1-10* (Figures II-10E and II-10G). *FON2* was expressed in a wider region in *fon2-3* than in wild type (Figure II-10F), consistent with the observation of increased stem cells in the *fon1* mutant (Suzaki et al., 2006). The *FON2* expression domain was markedly expanded in the enlarged inflorescence meristem of *fon2-3 asp1*, as compared with *fon2-3* (Figure II-10H). Collectively, these results indicate that the *asp1* mutation enhances the overproliferation of stem cells in *fon2*, and suggest that *FON2* and *ASP1* together negatively regulate stem cell proliferation in the inflorescence meristem in wild type.

TAB1* is unrelated to extreme enlargement of the inflorescence meristem in *fon2 asp1

In Arabidopsis, loss-of-function *clv3* mutants exhibit overproliferation of stem cells due to an expansion of the *WUS* expression domain (Brand et al., 2000; Schoof et al., 2000). I therefore explored whether the overproliferation of stem cells in *fon2 asp1* is related to *WUS*-like function in rice. The rice *WU*S ortholog is *TAB1*, which has been shown to be required for axillary meristem formation but not meristem maintenance (Tanaka et al., 2015).

Because *TAB1* is not expressed in the vegetative SAM, I first characterized its

expression in the inflorescence meristem. In wild type, *TAB1* was expressed in a small group of cells below the *FON2* expression domain (Figures II-11A and II-10E). The pattern of *TAB1* expression was comparable in *asp1-10* and wild type (Figure II-11C), but it was markedly expanded in the inflorescence meristem of both *fon2-3* and *fon2-3 asp-1-1* (Figures II-11B and II-11D). These results raised two possibilities: either the enhanced expression of *TAB1* caused the overproliferation of stem cells in *fon2* and *fon2 asp1*, or the expanded *TAB1* expression domain was a secondary effect due to enlargement of the inflorescence meristems in *fon2* and *fon2 asp1*.

To test these possibilities, I examined the genetic interaction of *tab1* with *fon2* and *asp1*. The *tab1-1* mutant, harboring a loss-of-function mutation at the splice site (Tanaka et al., 2015), showed a slightly larger and oval-shaped inflorescence meristem (Figures II-11E and II-11I). Slight enlargement of the meristem was also observed in *fon2-3 tab1-1* and *asp1-10 tab1-1* (Figures II-11F, II-11G, II-11J, and II-11K). In the *fon2-3 asp1-fe tab1-1* triple mutant, the size of the inflorescence meristem varied among plants, but it was generally larger than that of the *fon2-3 asp1-fe* double mutant (Figures II-11H and II-11L). The phenotype of the mature inflorescence of these mutants was consistent with that of the inflorescence meristem: namely, a fasciated inflorescence was formed in the *fon2-3 asp1-fe tab1-1* triple mutant as in the *fon2-3 asp1-fe* double mutant (Figure II-12). These results indicated that the *tab1* mutation did not suppress the inflorescence phenotypes of *fon2* and *fon2 asp1*. Thus, expansion of the *TAB1* expression domain in *fon2* and *fon2 asp1* did not cause the observed overproliferation of stem cells, but instead was a secondary effect due to enlargement of the inflorescence

meristem.

ASP1 regulates multiple genes controlled by *FON2* in the meristem

To determine the effects of *FON2* and *ASP1* on meristem maintenance in terms of gene expression, I carried out transcriptome profiling. Just after primary branch meristem initiation, the inflorescence meristems were dissected micro-surgically from the shoot apices of wild type, *fon2-3*, *asp1-10*, and *fon2-3 asp1-fe*, and subjected to microarray analysis.

I identified differentially expressed genes (DEGs) between wild type and mutants (FDR <0.05, fold change >1.5) in three biological replicates. As compared with wild type, 852 genes were upregulated in *fon2*, 165 genes in *asp1*, and 785 genes in *fon2 asp1* (Figure II-13A); these DEGs were termed "*fon2* up", "*asp1* up", and "*fon2 asp1* up", respectively. Downregulated genes were similarly identified in each mutant ("*fon2* down", "*asp1* down", and "*fon2 asp1* down"; Figure II-13B). Notably, about 35% (58/165) of the genes in "*asp1* up" were common to both "*fon2* up" and "*fon2 asp1* up", indicating that a considerable proportion of the genes repressed by *ASP1* were also negatively regulated by FON signaling. Consistent with the *in situ* hybridization analysis, *FON2* was upregulated about 2.4-fold in *fon2* and 4.9-fold in *fon2 asp1* (Figure II-14A).

Next, I performed Gene Ontology (GO) enrichment analysis of the DEGs by using agriGO v2.0 (Tian et al., 2017; http://systemsbiology.cau.edu.cn/agriGOv2/), and found that 21, 39 and 39 GO terms were significantly overrepresented in "*asp1* up", "*fon2* up",

and "*fon2 asp1* up", respectively (Figure II-13C). Notably, all GO terms enriched in "*asp1* up" were shared by "*fon2* up", and most GO terms that were enriched in "*fon2* up" were more significantly overrepresented in "*fon2 asp1* up". By contrast, this tendency was not observed for the GO terms enriched among the downregulated genes. Taken together, these results suggest that *ASP1* and FON signaling negatively regulate a set of genes with similar functions.

I visualized the results of the GO analysis on the basis of semantic similarity (Figure II-13D), which is defined by closeness in meaning among the GO terms (reviewed in Pesquita et al., 2009). In most cases, the GO terms enriched among the upregulated genes were located close to each other, but distant from those enriched among the downregulated genes. Most of the GO terms that were more significantly overrepresented in "*fon2 asp1* up" than in "*fon2* up" were in close proximity and constituted a cluster, marked "Gene expression" in Figure II-13D. This cluster was rich in GO terms related to transcriptional regulation including "gene expression" and "transcription, DNA-templated" (Figures II-13C and II-13D). Thus, the phenotype enhancement caused by the *asp1* mutation in *fon2* is likely to be closely associated with transcriptional regulation.

Next, I checked the expression of rice *WOX* genes in the microarray data (Nardmann et al., 2007), but no significant change in the expression of any *WOX* gene was detected in wild-type or the mutants (Figure II-14B). Therefore, similar to *TAB1*, these *WOX* genes do not seem to contribute to the *fon2 asp1* phenotype. A phytohormone, cytokinin, is also known to be involved in stem cell maintenance

(Leibfried et al., 2005; Gordon et al., 2009; Chickarmane et al., 2012); again, however, the expression of cytokinin-related genes (Tsai et al., 2012) showed no synergistic changes in *fon2 asp1* (Figure II-14C).

I also considered whether any of the genes so far reported to control inflorescence architecture are involved in the marked inflorescence phenotypes of *fon2 asp1* (Komatsu et al., 2003; Ikeda et al., 2007; Rao et al., 2008; Huang et al., 2009; Jiao et al., 2010; Miura et al., 2010; Ookawa et al., 2010; Tabuchi et al., 2011; Ikeda-Kawakatsu et al., 2012; Yoshida et al., 2013). Some genes, including *APO2*, *DEP1*, and *LAX2*, were upregulated in *fon2*, *asp1*, or *fon2 asp1*, indicating that they are negatively regulated by *FON2* or *ASP1* (Figure II-14A). However, no synergistic effect of the *fon2* and *asp1* mutations on these genes was detected.

Collectively, it seems that the marked inflorescence phenotypes of the *fon2 asp1* mutant cannot be explained simply by the expression of known genes required for stem cell maintenance and proper inflorescence development.

FON2 and _ASP1_ are involved in stem cell maintenance also in the vegetative SAM

Lastly, I examined the effect of the *fon2* and *asp1* mutations on vegetative development. At the late vegetative stage, *fon2-3 asp1-fe* often showed a switch from an alternate to an opposite phyllotaxy, which was rarely observed in wild type, *fon2-3*, or *asp1-10* (Figures II-15A to II-15D).

Because defects in phyllotaxy are sometimes attributed to a perturbation of meristem size (Jackson and Hake, 1999), I measured the size of the vegetative SAM in

each strain. The SAM of *fon2-3* was a little larger than that of wild type and *asp1-10* (Figures II-15E to II-15G, II-15M, and II-15N), and *FON2* was widely expressed in the apical region of the SAM of *fon2* (Figures II-15I to II-15K). The expression domain of *FON2* was extremely expanded in the SAM of *fon2-3 asp1-fe* (Figures II-15H, II-15L, II-15M, and II-15N). These results suggest that *FON2* is involved in stem cell maintenance not only in the flower and inflorescence meristems, but also in the vegetative SAM. In addition, *ASP1* seems to act together with *FON2* in the vegetative SAM, as in the reproductive meristems.

Discussion

asp1 is a genetic enhancer of *fon2*

It is previously reported that maintenance of the floral meristem is negatively regulated by FON signaling in rice, similar to CLV signaling in Arabidopsis (Suzaki et al., 2004, 2006, 2009). To identify new genes involved in stem cell maintenance, I used a genetic approach and found that the *fon2* flower phenotype was enhanced by a mutation in the *ASP1* gene, which encodes a transcriptional repressor similar to Arabidopsis TPL. The characteristic phenotype of *fon2*, namely an increase in pistil number, was synergistically enhanced in the *fon2 asp1* double mutant. *ASP1* was found to be expressed in various types of above-ground meristems, and its expression domain overlaps with that of *FON2* (Suzaki et al., 2006), suggesting that *ASP1* functions in stem cells together with *FON2*. Introduction of an *asp1* mutation into the *fon1* mutant also resulted in an enhanced flower phenotype, that is, increased pistil number. These results suggest that ASP1and FON signaling act synergistically in the regulation of meristem maintenance in rice.

The *fon2 asp1* double mutant exhibited bifurcation of the rachis, a marked phenotype that, to my knowledge, has not previously been observed in inflorescence mutants. This bifurcation seems to result from extreme enlargement of the inflorescence meristem (see below). This result was unexpected, because the single *fon2* mutation did not lead to obvious defects in the inflorescence meristem (Suzaki et al., 2006, and this study). It is previously reported that *FON2 SPARE1* (*FOS1*), a paralog of *FON2*,

functions redundantly with *FON2* in maintaining the flower meristem in wild rice species and *O. sativa indica*, and that *FOS1* is not functional in all strains of *O. sativa japonica* (Suzaki et al., 2009). The flower meristem is sensitive to *fon2* mutation, because the strains used in my developmental studies were *japonica*. Thus, it is possible that an unknown gene acts redundantly in the inflorescence meristem, similarly to *FOS1* in the flower meristem, and that the inflorescence meristem is regulated more robustly than the flower meristem even in *japonica* strains. The massive enlargement of the inflorescence meristem in *fon2 asp1* indicates that the *asp1* mutation might disrupt this robust maintenance mechanism in the inflorescence meristem.

In the vegetative phase, leaves initiated in an opposite phyllotaxy in the *fon2 asp1* mutant, as compared with an alternate phyllotaxy in wild-type rice. In maize, the pattern of leaf initiation changes from an alternate to an opposite phyllotaxy in *abphyl* mutants owing to enlargement of the SAM (Jackson and Hake, 1999; Yang et al., 2015). Consistent with this, the vegetative SAM was enlarged in the *fon2 asp1* double mutant, relative to wild type and the *fon2* single mutant. Close examination indicated that the SAM was in fact slightly enlarged in the *fon2* single mutant, unlike the previous observation by Suzaki et al. (2006). Because *FCP1* and *FCP2* act as major factors in negative regulation of the SAM (Suzaki et al., 2008; Ohmori et al., 2013), the *fon2* mutation seems to have less effect on this meristem as compared with the flower meristem.

Taken altogether, my observations indicate that *ASP1* is involved in maintaining various types of above-ground meristem; however, the extent of the effect of *asp1*

seems to depend on the type of the meristem, probably due to genetic redundancy complementing the *fon2* mutation. Alternatively, the exaggerated inflorescence phenotype of *fon2 asp1* might be associated with artificial selection to obtain large inflorescences producing a large number of grains during domestication and improvement of rice as a crop, similar to maize ear, which is highly sensitive to *td1* or *fea2* mutation (Taguchi-Shiobara et al., 2001; Bommert et al., 2005, 2013).

ASP1 negatively regulates stem cell proliferation in the meristem

SEM analysis showed that the inflorescence meristem was enlarged and fasciated in the *fon2 asp1* double mutant and split into two or three meristems at subsequent stages when the primary branch meristems were initiated. The expression domain of *FON2* was vastly expanded in the inflorescence meristem of *fon2 asp1*, relative to *fon2*, suggesting that the stem cells have overproliferated. These findings indicate that the *asp1* mutation synergistically enhances the enlarged inflorescence meristem in *fon2*, and suggest that *ASP1* plays an important role in stem cell maintenance. This represents the clear direct evidence that *TPL*-like genes are involved in stem cell maintenance in the above-ground meristems, although a mutation in the *TPL*-like gene *REL2* was recently shown to be involved in the control of the inflorescence meristem size in maize (Liu et al., 2019). Interestingly, in the root apical meristem, *WOX5* prevents precocious differentiation of columella stem cells by recruiting TPL to the target gene *CYCLING DOF FACTOR4*, which promotes cell differentiation (Pi et al., 2015).

Microarray analysis of RNAs isolated from micro-dissected inflorescence

meristems revealed that a large number of genes are up- or down-regulated in *fon2* and *fon2 asp1*, as compared with wild type. By contrast, the up- or down-regulated genes were not so many in *asp1*. This result was unexpected because TPL-like corepressors are thought to regulate a large number of genes by interacting with various transcription factors (Causier et al., 2012). *ASP1-RELATED1* (*ASPR1*), a paralog of *ASP1*, was upregulated in *asp1* (Figure II-14A), raising the possibility that loss of *ASP1* function may be compensated by upregulation of *ASPR1*. It seems, therefore, that a single *asp1* mutation would not necessarily lead to marked changes in gene expression and meristem enlargement.

Approximately half (73 of 165) of the genes upregulated in *asp1* were also upregulated in *fon2*, as compared with wild type. This suggests that a substantial proportion of the genes repressed by the corepressor action of ASP1 are also repressed directly or indirectly by FON signaling. In addition, GO analysis indicated that *ASP1* and *FON2* negatively regulate genes involved in similar biological processes, such as transcriptional regulation. FON signaling probably acts in the fine-tuning of the expression of genes responsible for stem cell proliferation in rice, similar to CLV signaling in Arabidopsis (Schoof et al., 2000; Reddy and Meyerowitz, 2005). It is possible that moderate expression levels of those genes are effective for this fine-tuning. In this scenario, genes regulated by FON signaling might be partially suppressed by *ASP1* expression at a moderate level in wild type. In the *fon2 asp1* double mutant, release from both negative fine-tuning by FON and moderate suppression by ASP1 might lead to the observed synergistic effect on the meristem. Alternatively, it is

possible that the synergistic effect of *fon2* and *asp1* mutations might result from the defects in other complex genetic networks including an ASP1 action independent of FON signaling.

The expression of neither *WOX* nor cytokinin-related genes was altered in these mutants as compared with wild type. Similarly, the expression of genes associated with large meristems and inflorescences, such as *APO1*/*SCM2* and *DEP1* (Ikeda et al., 2007; Huang et al., 2009; Ookawa et al., 2010), was not affected by *fon2* and *fon2 asp1* mutations. It is possible that, by physically interacting with various transcription factors, *ASP1* may repress multiple genes at a relatively low level; in this case, the effect of *asp1* mutation on individual genes might be difficult to observe, but the cumulative effect would result in a marked enlargement of the inflorescence meristem in *fon2 asp1*.

Putative roles of *TAB1* and *ASP1* in stem cell maintenance

In Arabidopsis, *WUS* promotes stem cell proliferation in all types of above-ground meristem, such as the vegetative SAM and inflorescence/flower meristems, and its expression domain is expanded in the enlarged meristems of the *clv1* and *clv3* mutants (Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000). By contrast, *TAB1*, the rice ortholog of *WUS*, is not expressed in the vegetative SAM, and loss of function of *TAB1* does not affect primary shoot growth, suggesting that *TAB1* is not involved in vegetative SAM maintenance (Tanaka et al., 2015). Instead, *TAB1* has a critical role in the initial stages of axillary meristem formation.

In contrast to the vegetative SAM, however, *TAB1* was expressed in the

inflorescence meristem and its expression domain was expanded in *fon2 asp1*. Thus, I hypothesized that overexpression *TAB1* might be a major factor in the extreme enlargement of the inflorescence meristem in *fon2 asp1*. Surprisingly, however, genetic analysis revealed that this meristem abnormality was not suppressed in the *fon2 asp1 tab1* triple mutant, indicating that my hypothesis should be dismissed. The *fon2 asp1* phenotype seems to result from cumulative changes in multiple genes due to the lack of ASP1 function as a transcriptional corepressor, as discussed above.

In general, *WUS*-like gene promotes stem cell activity and its loss-of-function mutation leads to premature termination of the meristem (Laux et al., 1996; Mayer et al., 1998; Kieffer et al., 2006). Notably, however, genetic introduction of the *tab1* mutation led to enlargement of the inflorescence meristem in wild type, *fon2*, *asp1*, and *fon2 asp1*, as compared with the respective original strains. These results clearly indicated that *TAB1* is not involved in promoting stem cell proliferation in the inflorescence meristem, even though it is expressed in this meristem. In Arabidopsis, a genome-wide study revealed that *WUS* regulates the expression of several genes, including *CLV1*, *TPL*, and *TPR*s, suggesting that SAM maintenance is modulated by a complex regulatory network (Busch et al., 2010). In addition, WUS physically interacts with TPL-like corepressors (Kieffer et al., 2006). Therefore, the enlargement of the inflorescence meristem observed in mutant strains containing the *tab1* mutation might be associated with WUS-like functions across complex genetic and protein networks, rather than specific functions in a WUS–CLV-like pathway. In fact, the physical interaction of TAB1 and TPL-like proteins has been reported in rice (Lu et al., 2015).

Similar to rice, *REL2* is associated with inflorescence architecture in maize. In addition, the recent report suggested the possibility that *REL2* is involved in the control of meristem maintenance (Liu et al., 2019), as described above. It will be of great interest to determine how *REL2* interacts genetically with regulators of stem cell maintenance such as *FEA2* and *TD1* in maize. Genetic redundancy among *TPL*-like genes seems to be relatively low in rice and maize, because single mutation and/or combination with another gene results in phenotypic alterations (Gallavotti et al., 2010; Yoshida et al., 2012; Liu et al., 2019). It is therefore expected that our understanding of the function of individual *TPL*-like genes in stem cell maintenance and other developmental processes will progress in rice and maize, in analogy to the discovery of a new signaling pathway that regulates stem cell maintenance in addition to CLV signaling (Suzaki et al., 2008; Je et al., 2016).

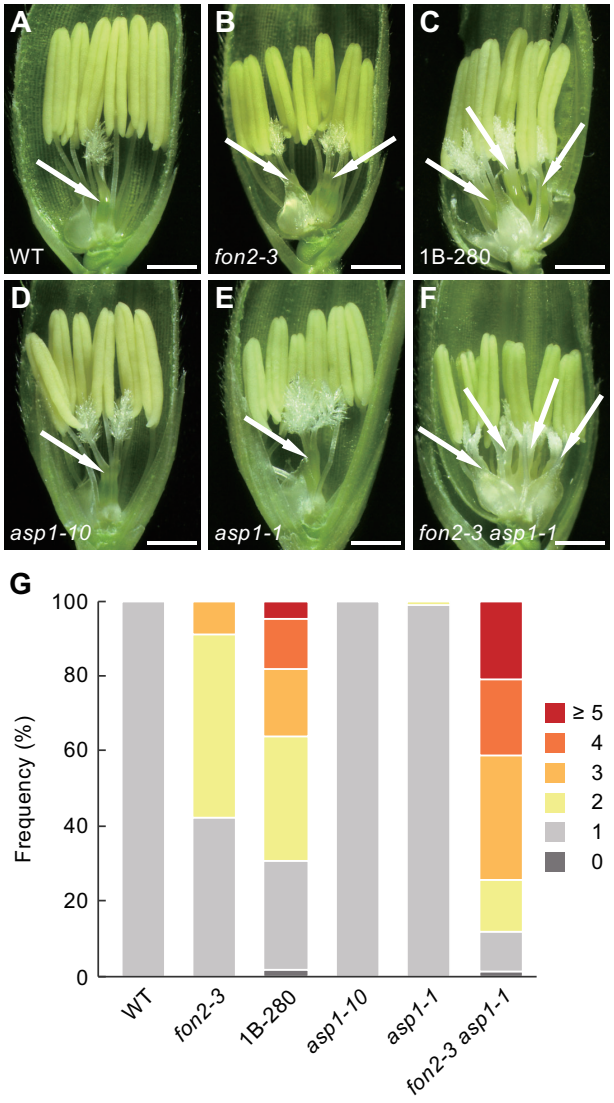


Figure II-1. Flower phenotypes.
(A) to **(F)** Flower phenotypes of wild type and the indicated mutants. Arrows indicate the pistils. Bars = 1 mm.
(G) Frequency of pistil number per flower. n = 50 (wild type), 100 (*fon2-3*), 160 (1B-280), 100 (*asp1-10*), 100 (*asp1-1*), 195 (*fon2-3 asp1-1*); three plants for each mutant.

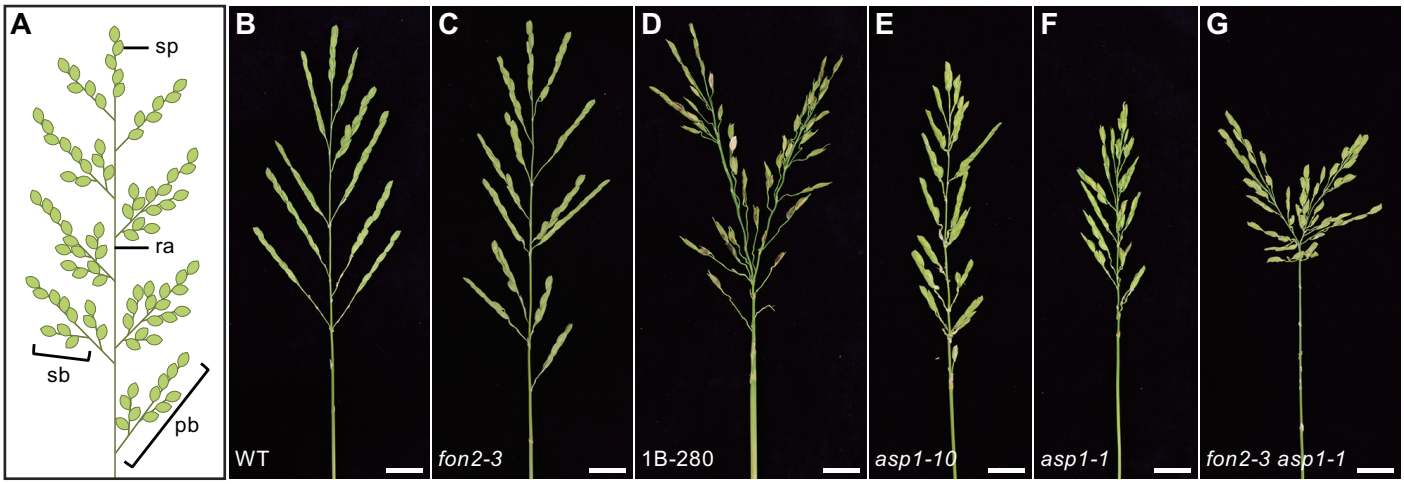


Figure II-2. Inflorescence phenotypes.
(A) Schematic representation of the wild-type inflorescence in rice.
(B) to **(G)** Inflorescence phenotypes. Bifurcation of the rachis is observed in 1B-280 **(D)** and *fon2-3 asp1-1* **(G)**.
pb, primary branch; ra, rachis; sb, secondary branch; sp, spikelet. Bars = 2 cm.

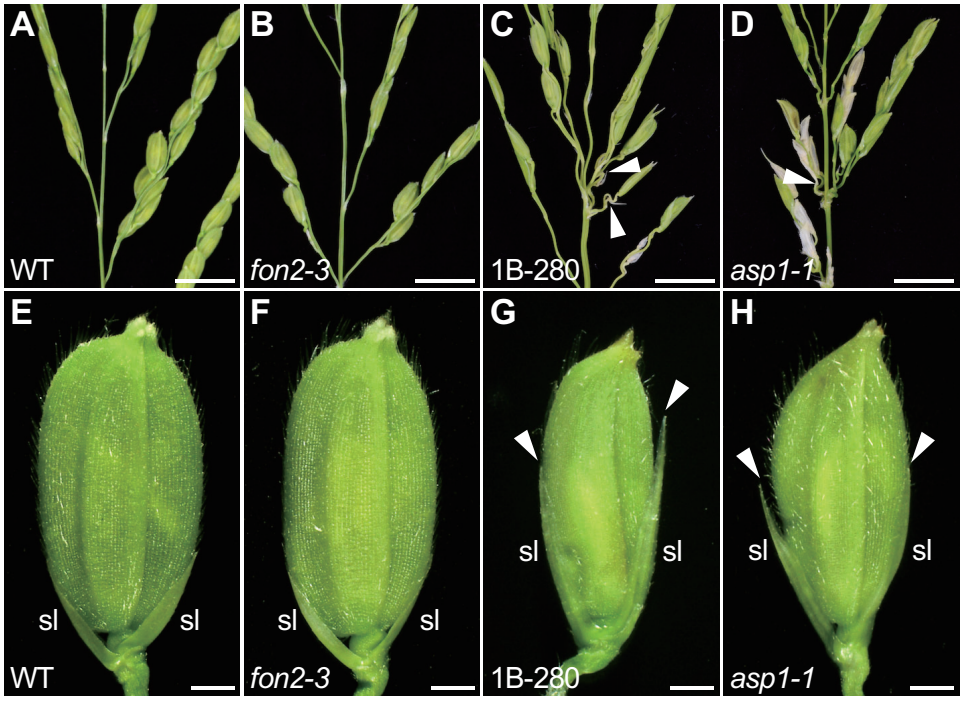


Figure II-3. *asp1*-like phenotypes in rice strain 1B-280.
(A) to **(D)** Phenotypes of branches. Arrowheads indicate the acute curvature of primary branch stems in 1B-280 and *asp1-1*.
(E) to **(H)** Phenotypes of spikelets. Arrowheads indicate the elongated sterile lemmas in 1B-280 and *asp1-1*.
sl, sterile lemma. Bars = 1 cm in **(A)** to **(D)**; 1 mm in **(E)** to **(H)**.

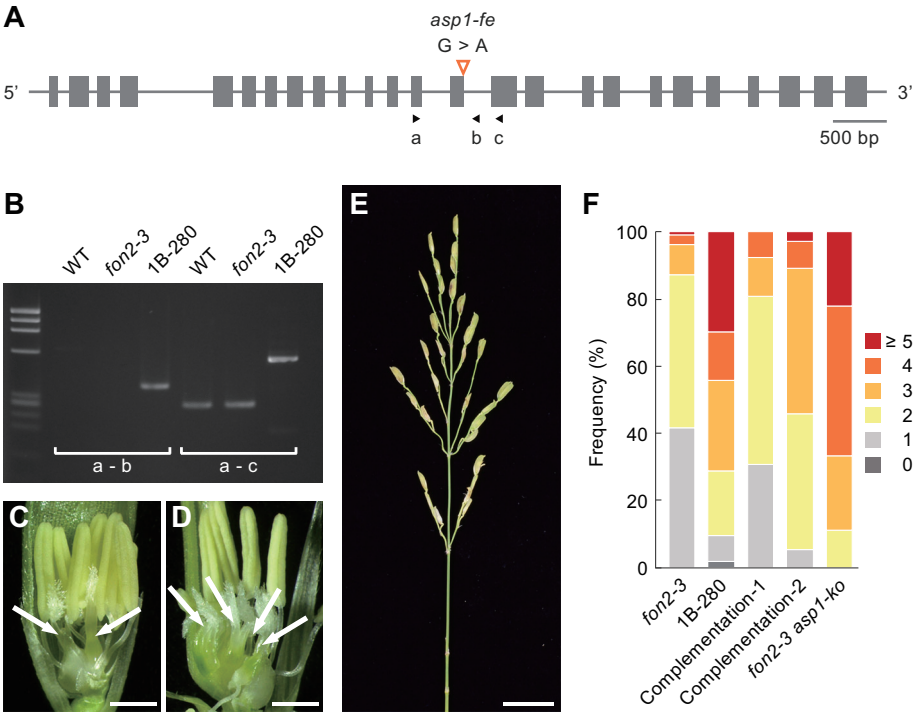


Figure II-4. Identification of the gene responsible for the enhanced phenotype of *fon2* in 1B-280.

(A) Structure of the *ASP1* gene. Boxes and lines between boxes represent exons and introns, respectively. A nucleotide substitution (open arrowhead) is present in the 5' splice site of intron 14 of *ASP1* in 1B-280 (designated *asp1-fe*). Closed arrowheads labeled a–c indicate the positions of the primers used for RT-PCR in **(B)**.

(B) RT-PCR analysis of *ASP1* mRNA. a–b and a–c indicate the primer pairs used for each analysis.

(C) A *fon2*-like flower rescued by introduction of *gASP1-GFP* into 1B-280, showing a decrease in pistil number as compared with the original strain 1B-280. Arrows indicate pistils.

(D) A flower in an *ASP1*-knockout line produced by CRISPR-Cas9 technology, showing an increase in pistil number as compared with the *fon2-3* mutant. Arrows indicate pistils.

(E) An inflorescence rescued by introduction of *gASP1-GFP* into 1B-280. The rachis bifurcation phenotype was not observed.

(F) Frequency of pistil number per flower in each strain. Complementation-1 and Complementation-2 represent independent lines expressing *gASP1-GFP* in the 1B-280 background. n = 212 (*fon2-3*), 104 (1B-280), 26 (Complementation-1), 37 (Complementation-2), 9 (*fon2-3 asp1-ko*).

Bars = 1 mm in **(C)** and **(D)**; 2 cm in **(E)**.

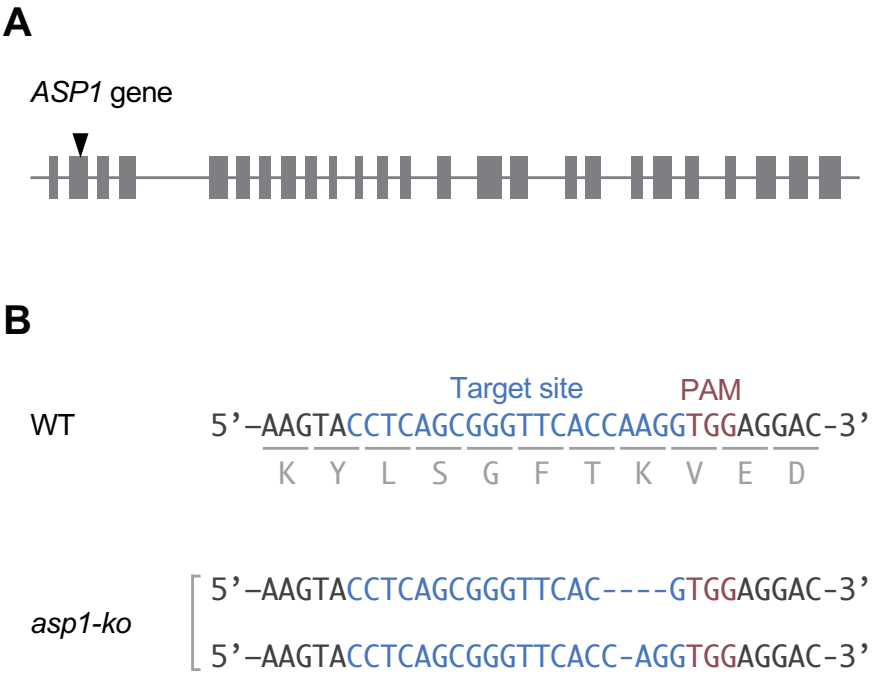


Figure II-5. Generation of a knockout line of *ASP1* in the *fon2-3* mutant by the CRISPR-Cas9 system.
(A) Position of the gRNA target site in *ASP1* (black arrowhead). The gRNA was designed to target the second exon **(B)**.
(B) Sequences of the resulting biallelic *asp1-ko* mutant shown in Figure II-4D.

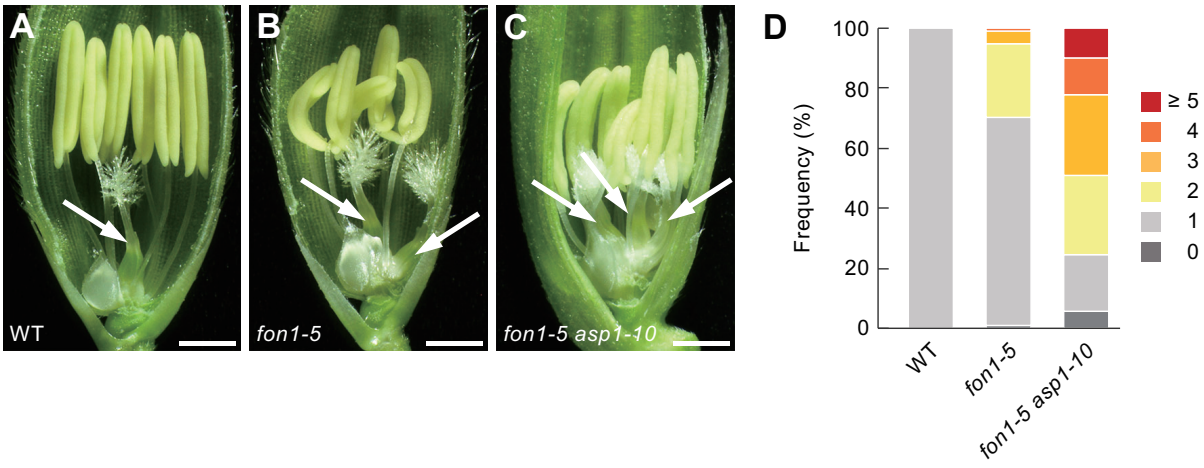


Figure II-6. Phenotypes of *fon1-5 asp1-10*.
(A) to **(C)** Flower phenotypes. Arrows indicate pistils. Bars = 1 mm.
(D) Proportion of pistil number per flower in each strain. *n* = 50 (wild type), 100 (*fon1-5*), 160 (*fon1-5 asp1-10*).

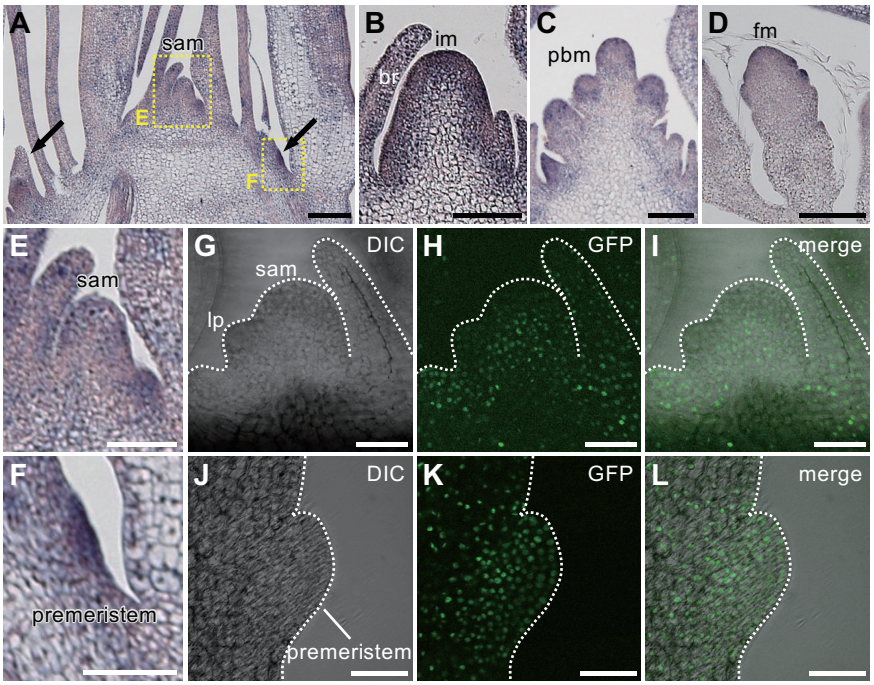


Figure II-7. Spatial expression patterns of *ASP1* transcripts and ASP1-GFP in the meristem.

(A) *In situ* localization of *ASP1* transcripts in the vegetative shoot apex **(A)**. Arrows indicate the established axillary meristem (left) and the premeristem zone (right).

(B) to **(D)** *In situ* localization of *ASP1* transcripts in the inflorescence meristem **(B)**, primary branch meristems **(C)**, and floral meristems **(D)**.

(E) and **(F)** Close-up views of the SAM and premeristem zone, indicated by the boxes in **(A)**.

(G) to **(L)** Localization of ASP1-GFP in the vegetative SAM (**(G)** to **(I)**) and premeristem zone in axillary meristem formation (**(J)** to **(L)**). The SAM, leaf primordia, and premeristem are outlined. Transgenic plants expressing *gASP1-GFP* in the *asp1-10* background, showing complete rescue of the mutant phenotype, were analyzed.

br, bract; fm, floral meristem; im, inflorescence meristem; lp, leaf primordium; pbm, primary branch meristem; sam, shoot apical meristem. Bars = 100 µm in **(A)** to **(D)**; 50 µm in **(E)** to **(L)**.

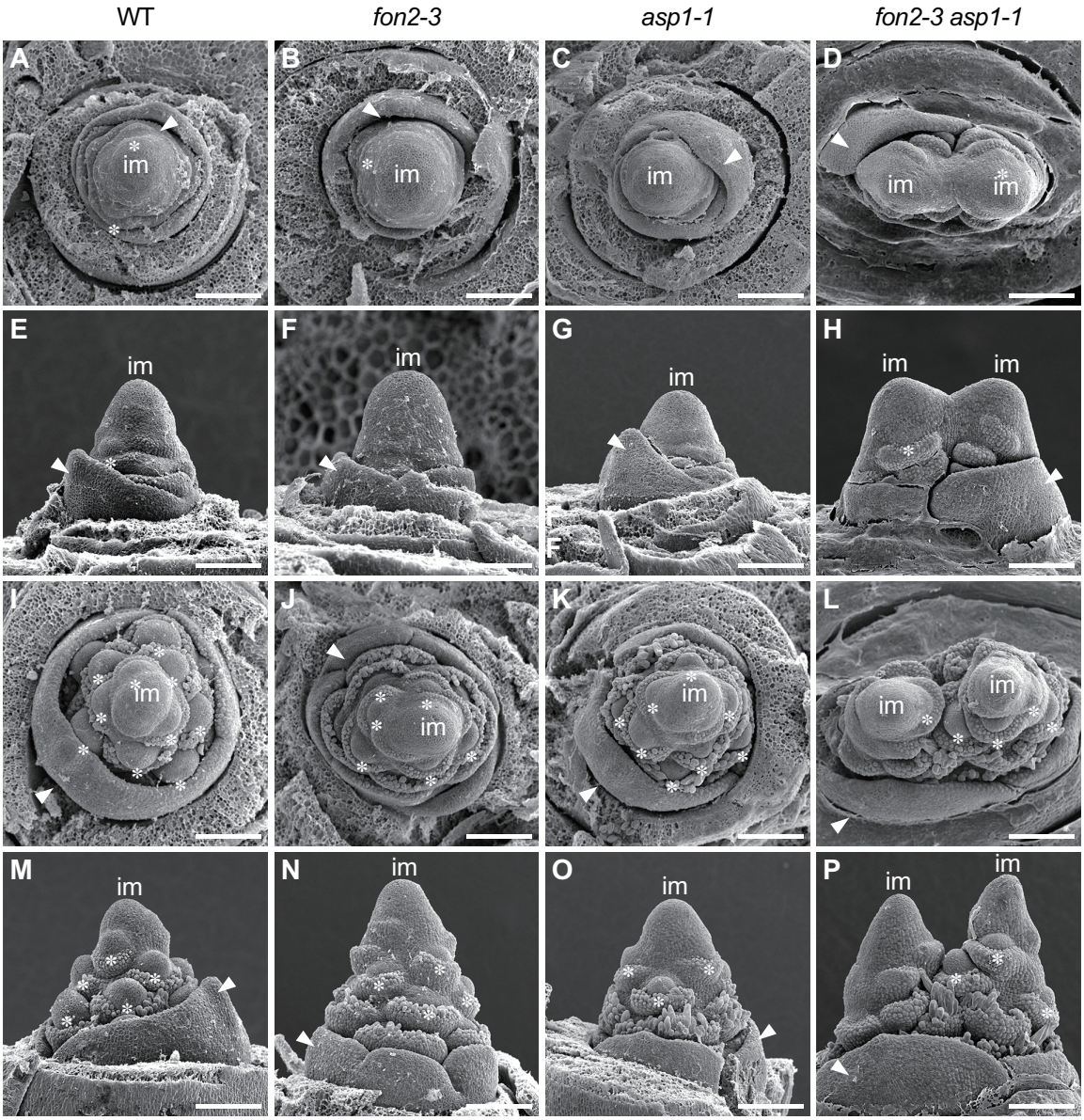


Figure II-8. SEM images of developing inflorescences.
(A) to **(H)** Developing inflorescences at an early stage of primary branch meristem initiation.
(I) to **(P)** Developing inflorescences at a later stage of primary branch meristem initiation.
Asterisks indicate primary branch meristems. Arrowheads indicate bracts. im, inflorescence meristem. Bars = 100 μm.

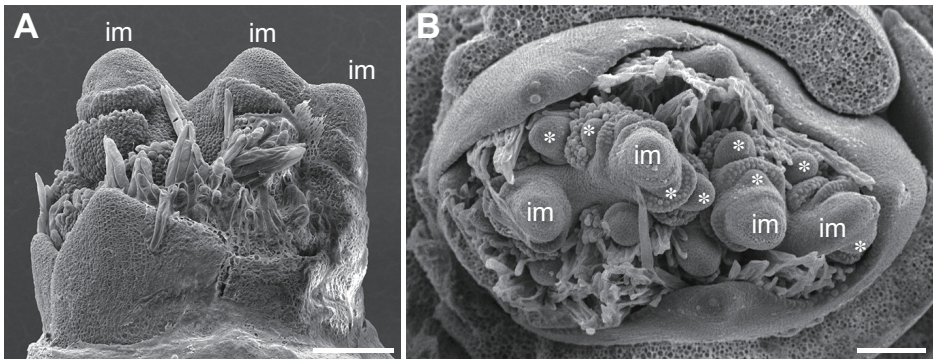


Figure II-9. Split inflorescence meristems in *fon2-3 asp1-fe*.
(A) An inflorescence meristem splitting into three independent meristems.
(B) An inflorescence bearing four inflorescence meristems.
Asterisks indicate primary branch meristems. im, inflorescence meristem. Bars = 100 μm.

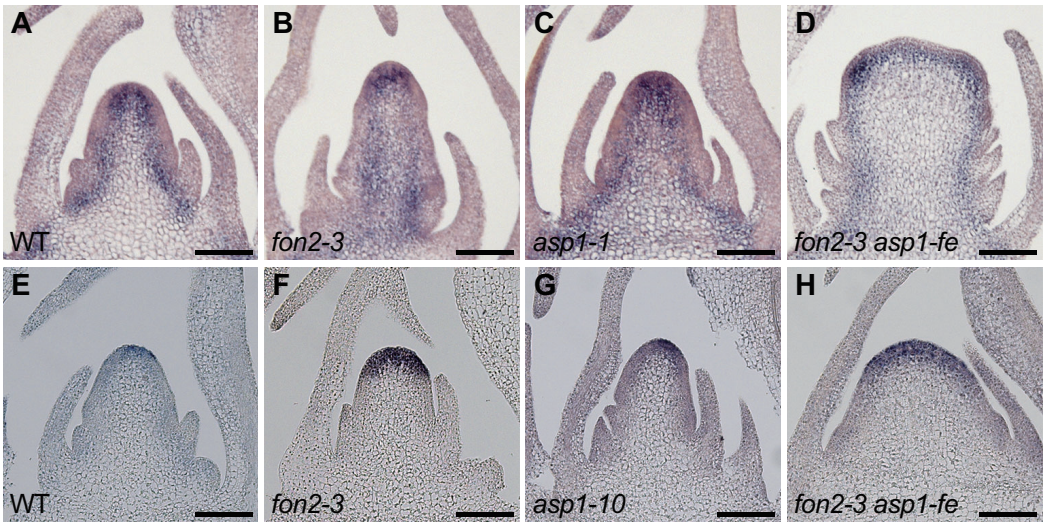


Figure II-10. Spatial expression patterns of marker genes in the inflorescence meristem.
(A) to **(D)** In situ localization of *OSH1* transcripts.
(E) to **(H)** In situ localization of *FON2* transcripts.
Bars = 100 µm.

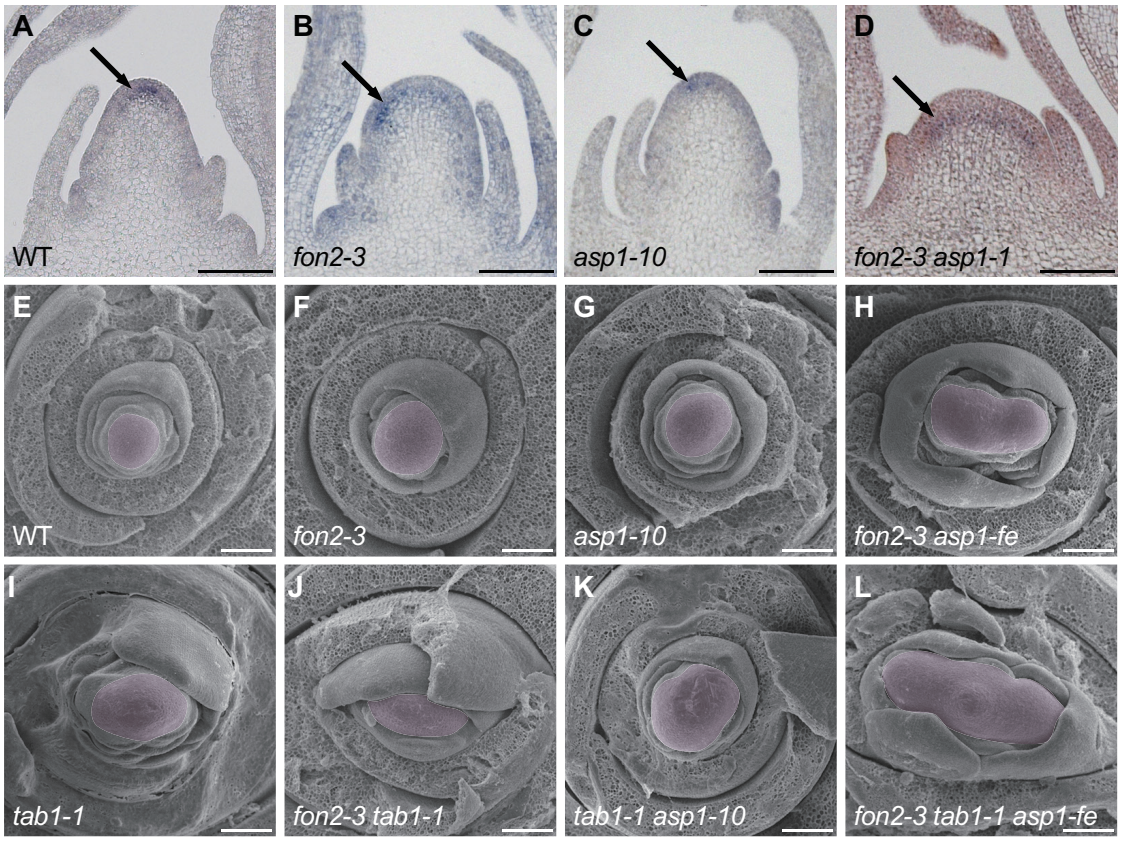


Figure II-11. *TAB1* expression patterns and the effect of *tab1* mutation on the inflorescence meristem.
(A) to **(D)** In situ localization of *TAB1* transcripts in the inflorescence meristem. Arrows indicate the signals of *TAB1* expression.
(E) to **(L)** SEM images of the developing inflorescence. Inflorescence meristems are highlighted in magenta.
Bars = 100 μm.

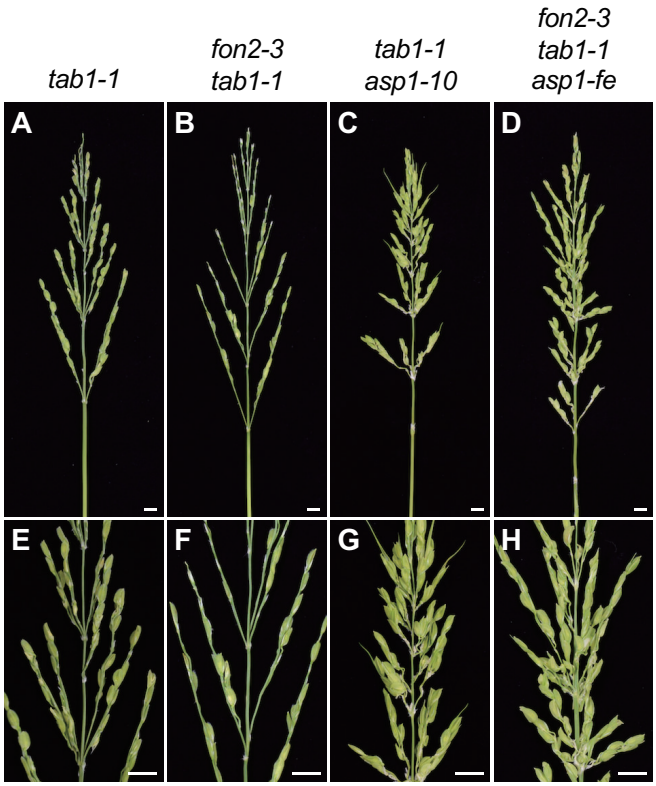


Figure II-12. Effects of the *tab1* mutation on inflorescence phenotype.
(A) to **(D)** Inflorescence phenotype of each strain.
(E) to **(H)** Respective close-up views of **(A)** to **(D)**.
Bars = 1 cm.

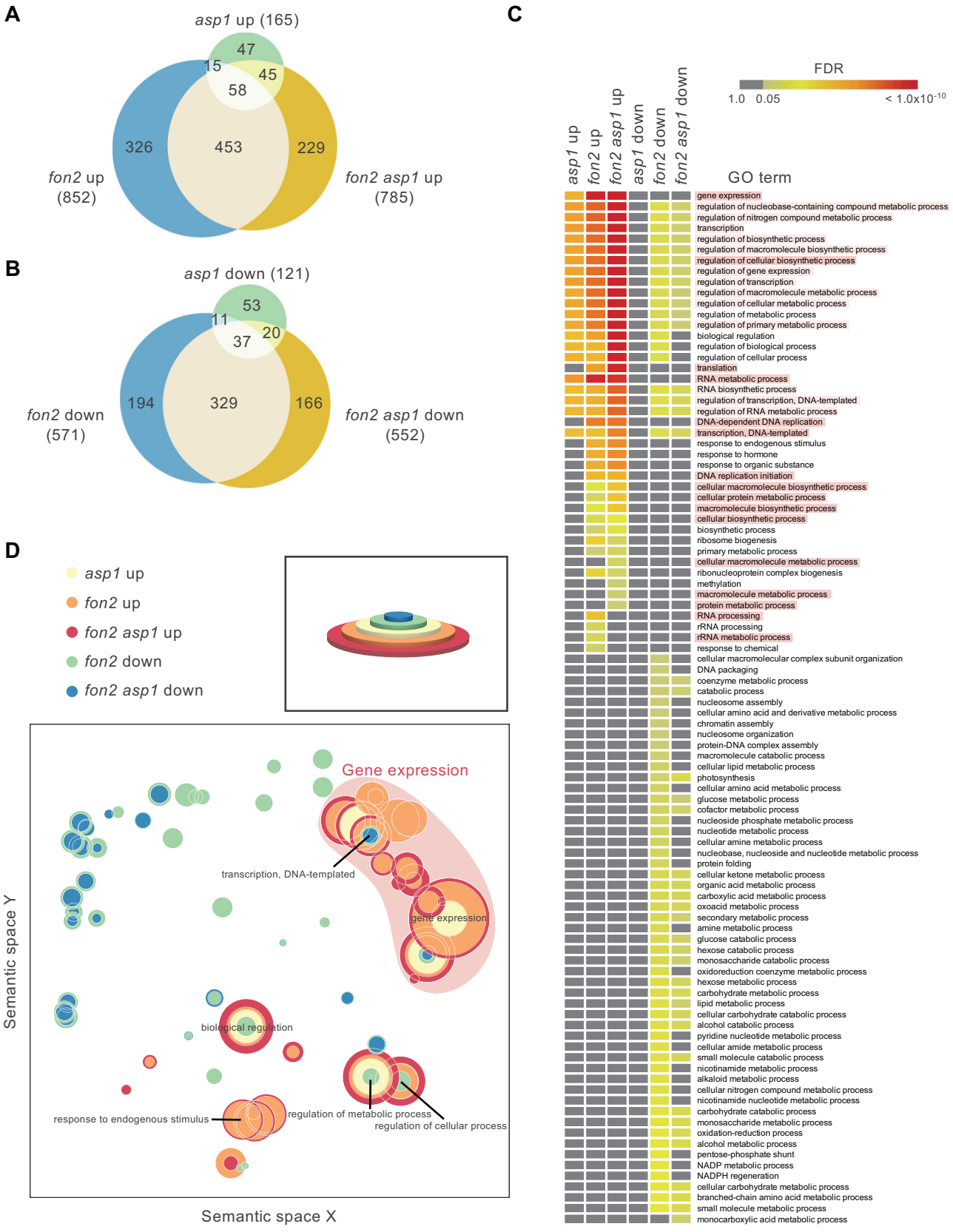

A
asp1 up (165)
47
15
45
58
326
453
229
fon2 up
(852)
fon2 asp1 up
(785)
B
asp1 down (121)
53
11
20
37
194
329
166
fon2 down
(571)
fon2 asp1 down
(552)
C
FDR
1.0 0.05
< 1.0x10-10
asp1 up
fon2 up
fon2 asp1 up
asp1 down
fon2 down
fon2 asp1 down
GO term
gene expression
regulation of nucleobase-containing compound metabolic process
regulation of nitrogen compound metabolic process
transcription
regulation of biosynthetic process
regulation of macromolecule biosynthetic process
regulation of cellular biosynthetic process
regulation of gene expression
regulation of transcription
regulation of macromolecule metabolic process
regulation of cellular metabolic process
regulation of metabolic process
regulation of primary metabolic process
biological regulation
regulation of biological process
regulation of cellular process
translation
RNA metabolic process
RNA biosynthetic process
regulation of transcription, DNA-templated
regulation of RNA metabolic process
DNA-dependent DNA replication
transcription, DNA-templated
response to endogenous stimulus
response to hormone
response to organic substance
DNA replication initiation
cellular macromolecule biosynthetic process
cellular protein metabolic process
macromolecule biosynthetic process
cellular biosynthetic process
biosynthetic process
ribosome biogenesis
primary metabolic process
cellular macromolecule metabolic process
ribonucleoprotein complex biogenesis
methylation
macromolecule metabolic process
protein metabolic process
RNA processing
rRNA processing
rRNA metabolic process
response to chemical
cellular macromolecular complex subunit organization
DNA packaging
coenzyme metabolic process
catabolic process
nucleosome assembly
cellular amino acid and derivative metabolic process
chromatin assembly
nucleosome organization
protein-DNA complex assembly
macromolecule catabolic process
cellular lipid metabolic process
photosynthesis
cellular amino acid metabolic process
glucose metabolic process
cofactor metabolic process
nucleoside phosphate metabolic process
nucleotide metabolic process
cellular amine metabolic process
nucleobase, nucleoside and nucleotide metabolic process
protein folding
cellular ketone metabolic process
organic acid metabolic process
carboxylic acid metabolic process
oxoacid metabolic process
secondary metabolic process
amine metabolic process
glucose catabolic process
hexose catabolic process
monosaccharide catabolic process
oxidoreduction coenzyme metabolic process
hexose metabolic process
carbohydrate metabolic process
lipid metabolic process
cellular carbohydrate catabolic process
alcohol catabolic process
pyridine nucleotide metabolic process
cellular amide metabolic process
small molecule catabolic process
nicotinamide metabolic process
alkaloid metabolic process
cellular nitrogen compound metabolic process
nicotinamide nucleotide metabolic process
carbohydrate catabolic process
monosaccharide metabolic process
oxidation-reduction process
alcohol metabolic process
pentose-phosphate shunt
NADP metabolic process
NADPH regeneration
cellular carbohydrate metabolic process
branched-chain amino acid metabolic process
small molecule metabolic process
monocarboxylic acid metabolic process
D
asp1 up
fon2 up
fon2 asp1 up
fon2 down
fon2 asp1 down
Gene expression
transcription, DNA-templated
gene expression
biological regulation
response to endogenous stimulus
regulation of metabolic process
regulation of cellular process
Semantic space Y
Semantic space X

Figure II-13. Transcriptome analysis.
(A) and **(B)** Venn diagrams of genes that were upregulated **(A)** and downregulated **(B)** in each mutant relative to wild type.
(C) GO terms overrepresented among the DEGs in each mutant. No GO terms were overrepresented among the DEGs in *asp1* down. GO terms highlighted in pink or pale pink were included in the "Gene expression" category in the REVIGO analysis in **(D)**, but those in pale pink were removed by REVIGO from the scatter plot in **(D)**.
(D) Results of the GO analysis visualized by using the REVIGO web tool (Supek et al., 2011; http://revigo.irb.hr/). Yellow, orange, red, green, and blue circles represent GO terms enriched among the DEGs in *asp1* up, *fon2* up, *fon2 asp1* up, *fon2* down, and *fon2 asp1* down, respectively. Circle size reflects the –log10 (FDR) value; the larger circle is arranged to be located in the lower layer for the same GO terms (see upper right panel). Semantically similar GO terms are presented close together.

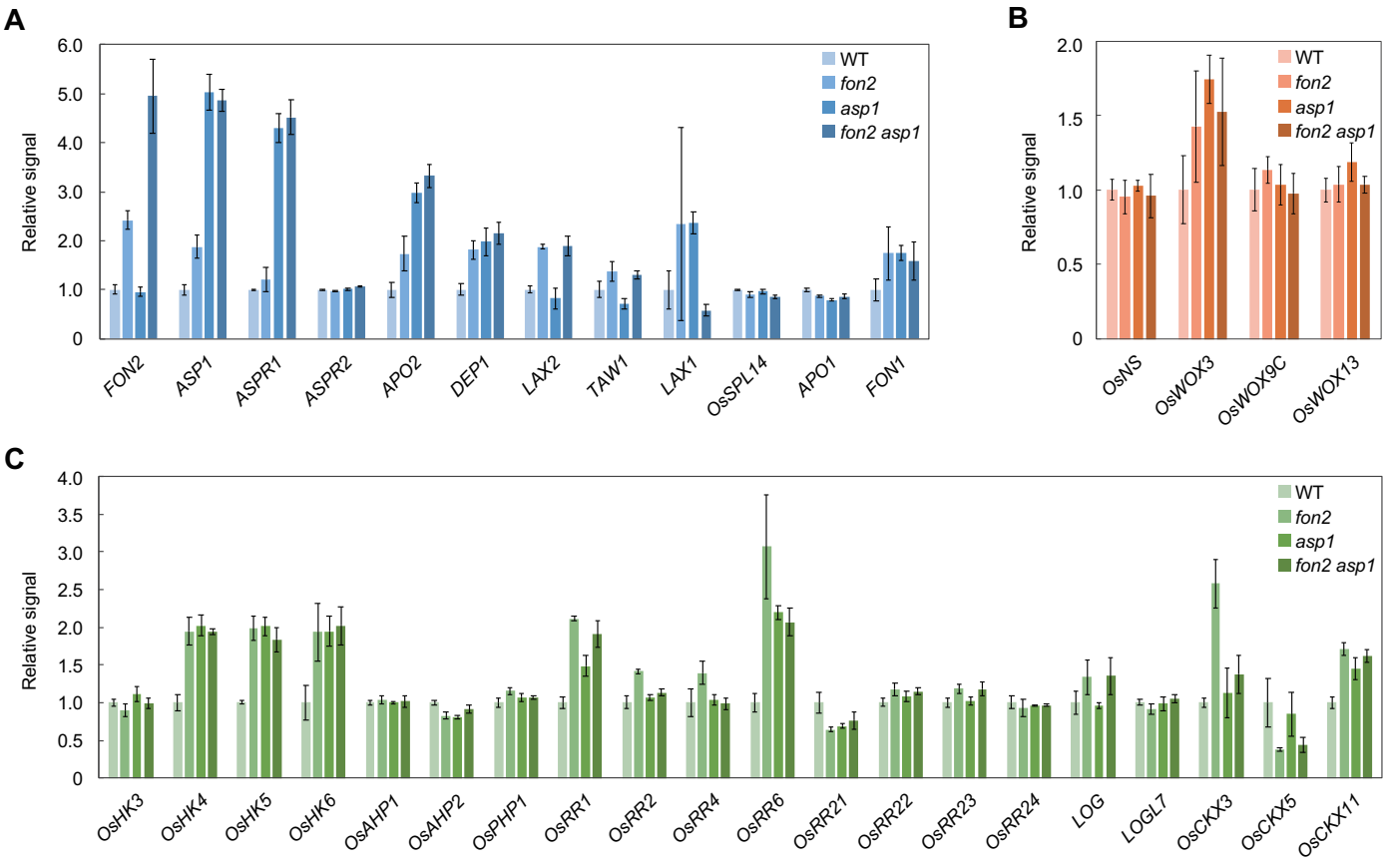


Figure II-14. Relative expression levels of genes from microarray data.
(A) Expression levels of genes reported to be associated with inflorescence architecture.
(B) Expression levels of genes in the *WOX* family.
(C) Expression levels of genes known to be related to cytokinin action.
Error bars indicate SE.

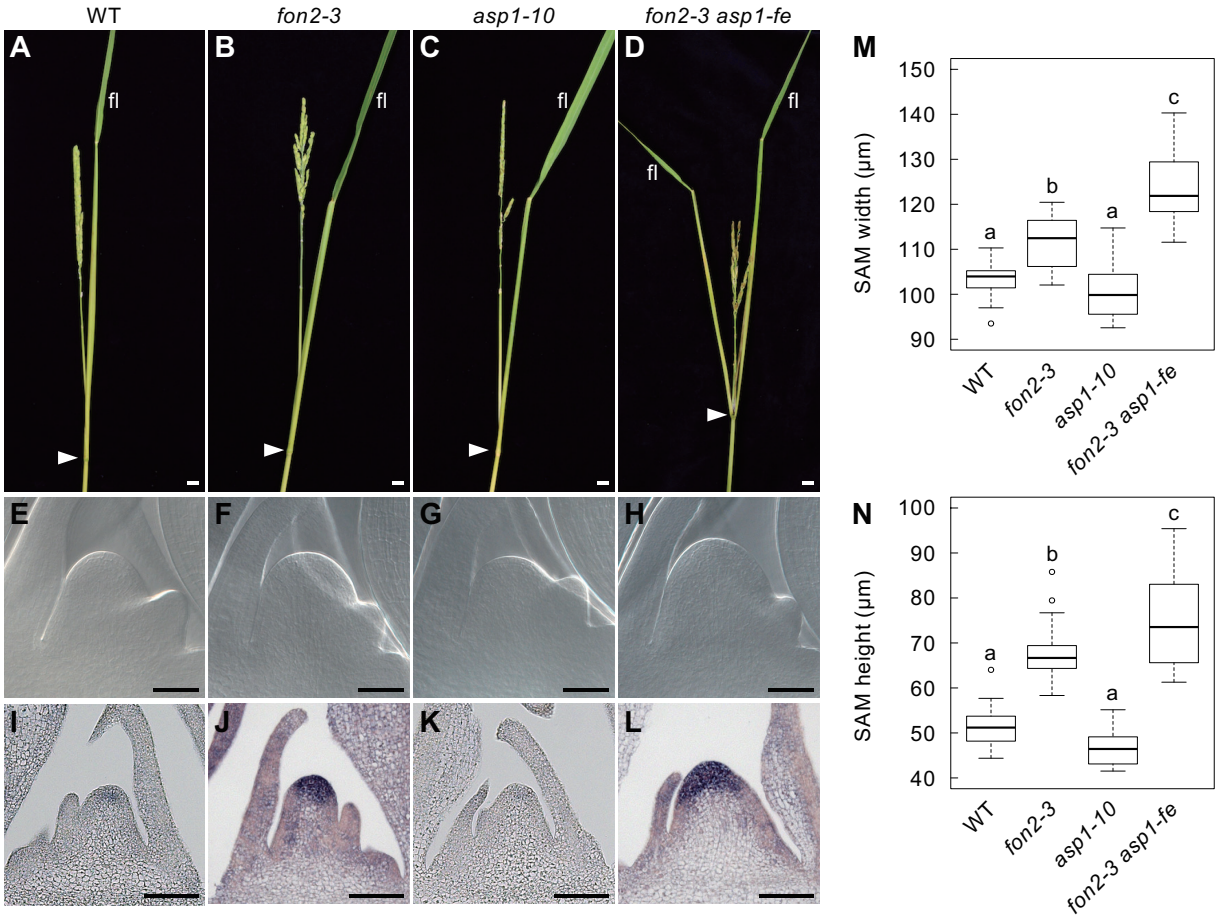


Figure II-15. Phenotypes of the vegetative SAM.
(A) to **(D)** Phyllotaxy phenotypes. Arrowheads indicate the nodes of the flag leaves.
(E) to **(H)** The SAM after treatment with a clearing agent.
(I) to **(L)** In situ localization of *FON2* transcripts.
(M) and **(N)** Quantification of SAM width **(M)** and height **(N)**. n = 20 (wild type), 18 (*fon2-3*), 16 (*asp1-10*), 20 (*fon2-3 asp1-fe*). Different letters (a, b, and c) indicate significant differences between samples ($P < 0.01$, Tukey's test).
fl, flag leaf. Bars = 1 cm in **(A)** to **(D)**; 50 µm in **(E)** to **(H)**; 100 µm in **(I)** to **(L)**.

Chapter III

Analysis of a rice *fickle spikelet1* mutant that displays an increase in flower and spikelet organ number

Introduction

Angiosperms generate diverse flowers whose morphology and size are variable. Grass flowers are formed in a unique inflorescence unit, called the spikelet. Each spikelet consists of a pair of glumes and one or more florets, the number of florets per spikelet varying among grass species. *Oryza sativa* (rice) generates a single floret in a spikelet, together with two rudimentary glumes and two sterile lemmas (reviewed in Tanaka et al., 2013, 2014; Hirano et al., 2014). The sterile lemma is specific to the genus *Oryza*, and is thought to be evolutionally derived from the lemma of two degenerated lateral florets (Arber, 1934; Yoshida et al., 2009). The floret consists of the lemma, palea, and floral organs such as lodicules, stamens and carpels.

Floral organ specification is explained by the ABC model, which is based on genetic analyses of *Arabidopsis thaliana* (Arabidopsis) and *Antirrhinum majus* (Coen and Meyerowitz, 1991). Molecular genetic studies of rice flower development have shown that the ABC model can largely be applied to rice flowers (reviewed in Hirano et al., 2014; Tanaka et al., 2014), although some modifications are required: for example, the *YABBY* gene *DROOPING LEAF*, but not the class C MADS-box gene, is required for carpel specification (Yamaguchi et al., 2004).

The number of floral organs is affected by mutations in genes related to regulation of stem cell maintenance in the floral meristem. In Arabidopsis, stem cell population in the floral meristem is stably maintained by the WUSCHEL–CLAVATA (WUS–CLV) feedback loop (Brand et al., 2000; Schoof et al., 2000; reviewed in Ha et al., 2010; Aichinger et al., 2012; Somssich et al., 2016). The CLV3 peptide negatively regulates stem cell proliferation via receptors, such as CLV1, and downstream signaling, whereas WUS, a homeodomain transcription factor, positively regulates stem cell proliferation. In the loss-of-function *clv* mutants, enlargement of the flower meristem due to the overproliferation of stem cells is observed and leads to an increase in floral organ number (Clark et al., 1993, 1995). In contrast, the loss of function of *WUS* results in the shortage of stem cells and a reduction in floral organ number (Laux et al., 1996).

In rice, *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2* negatively regulate stem cell proliferation in the floral meristem (Suzaki et al., 2004, 2006). *FON1* encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) orthologous to Arabidopsis CLV1, and *FON2* encodes a CLE peptide orthologous to CLV3. Overexpression analysis of *FON2* in *fon1* and genetic analysis using *fon1 fon2* double mutants indicate that FON2 acts as a ligand of FON1 (Suzaki et al., 2006). The loss of function of either *FON1* or *FON2* gives rise to an enlargement of the flower meristem, resulting in an increased number of floral organs, such as carpels and stamens, like *clv* mutants in Arabidopsis. In contrast to the increase in floral organ number, the number of organs specific to the spikelet (spikelet organs), such as the lemma and palea, is less affected in the *fon* mutants.

The number of spikelet organs and spikelet morphology are regulated by other genes, such as *EXTRA GLUME1* (*EG1*) (Li et al., 2009; Cai et al., 2014), *DEPRESSED PALEA1* (*DP1*) (Jin et al., 2011) and *TRIANGULAR HULL1* (*TH1*) (Li et al., 2012; Sato et al., 2014). In the *eg1* mutant, an extra glume similar to the lemma is generated outside the floret (Li et al., 2009). Palea growth is strongly inhibited in the *dp1* spikelet (Jin et al., 2011). By contrast, *TH1* is involved in the fine-tuning of lemma and palea morphology (Li et al., 2012; Sato et al., 2014).

In this chapter, in order to gain further insight into stem cell maintenance in rice, I tried to search new genes involved in stem cell regulation by using a genetic approach. I focused on a new spikelet mutant, *fickle spikelet1* (*fsp1*), displaying a unique feature related to a defect in stem cell maintenance. Like the *fon* mutants, *fsp1* showed an increase in floral organ number. Moreover, *fsp1* exhibited an increase in spikelet organ number, which is hardly observed in the *fon* mutants, raising the possibility that the gene responsible for the *fsp1* mutation is related to FON-independent stem cell maintenance. *fsp1* also had a defect in pollen development, often resulting in sterility. In addition, the phenotype of *fsp1* appears to be strongly affected by environmental conditions. Unexpectedly, I found that *fsp1* has a mutation in the essential amino acid in FON1. However, the *fsp1* phenotypes cannot be fully explained by the *fon1* mutation, raising the possibility that *fsp1* might have another mutation other than *fon1*.

Results

fsp1 displays an increase in both flower and spikelet organ number

The rice floret is composed of one lemma, one palea, two lodicules, six stamens and one pistil (Figures III-1A and III-1B). The lemma and palea are arranged in an alternate phyllotaxy and the two lodicules are located at the lemma side. Inside the two lodicules, six stamens surround one pistil (Figure III-1C).

The *fsp1* mutant exhibited pleiotropic abnormalities in the number of flower and spikelet organs. The *fsp1* mutation was inherited as a recessive trait, and I analyzed spikelet phenotypes in plants homozygous for *fsp1* by comparing them with those in wild type. In some *fsp1* spikelets, an extra organ, which is considered to be a lemma as inferred from its marginal structures, was formed instead of the palea (Figures III-1D and III-1L). I named this spikelet the "Double Lemma (L)-type". In the L-type spikelet, two ectopic organs, which I consider to be palea-like organs, were formed on the lateral side of the spikelet. Thus, both the lemma and palea were doubled. In addition, the number of floral organs was increased. In an extreme case, the L-type spikelets had four lodicules, thirteen stamens and three pistils (Figure III-1E). Thus, all organs constituting the floret were increased in number in the L-type spikelet.

In contrast to the L-type spikelet, some *fsp1* spikelets had a degenerated palea (Figures III-1F and III-1L). I named such spikelets "Degenerated Palea (P)-type". Despite the degeneration of the palea, the number of floral organs was higher in the P-type, as in the L-type. In a few cases, the *fsp1* spikelet produced an extra lemma-like

organ outside the floret (Extra Glume (G)-type) (Figures III-1G and III-1L). In G-type, a single lemma and palea and a pair of sterile lemmas were formed, as in wild type, but there were more floral organs, as in the cases of the L- and P-type spikelets. The fourth type was a spikelet in which no spikelet organs were affected, but floral organs were increased in number (Increased Floral Organ (F)-type) (Figure III-1L). Spikelets showing severe morphological abnormality were seldom observed in any type of *fsp1* spikelet.

All four types of the *fsp1* spikelets showed an increase in spikelet or flower organ number. Then, I counted the number of each organ in *fsp1* (Figure III-2). About 16.1% of the *fsp1* spikelets exhibited an increase in palea/lemma, 24.3% in lodicule, 22.9% in stamen, and 12.9% in pistil number.

The flower meristem was enlarged in *fsp1*

In order to determine the cause of the *fsp1* phenotype, I investigated developing spikelets at the early developmental stage by using scanning electron microscopy (SEM). In wild type, the spikelet in which the flower meristem initiated six stamen primordia was observed (Figure III-3A). In Figure III-3B, developing F-type (left) and G-type (right) *fsp1* spikelets were observed. The both type of spikelets had larger flower meristems as compared with the wild-type spikelet. The F-type spikelet initiated seven stamen primordia in the same whorl, which were surrounded by the normal lemma and palea. By contrast, in the G-type spikelet, the initiation pattern of the stamen primordia was somewhat disturbed, and an extra lemma-like organ primordium was produced

outside the palea.

In general, an increase in organ number is tightly associated with an enlargement of the meristem. Therefore, an increase in the number of flower organs observed in *fsp1* was probably caused by enlarged flower meristem, raising the possibility that the gene responsible for the *fsp1* phenotype is associated with meristem maintenance.

The expressivity of the *fsp1* phenotype was inconstant

I found that the expressivity of the *fsp1* mutant phenotype was variable. For example, all spikelets in a panicle exhibited the mutant phenotype in a plant, whereas, in another plant, only about 10% of spikelets in a panicle were affected (Figures III-4A and III-4B). In addition, the expressivity of the mutant phenotype was somewhat variable among panicles even within the same plant.

In addition, I found that the phenotype of the *fsp1* spikelet was enhanced when plants were cultivated in a growth chamber in winter. The frequency of spikelets showing the mutant phenotype was about 33%, in 413 spikelets examined, when plants were grown in the field in summer. By contrast, all spikelets except one exhibited the mutant phenotype when plants were grown in a growth chamber in winter (n = 147) (Figures III-4C and III-4D). In addition, the frequencies of each type of *fsp1* spikelet differed in the plants grown in the two environments. Thus, spikelet development seems to be affected by environmental conditions in the *fsp1* mutant, although it is not clear whether the variable effect on spikelet phenotype is due to the difference between the growth conditions related to the field and a growth chamber or to that between some

factor(s) related to the season, such as day length.

The *fsp1* spikelet showed other defects when plants were cultivated in a growth chamber in winter. Almost all *fsp1* spikelets produced pale yellow or white anthers in these plants (Figures III-1H and III-1I), whereas they produced normal vivid yellow anthers in plants grown in summer (Figure III-1E). Pale yellow or white anthers imply that pollen development is defective, and abnormal pollen generally causes sterility. Indeed, fertility in the *fsp1* mutant was greatly reduced when plants were grown in a growth chamber in winter. A reduction in fertility was also observed in some panicles in the plants grown in summer. Some *fsp1* panicles exhibited fertility comparable to the wild-type panicles, whereas others showed high sterility, as inferred from their upright rather than bent appearance even after maturation of the seeds (Figures III-1J and III-1K). Notably, these distinct panicle shapes were observed in the same plant. These results suggest that the effect of the *fsp1* mutation on pollen development also varies among panicles, as with types.

fsp1 has a mutation in the *FON1* gene

To identify the gene responsible for the *fsp1* mutation, I carried out map-based cloning. Using 40 F2 plants produced by a cross between *fsp1* and Kasalath, an *indica* strain, I mapped the *fsp1* locus to the ~1.3-Mb region between two markers, 883M and R1608, on chromosome 6 (Figure III-5A). I searched a putative gene, whose mutation is related to the *fsp1* phenotype, and found that the *FON1* gene is located in this region. *FON1* is known to be involved in stem cell maintenance, and its loss of function causes an

increase in flower organ number due to an enlargement of the flower meristem (Suzaki et al., 2004). Thus, it raises the possibility that the *fsp1* phenotype is caused by a *fon1* mutation.

Then, I sequenced *FON1* in *fsp1* and found a nucleotide substitution in exon 1, which results in an amino acid substitution (R417C; Figure III-5B). Arg417 of FON1 is located in the LRR domain, an extracellular receptor domain, and is highly conserved among other LRR-RLKs. It is therefore likely that FON1 in *fsp1* is not fully functional, and that the *fsp1* defects are at least partly caused by the *fon1* mutation.

Discussion

The *fsp1* phenotypes cannot be fully explained by the *fon1* mutation

It has been reported that the number of floral organs is increased in the *fon1* and *fon2* mutants (Suzaki et al., 2004, 2006). Like *clavata* mutants in Arabidopsis (Clark et al., 1993, 1995), excessive stem cell accumulation causes an increase in the number of inner floral organs such as carpels in the *fon* mutants, whereas spikelet organs are less affected.

Sequencing analysis revealed that *fsp1* has a mutation in *FON1*, consistent with the *fsp1* phenotypes of increased flower organ number and the enlarged flower meristem. The L-type spikelet is sometimes observed in the *fon1* mutant (Suzaki et al., 2004). However, *fsp1*, unlike *fon1*, showed an increase in spikelet organ number to a similar extent as that in flower organ number (Figure III-2; Suzaki et al., 2004). In addition, the P-type and G-type spikelets are rarely observed in the *fon1* mutants, and a defect in pollen development is not either. These results raise the possibility that *fsp1* has a mutation that could cause the *fsp1* phenotypes other than *fon1*. Further supporting evidence of this possibility was obtained through the analysis of the genotypes and spikelet phenotypes of F3 population for map-based cloning. Future isolation of the second site mutation will provide cues for interpretation of the *fsp1* phenotypes distinct from those of the known *fon1* mutant, and further insights into meristem maintenance in rice.

In the *dp1* mutant, palea development is severely compromised, and the P-type

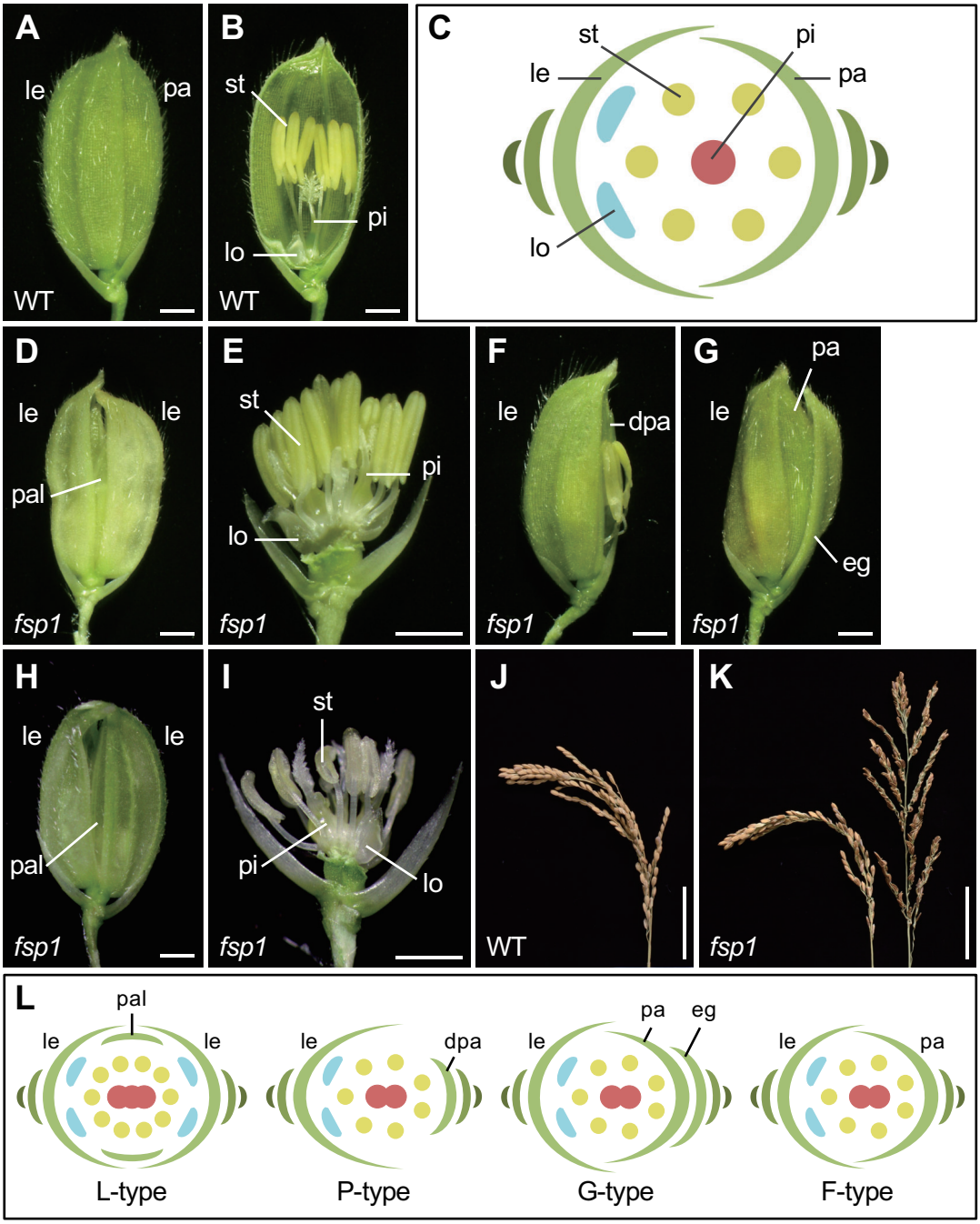
fsp1 spikelet is similar to the *dp1* spikelet (Jin et al., 2011). In addition, a double lemma phenotype (twin-flower), which is similar to the L-type *fsp1* spikelet, is also observed in the *dp1* spikelet (Jin et al., 2011). The G-type *fsp1* spikelets resemble those of the *eg1* and *eg2* mutants, which generate extra lemma-like organs outside the floret (Li et al., 2009; Cai et al., 2014). It will be of great interest to examine the interaction between *FON* and these genes, *DP1* and *EG*.

fsp1 has a mutation in the essential residue of FON1

The *fon1* mutation in *fsp1* results in an amino acid substitution from Arg to Cys at position 417. FON1 belongs to the LRR-RLK subfamily XI, in which an Arabidopsis CLE receptor, TDIF RECEPTOR (TDR), is included (Liu et al., 2017). TDR regulates vascular stem cell fate through the perception of tracheary element differentiation inhibitory factor (TDIF) (Hirakawa et al., 2008), and its detailed crystal structure was reported recently (Morita et al., 2016; Zhang et al., 2016). Arg417 of FON1 corresponds to Arg421 of TDR. Arg421 of TDR hydrogen bonds with the Asn12 of TDIF, and R421A mutation slightly reduced the TDIF-binding activity (Morita et al., 2016; Zhang et al., 2016). It is therefore possible that Arg417 of FON1 has a critical role for the physical interaction with FON2, a putative ligand of FON1.

A characteristic feature of the *fsp1* mutant is that the expressivity of the mutant phenotype was variable among plants and among panicles within the same plant. This suggests that the mutation responsible for the *fsp1* phenotype is highly susceptible to a slight change in the environment, consistent with the *fon1* mutation in *fsp1*. The R417C

mutation of FON1 in *fsp1* might be incomplete loss-of-function, considered from the fact that the R421A mutation of TDR does not fully disrupt the interaction between TDR and TDIF (Morita et al., 2016). Thus, the interaction between FON1 and FON2 in *fsp1* might be incomplete and susceptible to the environmental condition.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G-type
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pa
F-type

Figure III-1. Phenotypes of the spikelet and inflorescence in *fsp1* and wild type.
(A) and **(B)** Wild-type spikelets. Part of the lemma and palea is removed in **(B)**.
(C) Schematic representation of a wild-type spikelet.
(D) to **(G)** Spikelet types of the *fsp1* mutant grown in the field in summer. L-type (**(D)** and **(E)**), P-type **(F)**, and G-type **(G)**. The lemmas and palea-like organs are removed from the spikelet **(D)** in **(E)**.
(H) and **(I)** An L-type spikelet of the *fsp1* mutant grown in a growth chamber in winter. The lemmas and palea-like organs are removed from the spikelet **(H)** in **(I)**.
(J) and **(K)** Mature panicles of plants grown in the field in summer. Wild type **(J)** and *fsp1* **(K)**. The left panicle in **(K)** shows fertility similar to that in the wild-type panicle **(J)**, whereas the right panicle, standing upright, shows that fertility is severely compromised.
(L) Schematic representation of the four types of *fsp1* spikelet.
le, lemma; pa, palea; lo, lodicule; st, stamen; pi, pistil; pal, palea-like organ; dpa, degenerated palea; eg, extra glume. Bars = 1 mm in **(A)**, **(B)** and **(D)** to **(I)**; 5 cm in **(J)** and **(K)**.

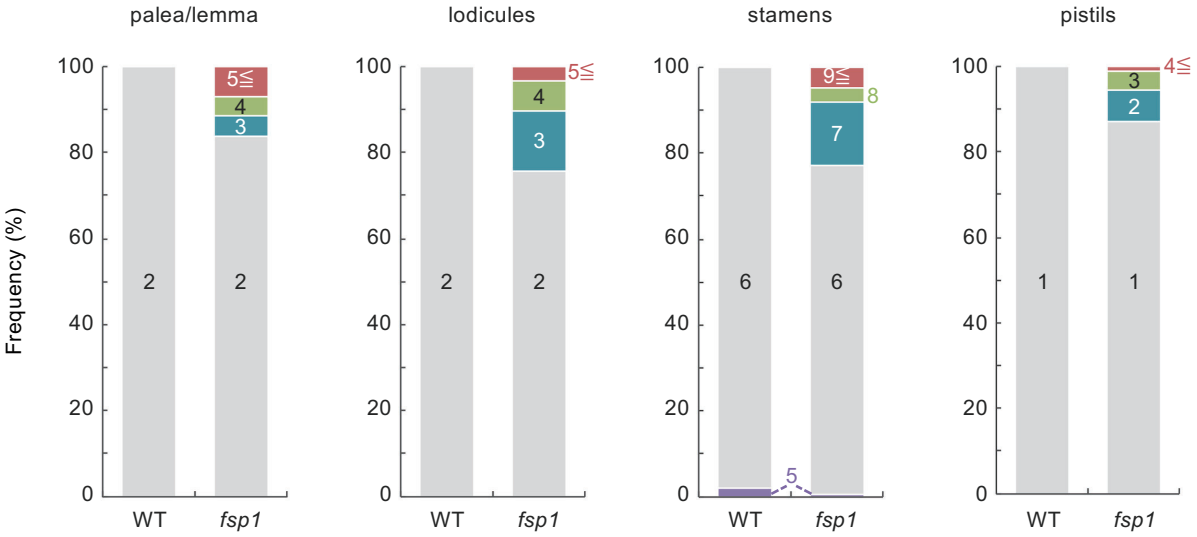

Figure III-2. Number of flower organs in *fsp1* and wild type.
For wild type and *fsp1*, 50 and 411 flowers were examined, respectively.

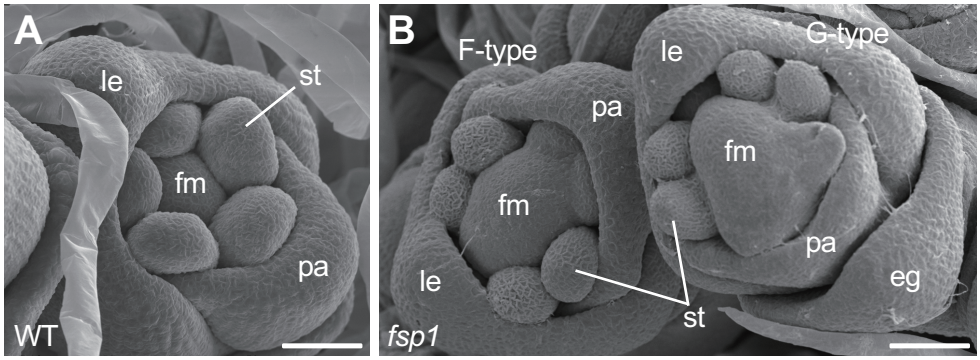

Figure III-3. SEM images of developing spikelets.
(A) and **(B)** Developing spikelets at the stage of stamen initiation. Wild type **(A)** and *fsp1* **(B)**.
eg, extra glume; fm, flower meristem; le, lemma; pa, palea; st, stamen.
Bars = 50 μm.

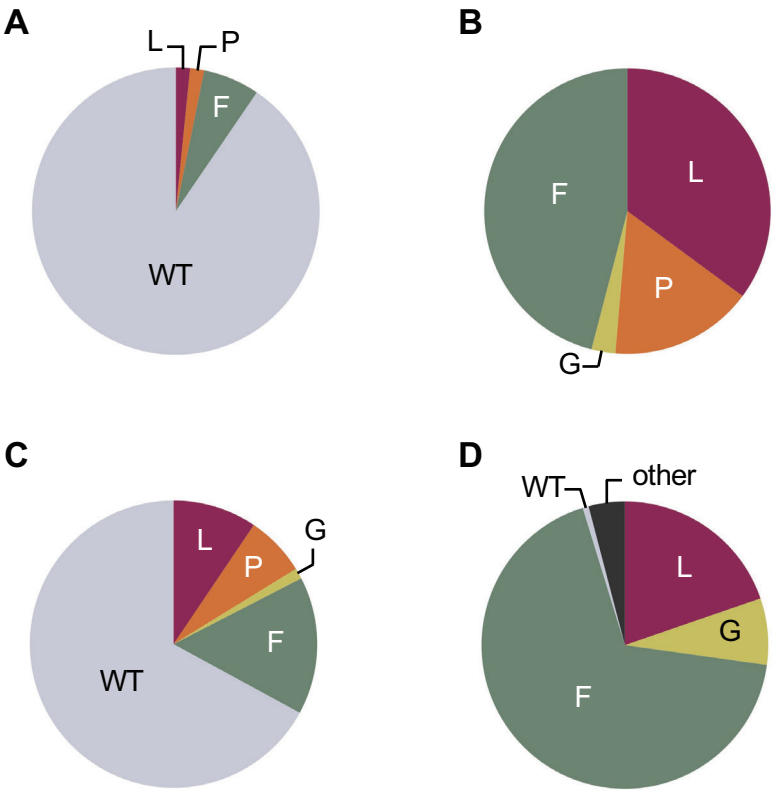

Figure III-4. Frequency of spikelet types in the *fsp1* mutant.
(A) and **(B)** The proportion of each type of *fsp1* spikelet in a panicle in two different plants, which were grown in the field. n = 63 spikelets in **(A)** and 37 in **(B)**.
(C) The proportion of each type of *fsp1* spikelet in nine panicles in plants grown in the field in summer. n = 413.
(D) The proportion of each type of *fsp1* spikelet in four panicles in plants grown in a growth chamber in winter. n = 147.
F, F-type; G, G-type; L, L-type; other, unclassified spikelet type; P, P-type; WT, wild type.

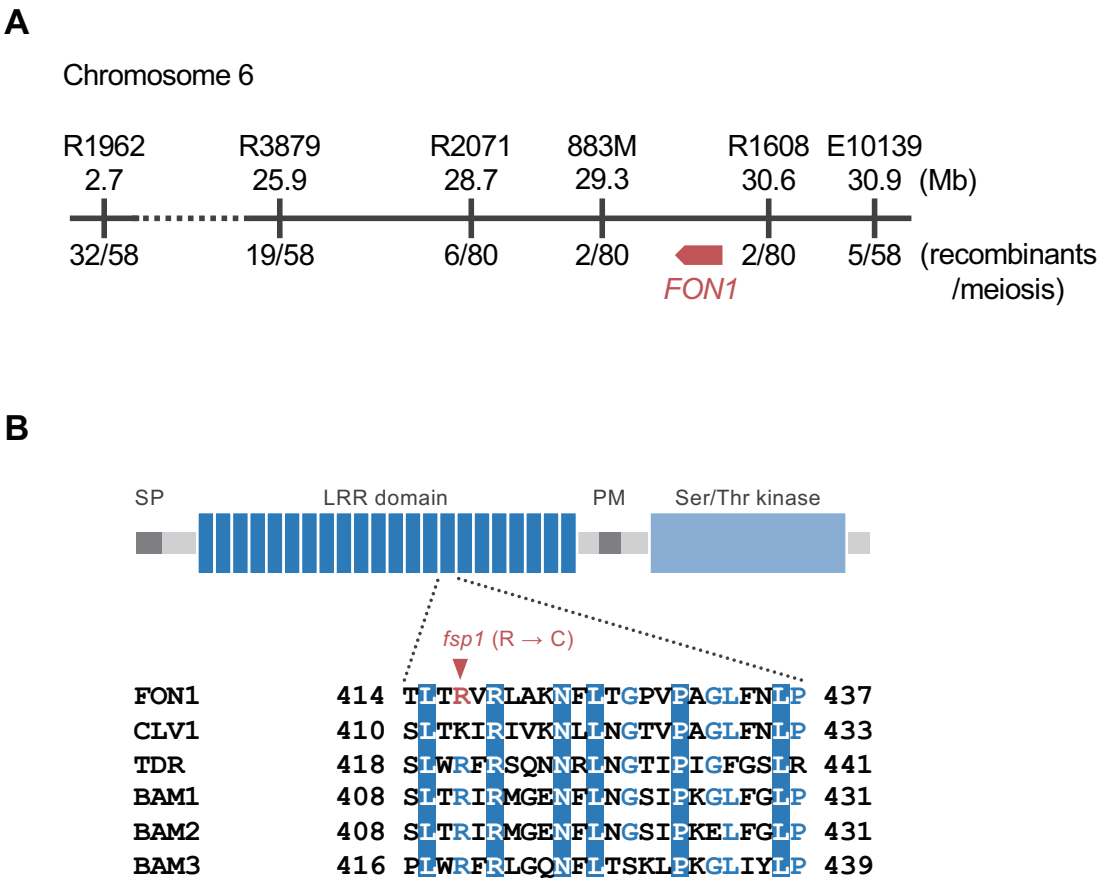

Figure III-5. Map-based cloning and the *fon1* mutation in *fsp1*.
(A) Genetic map of the *fsp1* locus.
(B) Protein structure of FON1 and amino acid alignment of the LRR15 repeats. *fsp1* has a nucleotide substitution in exon 1 of *FON1* which is predicted to result in an amino acid substitution (arrowhead) in the LRR15 repeat.
SP, signal peptide; TM, transmembrane domain.

Chapter IV

Materials and methods

Plant materials

Oryza sativa L. ssp. *japonica* variety Taichung 65 (T65) was used as the wild type (WT). The *fon2-3*, *asp1-1*, *fon1-5*, and *tab1-1* mutants have been reported previously (Yamaki et al., 2005; Suzaki et al., 2006, 2008; Yoshida et al., 2012; Tanaka et al., 2015). *fon2-3* was isolated from M2 population obtained by *N*-methyl-*N*-nitrosourea (MNU) treatment of T65 and has a nucleotide change, leading to an amino acid substitution from Val to Met at the third position of the mature FON2 peptide (Yamaki et al., 2005; Suzaki et al., 2006). The phenotype of *fon2-3* is almost as severe as those of complete loss-of-function mutants (Suzaki et al., 2006). *asp1-1* is a loss-of-function null mutant and has a frame shift mutation, leading to a premature stop codon in the first β-propeller domain (Yoshida et al., 2012). *fon1-5* is a loss-of-function null mutant having a nonsense mutation in the LRR domain (Suzaki et al., 2008). *tab1-1* has a nucleotide substitution at the splice site of the first intron, leading to premature termination of the protein (Tanaka et al., 2015). 1B-280 (*fon2-3 asp1-fe*) was identified in the genetic screen described by Yasui et al. (2017). *asp1-10* (TCM697) was identified as a mutant showing acute curvature of branches in rice genetic resources (Oryzabase; https://shigen.nig.ac.jp/rice/oryzabase/) obtained by MNU treatment of T65 and subsequently confirmed to contain a mutation in *ASP1* by direct sequencing.

fsp1 (CM883) was identified as a mutant showing pleiotropic flower phenotypes,

such as an increase in spikelet and flower organ number, in genetic resources (Oryzabase) obtained by MNU treatment of Kinmaze.

RT-PCR analysis

For RT-PCR analysis of *ASP1* mRNA, total RNA was extracted from T65, *fon2-3*, and *fon2-3 asp1-fe* using TRIsure (BIOLINE). After DNase I treatment, first-strand cDNA was synthesized from 150 ng of total RNA using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and the oligo(dT)15 primer. PCR was then performed with primers 5'-CGAAGCTCCAGTATATTCTG-3', 5'-TTGCAACTAGAGAAGCTACG-3', and 5'-CTTCCTGAAACCATTGTATG-3'.

Plasmid construction and transformation

To construct *gASP1-GFP*, a 5-kb genomic fragment of the *ASP1* upstream region was amplified with the primers 5'-CACCCTCACACGGCCGATGGTACG-3' and 5'-GGCTCCGCCGATCCCAGCCT-3', and the PCR product was cloned into a pENTR/D-TOPO vector (Thermo Fisher Scientific). The resulting plasmid, designated pENTR-pASP1, was digested with *Sac*I (in the 5' UTR region of *ASP1*), blunted, and then digested with *Asc*I (just upstream of the *att*L2 site in the original vector) for subsequent ligation. Next, an approximate 7.8-kb genomic fragment containing the entire *ASP1* gene (25 exons and 24 introns) except the stop codon was amplified with the primers 5'-AGCTCTGGGTTTATTAATTTTTTTTGG-3' and 5'-AAAAGGCGCGCCCGACTTCTGGTTTGTTAGCTG-3' (the *Asc*I site is underlined).

The blunt-end PCR product, digested with *Asc*I, was ligated with pENTR-pASP1. The resulting plasmid was confirmed to carry an approximate 12.6-kb genomic sequence comprising the 5-kb upstream region and the entire *ASP1* gene except for the stop codon by direct sequencing. The 12.6-kb fragment was inserted into pGWB4, a binary vector carrying an *sGFP* gene (Nakagawa et al., 2007), by the LR recombination (Thermo Fisher Scientific) to produce *gASP1-GFP*.

To prepare a construct for knockout of the *ASP1* gene using the CRISPR-Cas9 system, a 20-bp sequence in the second exon was selected as the gRNA target site (Supplemental Figure 2). The target sequence was then inserted into an all-in-one vector carrying the Cas9/gRNA expression cassette, derived from pU6gRNA-oligo and pZH_OsU3gYSA_MMCas9, according to the method of Mikami et al. (2015).

The constructs were introduced into *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) EHA101 strain and transformed into scutellum-derived calli according to the method of Hiei et al. (1994). *fon2-3 asp1-fe*, *asp1-10*, and *fon2-3* were used as hosts for complementation, observation of ASP1-GFP localization, and knockout of *ASP1*, respectively.

In situ **hybridization**

The two probes for detection of *ASP1* transcripts were prepared as follows. Partial cDNA fragments were amplified with the primer pair 5'-AATCGGGTCCAAAAAAACCAAAAGC-3' and 5'-TCCAGCTCTCAAAGCCGAACT-3' for probe1 (located in the 5'UTR), and 5'-

CATACTTACAACAACAGATTGTGACGG-3’ and 5’-GCTGGTTGCCACATTTGAGG-3’ for probe2 (located in the region between the two β-propeller domains). The PCR products were then cloned into a pCRII vector (Thermo Fisher Scientific). The resulting plasmids were linearized and transcribed with Sp6 and T7 RNA polymerase for probe1 and probe2, respectively, by using a DIG RNA Labeling Kit (Roche).

The *FON2* probe was transcribed as described previously (Suzaki et al., 2006), and then treated by alkaline hydrolysis. The *OSH1* and *TAB1* probes were prepared as described by Yasui et al. (2017) and Tanaka et al. (2015), respectively.

Plant tissues were fixed and dehydrated as described by Toriba and Hirano (2018). Next, the samples were embedded in Paraplast Plus (McCormick), and sectioned at a thickness of 10 μm using a microtome. *In situ* hybridization and immunological detection were carried out as described in Toriba and Hirano (2018).

Imaging and measurement of meristems

To observe ASP1-GFP fluorescence, shoot apices of transgenic plants (T0 generation) were embedded in 5% (w/v) agar and sliced into 30-μm sections using a vibratome. GFP fluorescence was observed with a confocal microscope (LSM510, Zeiss).

Inflorescence and flower meristems were observed by SEM as described by Tanaka et al. (2015). To measure SAM size, shoot apices of 8-week-old plants 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 SAM size was measured just above the P1 primordium by using ImageJ.

Microarray experiments

Inflorescence meristems at the In1~In2 stage (Itoh et al., 2005) including 1~2 bracts of each strain were pooled (nine per pool) and used for RNA isolation. Total RNA was extracted with TRIsure (BIOLINE) and treated with DNaseI. Microarray analysis was carried out using the Rice (US) gene 1.0 ST array (Thermo Fisher Scientific) as described by Yasui et al. (2018) with three biological replicates per assay. The resulting data were analyzed by using R software and the Bioconductor package limma (Ritchie et al., 2015). GO enrichment analysis was performed with agriGO v2.0 (Tian et al., 2017; http://systemsbiology.cau.edu.cn/agriGOv2/), and the results were visualized by REVIGO (Supek et al., 2011; http://revigo.irb.hr/) and the R package ggplot2 (Wickham, 2016).

Map-based cloning

The *fsp1* locus was mapped by using 40 F2 plants produced by a cross between *fsp1* and Kasalath (ssp. *indica*). The markers used for genetic mapping as follows: R1962 (5'-GCTTGGATTATGACATTTAG-3', 5'-TGAAGCAAGGAACAAACA-3'; *Eco*RI digestion), R3879 (5'-CACTAATCAAGCCACTTCGG-3', 5'-CGAAACTTGTTTTCCTTCCC-3'), R2071 (5'-TAACTCATTGCCTTTTGCC-3', 5'-CTTCCATTTCCTTGATTCAG-3'; *Sac*I digestion), 883M (5'-

GCAGTGCGTTTATTAAATAAGG-3', 5'-TGGAGTACAAGCCAGTACTACA-3';

*Hin*dIII digestion), R1608 (5'- TTAAGGAGTTGTGCTGTTGC-3', 5'-

GGCATAAAGACAATTAGTGC-3'), E10139 (5'-AATCTGGAGATCCTGGCTTG-3',

5'-TCTGGATGAAAGCCTGAAAC-3'; *Eco*RI digestion).

Chapter V

Concluding remarks

My aim in this thesis was to uncover the molecular mechanisms underlying stem cell maintenance in the shoot apical meristem using a model monocot, *Oryza sativa* (rice). In rice, FON signaling pathway, corresponding to CLV signaling in *Arabidopsis thaliana* (Arabidopsis), has been considered to regulate stem cell maintenance (Suzaki et al., 2004, 2006). In this study, I focused on two novel mutants: 1B-280 and *fsp1*, which exhibit enhanced *fon* phenotypes and partially similar phenotypes to *fon*, respectively. As a result, the analysis of 1B-280 revealed that the *ASP1* gene, encoding a TPL-related transcription corepressor, regulates stem cell regulation in concert with FON signaling. The analysis of *fsp1* showed that it has a mutation in the *FON1* gene; however, unlike the conventional *fon1* mutant, *fsp1* displayed an increase not only in spikelet organ number but also in flower organ number, raising the possibility that *fsp1* has another mutation other than *fon1*, which might be related to FON.

I revealed that the *asp1* mutation was responsible for the enhanced *fon* phenotype of 1B-280, which prompted me to analyze the relationship between *ASP1* and FON signaling. The loss-of-function *asp1* mutations synergistically enhanced the *fon2* phenotype: *fon2 asp1* showed a marked increase in stem cell number as compared with each single mutant, resulting in massive enlargement of the meristem. While FON signaling seems to provide negative fine-tuning, similar to CLV signaling in Arabidopsis, ASP1 appears to provide moderate suppression of multiple genes involved

in stem cell maintenance; thus, loss of function of both the *FON2* and *ASP1* genes results in marked overproliferation of stem cells. To my knowledge, this is the first direct evidence that *TPL*-like genes are involved in stem cell maintenance in the above-ground meristems. Future identification of genes directly regulated by ASP1 and its paralogs, ASPR1 and ASPR2, will help elucidate the mechanism of stem cell maintenance by TPL-like corepressors.

In Arabidopsis, *WUS* positively regulates stem cell proliferation downstream of CLV signaling. Surprisingly, however, the *WUS* ortholog *TAB1* was found to be not involved in the extreme enlargement of the inflorescence meristem in *fon2 asp1*; rather, loss of *TAB1* led to an increase in meristem size in wild type. In rice, therefore, *TAB1* might be associated with unknown functions involved in more complex genetic and protein networks, rather than in a WUS–CLV-like pathway. Future analysis of the difference between *TAB1* and *WUS* might reveal new aspects of the function of the *WUS*-like *WOX* gene in stem cell maintenance.

The Arabidopsis WUS–CLV feedback loop is a fundamental mechanism underlying the stem cell maintenance and is conserved in diverse plant species. In this study, I focused on a rice CLV-like signaling, FON signaling. The findings of this study will provide the key to further understanding the molecular mechanisms controlling stem cell maintenance not only in rice but also in other plants including Arabidopsis.

References

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

Arber, A. (1934). The gramineae: a study of cereal, bamboo, and grasses. (Cambridge: University Press).

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., and Matsuoka, M. (2005). Cytokinin oxidase regulates rice grain production. Science **309:** 741-745.

Betsuyaku, S., Takahashi, F., Kinoshita, A., Miwa, H., Shinozaki, K., Fukuda, H., and Sawa, S. (2011). Mitogen-activated protein kinase regulated by the CLAVATA receptors contributes to shoot apical meristem homeostasis. Plant Cell Physiol. **52:** 14-29.

Bleckmann, A., Weidtkamp-Peters, S., Seidel, C.A., and Simon, R. (2010). Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane. Plant Physiol. **152:** 166-176.

Bommert, P., Je, B.I., Goldshmidt, A., and Jackson, D. (2013). The maize Gα gene *COMPACT PLANT2* functions in CLAVATA signalling to control shoot meristem size. Nature **502:** 555-558.

Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S., and Werr, W. (2005). *thick tassel dwarf1* encodes a putative maize ortholog of the *Arabidopsis CLAVATA1* leucine-rich repeat receptor-like kinase. Development **132:** 1235-1245.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. Science **289:** 617-619.

Busch, W., Miotk, A., Ariel, F.D., Zhao, Z., Forner, J., Daum, G., Suzaki, T., Schuster, C., Schultheiss, S.J., Leibfried, A., Haubeiß, S., Ha, N., Chan, R.L., and Lohmann, J.U. (2010). Transcriptional control of a plant stem cell niche. Dev. Cell **18:** 841-853.

Cai, Q., Yuan, Z., Chen, M., Yin, C., Luo, Z., Zhao, X., Liang, W., Hu, J., and Zhang, D. (2014). Jasmonic acid regulates spikelet development in rice. Nat.

Commun. **5:** 3476.

Causier, B., Ashworth, M., Guo, W., and Davies, B. (2012). The TOPLESS interactome: a framework for gene repression in Arabidopsis. Plant Physiol. **158:** 423-438.

Chickarmane, V.S., Gordon, S.P., Tarr, P.T., Heisler, M.G., and Meyerowitz, E.M. (2012). Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci., USA **109:** 4002-4007.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. Development **119:** 397-418.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. Development **121:** 2057-2067.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89:** 575-585.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature **353:** 31-37.

Daum, G., Medzihradszky, A., Suzaki, T., and Lohmann, J.U. (2014). A mechanistic framework for noncell autonomous stem cell induction in *Arabidopsis*. Proc. Natl. Acad. Sci., USA **111:** 14619-14624.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. Science **283:** 1911-1914.

Gallavotti, A., Long, J.A., Stanfield, S., Yang, X., Jackson, D., Vollbrecht, E., and Schmidt, R.J. (2010). The control of axillary meristem fate in the maize ramosa pathway. Development **137:** 2849-2856.

Gordon, S.P., Chickarmane, V.S., Ohno, C., and Meyerowitz, E.M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci., USA **106:** 16529-16534.

Ha, C.M., Jun, J.H., and Fletcher, J.C. (2010). Shoot apical meristem form and function. Curr. Top. Dev. Biol. **91:** 103-140.

Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann,

M., and Laux, T. (2004). Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development **131:** 657-668.

Hiei, Y., Ohta, S., Komari, T., and 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.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., and Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. Proc. Natl. Acad. Sci., USA **105:** 15208-15213.

Hirano, H.-Y., Tanaka, W., and Toriba, T. (2014). Grass flower development. In Flower development - Methods and protocols, J.L. Riechmann and F. Wellmer, eds (New York: Springer), pp. 57-84.

Hu, C., Zhu, Y., Cui, Y., Cheng, K., Liang, W., Wei, Z., Zhu, M., Yin, H., Zeng, L., Xiao, Y., Lv, M., Yi, J., Hou, S., He, K., Li, J., and Gou, X. (2018). A group of receptor kinases are essential for CLAVATA signalling to maintain stem cell homeostasis. Nat. Plants **4:** 205-211.

Huang, X., Qian, Q., Liu, Z., Sun, H., He, S., Luo, D., Xia, G., Chu, C., Li, J., and Fu, X. (2009). Natural variation at the *DEP1* locus enhances grain yield in rice. Nat. Genet. **41:** 494-497.

Ikeda, K., Ito, M., Nagasawa, N., Kyozuka, J., and Nagato, Y. (2007). Rice *ABERRANT PANICLE ORGANIZATION 1*, encoding an F-box protein, regulates meristem fate. Plant J. **51:** 1030-1040.

Ikeda, M., Mitsuda, N., and Ohme-Takagi, M. (2009). *Arabidopsis* WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. Plant Cell **21:** 3493-3505.

Ikeda-Kawakatsu, K., Maekawa, M., Izawa, T., Itoh, J.-I., and Nagato, Y. (2012). *ABERRANT PANICLE ORGANIZATION 2/RFL*, the rice ortholog of Arabidopsis *LEAFY*, suppresses the transition from inflorescence meristem to floral meristem through interaction with *APO1*. Plant J. **69:** 168-180.

Ishida, T., Tabata, R., Yamada, M., Aida, M., Mitsumasu, K., Fujiwara, M., Yamaguchi, K., Shigenobu, S., Higuchi, M., Tsuji, H., Shimamoto, K., Hasebe, M., Fukuda, H., and Sawa, S. (2014). Heterotrimeric G proteins control

stem cell proliferation through CLAVATA signaling in *Arabidopsis*. EMBO rep. **15:** 1202-1209.

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

Jackson, D., and Hake, S. (1999). Control of phyllotaxy in maize by the *abphyl1* gene. Development **126:** 315-323.

Je, B.I., Xu, F., Wu, Q., Liu, L., Meeley, R., Gallagher, J.P., Corcilius, L., Payne, R.J., Bartlett, M.E., and Jackson, D. (2018). The CLAVATA receptor FASCIATED EAR2 responds to distinct CLE peptides by signaling through two downstream effectors. eLife **7:** e35673.

Je, B.I., Gruel, J., Lee, Y.K., Bommert, P., Arevalo, E.D., Eveland, A.L., Wu, Q., Goldshmidt, A., Meeley, R., Bartlett, M., Komatsu, M., Sakai, H., Jönsson, H., and Jackson, D. (2016). Signaling from maize organ primordia via FASCIATED EAR3 regulates stem cell proliferation and yield traits. Nat. Genet. **48:** 785-791.

Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the *CLAVATA1* receptor-like kinase. Plant Cell **11:** 1925-1934.

Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., and Li, J. (2010). Regulation of *OsSPL14* by OsmiR156 defines ideal plant architecture in rice. Nat. Genet. **42:** 541-544.

Jin, Y., Luo, Q., Tong, H., Wang, A., Cheng, Z., Tang, J., Li, D., Zhao, X., Li, X., Wan, J., Jiao, Y., Chu, C., and Zhu, L. (2011). An AT-hook gene is required for palea formation and floral organ number control in rice. Dev. Biol. **359:** 277-288.

Kayes, J.M., and Clark, S.E. (1998). *CLAVATA2*, a regulator of meristem and organ development in *Arabidopsis*. Development **125:** 3843-3851.

Kieber, J.J., and Schaller, G.E. (2018). Cytokinin signaling in plant development. Development **145**.

Kieffer, M., Stern, Y., Cook, H., Clerici, E., Maulbetsch, C., Laux, T., and Davies, B. (2006). Analysis of the transcription factor WUSCHEL and its functional homologue in *Antirrhinum* reveals a potential mechanism for their roles in meristem maintenance. Plant Cell **18:** 560-573.

Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H., and Sawa, S. (2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in *Arabidopsis*. Development **137:** 3911-3920.

Komatsu, K., Maekawa, M., Ujiie, S., Satake, Y., Furutani, I., Okamoto, H., Shimamoto, K., and Kyozuka, J. (2003). *LAX* and *SPA*: major regulators of shoot branching in rice. Proc. Natl. Acad. Sci., USA **100:** 11765-11770.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H., and Sakagami, Y. (2006). A plant peptide encoded by *CLV3* identified by in situ MALDI-TOF MS analysis. Science **313:** 845-848.

Krogan, N.T., Hogan, K., and Long, J.A. (2012). APETALA2 negatively regulates multiple floral organ identity genes in *Arabidopsis* by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. Development **139:** 4180-4190.

Laux, T., Mayer, K.F.X., Berger, J., and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. Development **122:** 87-96.

Leibfried, A., To, J.P.C., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438:** 1172-1175.

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

Li, H., Xue, D., Gao, Z., Yan, M., Xu, W., Xing, Z., Huang, D., Qian, Q., and Xue, Y. (2009). A putative lipase gene *EXTRA GLUME1* regulates both empty-glume fate and spikelet development in rice. Plant J. **57:** 593-605.

Li, X., Sun, L., Tan, L., Liu, F., Zhu, Z., Fu, Y., Sun, X., Sun, X., Xie, D., and Sun, C. (2012). *TH1*, a DUF640 domain-like gene controls lemma and palea development in rice. Plant Mol. Biol. **78:** 351-359.

Liu, P.-L., Du, L., Huang, Y., Gao, S.-M., and Yu, M. (2017). Origin and diversification of leucine-rich repeat receptor-like protein kinase (*LRR-RLK*) genes in plants. BMC Evol. Biol. **17:** 47.

Liu, X., Galli, M., Camehl, I., and Gallavotti, A. (2019). RAMOSA1 ENHANCER LOCUS2-mediated transcriptional repression regulates vegetative and reproductive architecture. Plant Physiol. **179:** 348-363.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in *Arabidopsis*. Science **312:** 1520-1523.

Long, J.A., Woody, S., Poethig, S., Meyerowitz, E.M., and Barton, M.K. (2002). Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. Development **129:** 2797-2806.

Lu, Z., Shao, G., Xiong, J., Jiao, Y., Wang, J., Liu, G., Meng, X., Liang, Y., Xiong, G., Wang, Y., and Li, J. (2015). *MONOCULM 3*, an ortholog of *WUSCHEL* in rice, is required for tiller bud formation. J. Genet. Genomics **42:** 71-78.

Matsuoka, M., Ichikawa, H., Saito, A., Tada, Y., Fujimura, T., and Kanomurakami, Y. (1993). Expression of a rice homeobox gene causes altered morphology of transgenic plants. Plant Cell **5:** 1039-1048.

Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell **95:** 805-815.

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

Miura, K., Ikeda, M., Matsubara, A., Song, X.J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., and Ashikari, M. (2010). *OsSPL14* promotes panicle branching and higher grain productivity in rice. Nat. Genet. **42:** 545-549.

Miwa, H., Betsuyaku, S., Iwamoto, K., Kinoshita, A., Fukuda, H., and Sawa, S. (2008). The Receptor-Like Kinase SOL2 Mediates CLE Signaling in *Arabidopsis*. Plant Cell Physiol. **49:** 1752-1757.

Morita, J., Kato, K., Nakane, T., Kondo, Y., Fukuda, H., Nishimasu, H., Ishitani, R., and Nureki, O. (2016). Crystal structure of the plant receptor-like kinase TDR in complex with the TDIF peptide. Nat. Commun. **7:** 12383.

Müller, R., Bleckmann, A., and Simon, R. (2008). The receptor kinase CORYNE of *Arabidopsis* transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. Plant Cell **20:** 934-946.

Müller, R., Borghi, L., Kwiatkowska, D., Laufs, P., and Simon, R. (2006). Dynamic and compensatory responses of Arabidopsis shoot and floral meristems to *CLV3* signaling. Plant Cell **18:** 1188-1198.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of

series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. **104:** 34-41.

Nardmann, J., Zimmermann, R., Durantini, D., Kranz, E., and Werr, W. (2007). *WOX* gene phylogeny in *Poaceae*: a comparative approach addressing leaf and embryo development. Mol. Biol. Evol. **24:** 2474-2484.

Ogawa, M., Shinohara, H., Sakagami, Y., and Matsubayashi, Y. (2008). *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. Science **319:** 294.

Oh, E., Zhu, J.Y., Ryu, H., Hwang, I., and Wang, Z.Y. (2014). TOPLESS mediates brassinosteroid-induced transcriptional repression through interaction with BZR1. Nat. Commun. **5:** 4140.

Ohmori, Y., Tanaka, W., Kojima, M., Sakakibara, H., and Hirano, H.-Y. (2013). *WUSCHEL-RELATED HOMEOBOX4* is involved in meristem maintenance and is negatively regulated by the CLE gene *FCP1* in rice. Plant Cell **25:** 229-241.

Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M., and Matsubayashi, Y. (2009). A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. Nat. Chem. Biol. **5:** 578-580.

Ookawa, T., Hobo, T., Yano, M., Murata, K., Ando, T., Miura, H., Asano, K., Ochiai, Y., Ikeda, M., Nishitani, R., Ebitani, T., Ozaki, H., Angeles, E.R., Hirasawa, T., and Matsuoka, M. (2010). New approach for rice improvement using a pleiotropic QTL gene for lodging resistance and yield. Nat. Commun. **1:** 132.

Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Pérez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., García-Casado, G., Witters, E., Inzé, D., Long, J.A., De Jaeger, G., Solano, R., and Goossens, A. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464:** 788-791.

Pesquita, C., Faria, D., Falcão, A.O., Lord, P., and Couto, F.M. (2009). Semantic similarity in biomedical ontologies. PLOS Comput. Biol. **5:** e1000443.

Pi, L., Aichinger, E., van der Graaff, E., Llavata-Peris, C.I., Weijers, D., Hennig, L., Groot, E., and Laux, T. (2015). Organizer-derived WOX5 signal maintains root columella stem cells through chromatin-mediated repression of *CDF4* expression. Dev. Cell **33:** 576-588.

Rao, N.N., Prasad, K., Kumar, P.R., and Vijayraghavan, U. (2008). Distinct

regulatory role for *RFL*, the rice *LFY* homolog, in determining flowering time and plant architecture. Proc. Natl. Acad. Sci., USA **105:** 3646-3651.

Reddy, G.V., and Meyerowitz, E.M. (2005). Stem-cell homeostasis and growth dynamics can be uncoupled in the *Arabidopsis* shoot apex. Science **310:** 663-667.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). *limma* powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. **43:** e47.

Ryu, H., Cho, H., Bae, W., and Hwang, I. (2014). Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. Nat. Commun. **5:** 4138.

Sato, D.-S., Ohmori, Y., Nagashima, H., Toriba, T., and Hirano, H.-Y. (2014). A role for *TRIANGULAR HULL1* in fine-tuning spikelet morphogenesis in rice. Genes Genet. Syst. **89:** 61-69.

Sato, Y., Hong, S.K., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y., and Matsuoka, M. (1996). A rice homeobox gene, *OSH1*, is expressed before organ differentiation in a specific region during early embryogenesis. Proc. Natl. Acad. Sci., USA **93:** 8117-8122.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jürgens, G., and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell **100:** 635–644.

Shinohara, H., and Matsubayashi, Y. (2015). Reevaluation of the CLV3-receptor interaction in the shoot apical meristem: dissection of the CLV3 signaling pathway from a direct ligand-binding point of view. Plant J. **82:** 328-336.

Somssich, M., Je, B.I., Simon, R., and Jackson, D. (2016). CLAVATA-WUSCHEL signaling in the shoot meristem. Development **143:** 3238-3248.

Song, S.-K., Lee, M.M., and Clark, S.E. (2006). POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for *Arabidopsis* shoot and floral stem cells. Development **133:** 4691-4698.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. (Cambridge, UK: Cambridge University Press).

Supek, F., Bosnjak, M., Skunca, N., and Smuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. PLOS one **6:** e21800.

Suzaki, T., Yoshida, A., and Hirano, H.-Y. (2008). Functional diversification of CLAVATA3-related CLE proteins in meristem maintenance in rice. Plant Cell **20:** 2049-2058.

Suzaki, T., Ohneda, M., Toriba, T., Yoshida, A., and Hirano, H.-Y. (2009). *FON2 SPARE1* redundantly regulates floral meristem maintenance with *FLORAL ORGAN NUMBER2* in rice. PLOS Genet. **5:** e1000693.

Suzaki, T., Sato, M., Ashikari, M., Miyoshi, M., Nagato, Y., and Hirano, H.-Y. (2004). The gene *FLORAL ORGAN NUMBER1* regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to *Arabidopsis* CLAVATA1. Development **131:** 5649-5657.

Suzaki, T., Toriba, T., Fujimoto, M., Tsutsumi, N., Kitano, H., and Hirano, H.-Y. (2006). Conservation and diversification of meristem maintenance mechanism in *Oryza sativa*: function of the *FLORAL ORGAN NUMBER2* gene Plant Cell Physiol. **47:** 1591-1602.

Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. Science **319:** 1384-1386.

Tabuchi, H., Zhang, Y., Hattori, S., Omae, M., Shimizu-Sato, S., Oikawa, T., Qian, Q., Nishimura, M., Kitano, H., Xie, H., Fang, X., Yoshida, H., Kyozuka, J., Chen, F., and Sato, Y. (2011). *LAX PANICLE2* of rice encodes a novel nuclear protein and regulates the formation of axillary meristems. Plant Cell **23:** 3276-3287.

Taguchi-Shiobara, F., Yuan, Z., Hake, S., and Jackson, D. (2001). The *fasciated ear2* gene encodes a leucine-rich repeat receptor-like protein that regulates shoot meristem proliferation in maize. Genes Dev. **15:** 2755-2766.

Tanaka, W., Toriba, T., and Hirano, H.-Y. (2014). Flower development in rice. In The molecular genetics of floral transition and flower development, F. Fornara, ed (Amsterdam: Elsevier), pp. 221-262.

Tanaka, W., Toriba, T., and 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., Pautler, M., Jackson, D., and Hirano, H.-Y. (2013). Grass meristems II: inflorescence architecture, flower development and meristem gate. Plant Cell

Physiol. **54:** 313-324.

Tanaka, W., Ohmori, Y., Ushijima, T., Matsusaka, H., Matsushita, T., Kumamaru, T., Kawano, S., and Hirano, H.-Y. (2015). Axillary meristem formation in rice requires the *WUSCHEL* ortholog *TILLERS ABSENT1*. Plant Cell **27:** 1173-1184.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., and Su, Z. (2017). agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. **45:** W122-W129.

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

Tsai, Y.C., Weir, N.R., Hill, K., Zhang, W., Kim, H.J., Shiu, S.H., Schaller, G.E., and Kieber, J.J. (2012). Characterization of genes involved in cytokinin signaling and metabolism from rice. Plant Physiol. **158:** 1666-1684.

Wickham, H. (2016). ggplot2: elegant graphics for data analysis. (Springer).

Williams, R.W., Wilson, J.M., and Meyerowitz, E.M. (1997). A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. Proc. Natl. Acad. Sci., USA **94:** 10467-10472.

Yadav, R.K., Perales, M., Gruel, J., Girke, T., Jönsson, H., and Reddy, G.V. (2011). WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex. Genes Dev. **25:** 2025-2030.

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

Yamaguchi, Y.L., Ishida, T., and Sawa, S. (2016). CLE peptides and their signaling pathways in plant development. J. Exp. Bot. **67:** 4813-4826.

Yamaki, S., Satoh, H., and Nagato, Y. (2005). *Gypsy embryo* specifies ovule curvature by regulating ovule/integument development in rice. Planta **222:** 408-417.

Yang, F., Bui, H.T., Pautler, M., Llaca, V., Johnston, R., Lee, B.H., Kolbe, A., Sakai, H., and Jackson, D. (2015). A maize glutaredoxin gene, *Abphyl2*, regulates shoot meristem size and phyllotaxy. Plant Cell **27:** 121-131.

Yasui, Y., Tanaka, W., Sakamoto, T., Kurata, T., and 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.

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

Yoshida, A., Suzaki, T., Tanaka, W., and Hirano, H.-Y. (2009). The homeotic gene *LONG STERILE LEMMA* (*G1*) specifies sterile lemma identity in the rice spikelet. Proc. Natl. Acad. Sci., USA **106:** 20103-20108.

Yoshida, A., Ohmori, Y., Kitano, H., Taguchi-Shiobara, F., and Hirano, H.-Y. (2012). *ABERRANT SPIKELET AND PANICLE1*, encoding a TOPLESS-related transcriptional co-repressor, is involved in the regulation of meristem fate in rice. Plant J. **70:** 327-339.

Yoshida, A., Sasao, M., Yasuno, N., Takagi, K., Daimon, Y., Chen, R., Yamazaki, R., Tokunaga, H., Kitaguchi, Y., Sato, Y., Nagamura, Y., Ushijima, T., Kumamaru, T., Iida, S., Maekawa, M., and Kyozuka, J. (2013). *TAWAWA1*, a regulator of rice inflorescence architecture, functions through the suppression of meristem phase transition. Proc. Natl. Acad. Sci., USA **110:** 767-772.

Yu, L.P., Simon, E.J., Trotochaud, A.E., and Clark, S.E. (2000). *POLTERGEIST* functions to regulate meristem development downstream of the *CLAVATA* loci. Development **127:** 1661-1670.

Zhang, H., Lin, X., Han, Z., Qu, L.J., and Chai, J. (2016). Crystal structure of PXY-TDIF complex reveals a conserved recognition mechanism among CLE peptide-receptor pairs. Cell Res. **26:** 543-555.

Zhou, Y., Yan, A., Han, H., Li, T., Geng, Y., Liu, X., and Meyerowitz, E.M. (2018). HAIRY MERISTEM with WUSCHEL confines CLAVATA3 expression to the outer apical meristem layers. Science **361:** 502-506.

Zhou, Y., Liu, X., Engstrom, E.M., Nimchuk, Z.L., Pruneda-Paz, J.L., Tarr, P.T., Yan, A., Kay, S.A., and Meyerowitz, E.M. (2015). Control of plant stem cell function by conserved interacting transcriptional regulators. Nature **517:** 377-380.