Rice
a SpringerOpen Journal

RESEARCH   **Open Access**

Gibberellin is not a regulator of *miR156* in rice juvenile-adult phase change

Nobuhiro Tanaka

Abstract

Plant hormone gibberellin (GA) promotes juvenile-adult phase change in higher plants. To confirm the functions of GA in rice, I used dwarf mutant *d18-dy*. *d18-dy* is a loss-of-function allele of *D18*, which encodes *GA3ox2*. *d18-dy* mutant exhibited long juvenile phase in morphological traits such as the size of the shoot apical meristem (SAM), shape of leaf blades, presence or absence of midribs and node–internode differentiation in stem. In contrast, expression patterns of juvenile-adult phase change markers *miR156* and *miR172* were similar between wild type and *d18-dy*. In addition, *d18-dy* mutation and GA did not affect expression levels of downstream genes of *miR156*. GA does not function upstream of *miR156* in juvenile-adult phase change.

Keywords: Gibberellin, *miR156*, *miR172*, Os*SPLs*

Background

Juvenile and adult phases are distinguished by several morphological markers (Lawson and Poethig 1995; Telfer et al. 1997; Asai et al. 2002). Juvenile-adult phase change is regulated by *miR156*, *miR172* and gibberellin (GA) in many higher plants (Lawson and poethig 1995; Telfer et al. 1997; Wu and Poethig 2006; Wang et al. 2011; Tanaka et al. 2011). However, the molecular mechanism involved in the juvenile-adult phase change is still unclear.

In *Arabidopsis*, juvenile leaves are round without abaxial trichomes, and adult leaves are long and serrated with abaxial trichomes (Telfer et al. 1997). In maize, leaves have epicuticular wax in juvenile phase, and adult leaves have no wax (Lawson and Poethig 1995). From these morphological markers, a lot of juvenile-adult phase change related mutants are reported in *Arabidopsis* and maize (Moose and Sisco 1996; Chuck et al. 2007; Schwarz et al. 2008; Smith et al. 2009). The observation of these mutants revealed that *miR156* had significant roles in juvenile-adult phase change (Wu and Poethig 2006; Chuck et al. 2007; Wu et al. 2009). In the early vegetative stage, transcription level of *miR156* exceeds that of *miR172*, whereas in later vegetative stage, the inverse pattern is seen (Wu and Poethig 2006; Chuck et al.

2007). In *Arabidopsis*, overexpression of *miR156* causes a prolonged juvenile phase (Wu and Poethig 2006). In addition, *miR156* overexpressed mutant, *Corngrass1* (*Cg1*) shows long juvenile phase in maize (Chuck et al. 2007). The *glossy15* (*gl15*) mutant shortens the juvenile phase in the maize epidermis (Moose and Sisco 1996); *GL15* is an AP2-like gene that is the target of *miR172* (Lauter et al. 2005). In addition, *miR156* also controls juvenile-adult phase change in trees (Wang et al. 2011). *miR156* inhibits juvenile-adult phase change via repression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) family genes resulting in decrease of *miR172* (Wu et al. 2009). Thus, *miR156* and *miR172* are key regulators in the juvenile–adult phase change.

Plant hormone Gibberellin (GA) is involved in the regulation of plant growth. GA is also known as adult phase promoter in *Arabidopsis* and maize (Lawson and Poethig 1995; Telfer et al. 1997; Telfer and Poethig 1998). GA deficient mutant, *ga1-3* exhibits dwarfism and glabrous leaf without abaxial trichome in *Arabidopsis* (Telfer et al. 1997). In maize *d1* and *d3* mutants, the expression of leaf epidermal wax is prolonged and the expression of leaf epidermal hairs is delayed compared with wild type (Lawson and Poethig 1995).

There are a few reports that examine the relationship between *miR156* and GA (Schwarz et al. 2008; Wang et al. 2009). Because the expression level of *SPL9* was similar in wild type and *ga1-3* mutant in *Arabidopsis*

Correspondence: antanaka@mail.ecc.u-tokyo.ac.jp
Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

Springer

(Wang et al. 2009), *miR156* and GA related genes may function independently. However, the relationship between *miR156* and GA in juvenile-adult phase change is not confirmative.

In rice, a lot of GA-biosynthesis-deficient mutants are reported (Sasaki et al. 2002; Sakamoto et al. 2004). However no reports described how GA is related to juvenile-adult phase change in rice except Tanaka et al. (2011). Almost all molecular genetic studies of juvenile-adult phase change have been confined to *Arabidopsis* and maize. However, considerable number of morphological and physiological traits known to differ between the juvenile and adult phases are reported in rice, including the size of the shoot apical meristem (SAM), size and shape of leaf blades, presence of midribs, vascular orientation in the stem, node–internode differentiation, and photosynthetic rate (Itoh et al. 2005), thus rice is useful plant to understand the juvenile-adult phase change.

In this report, I examined *d18-dy* mutant from the stand point of juvenile-adult phase change. Expression patterns of *miR156* and *miR172* in *d18-dy* demonstrate that GA regulates juvenile-adult phase change independently of *miR156*-related pathway.

Results
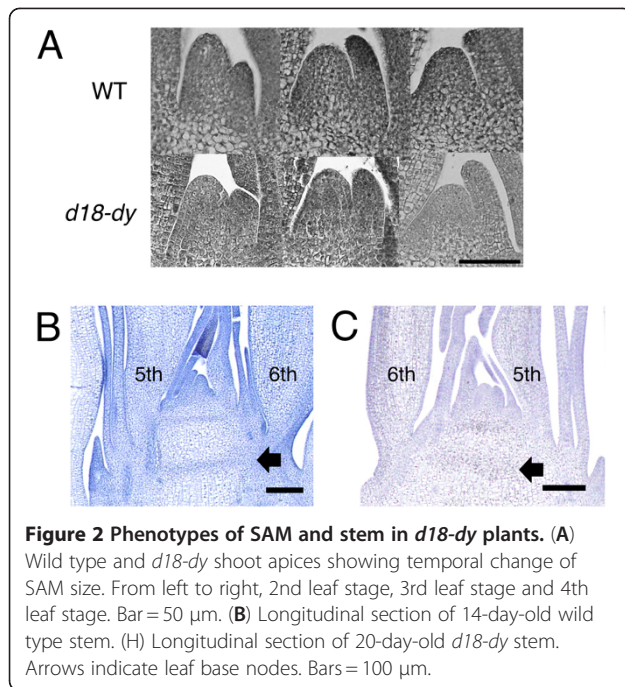
Vegetative phenotypes of *d18-dy*

To confirm the function of GA as adult phase promoter, I examined the phenotypes of GA deficient mutant, *d18-dy*. Rice *D18* encodes *GA3ox2*, and the loss of function allele *d18-dy* causes severe dwarfism (Figure 1A). *d18-dy* has 9 bp deletion in the first exon of *GA3ox2* gene (Sakamoto et al. 2004). GA 3-oxidase oxidizes GA_{20} and GA_9 to synthesize active GA, GA_1 and GA_4. The GA3ox deficient mutant is convenient to understand the function of GA in juvenile-adult phase change. First, I examined leaf shape that is defined as the ratio of leaf blade length to width. In wild type, the ratio drastically increased with elevation of leaf positions (Figure 1B). In *d18-dy*, even the ratio of the 7th leaf blade was below that of wild type 3rd leaf (Figure 1B). Second, I evaluated the presence of midrib in wild type and *d18-dy* leaf blades. The extent of midrib formation is a good marker for evaluating the juvenility in rice (Tanaka et al. 2011). I measured relative midrib length from the base of leaf blade. In *d18-dy* 3rd and 4th leaf blades, midrib covered approximately 20% and 40% of leaf blade, respectively (Figure 1C-E). These values were approximately half of those of wild type (Figure 1E). In both wild type and *d18-dy* 6th leaf, midrib covered more than 70% of leaf blade (Figure 1E). These results suggest that *d18-dy* shows prolonged juvenile phase, and it enters adult phase at around 6th leaf stage.

Next, I observed the changing pattern of SAM size during development. In *d18-dy*, SAM remained smaller than that of wild type till the 4th-leaf stage (Figure 2A). Additionally, *d18-dy* showed different node-internode differentiation. Above the 5th leaf, the stem has obvious node in wild type (Figure 2B). By contrast, node differentiation was not detectable until the insertion of 6th leaf in *d18-dy* (Figure 2C). These stem structures also indicate that *d18-dy* has long juvenile phase. From these
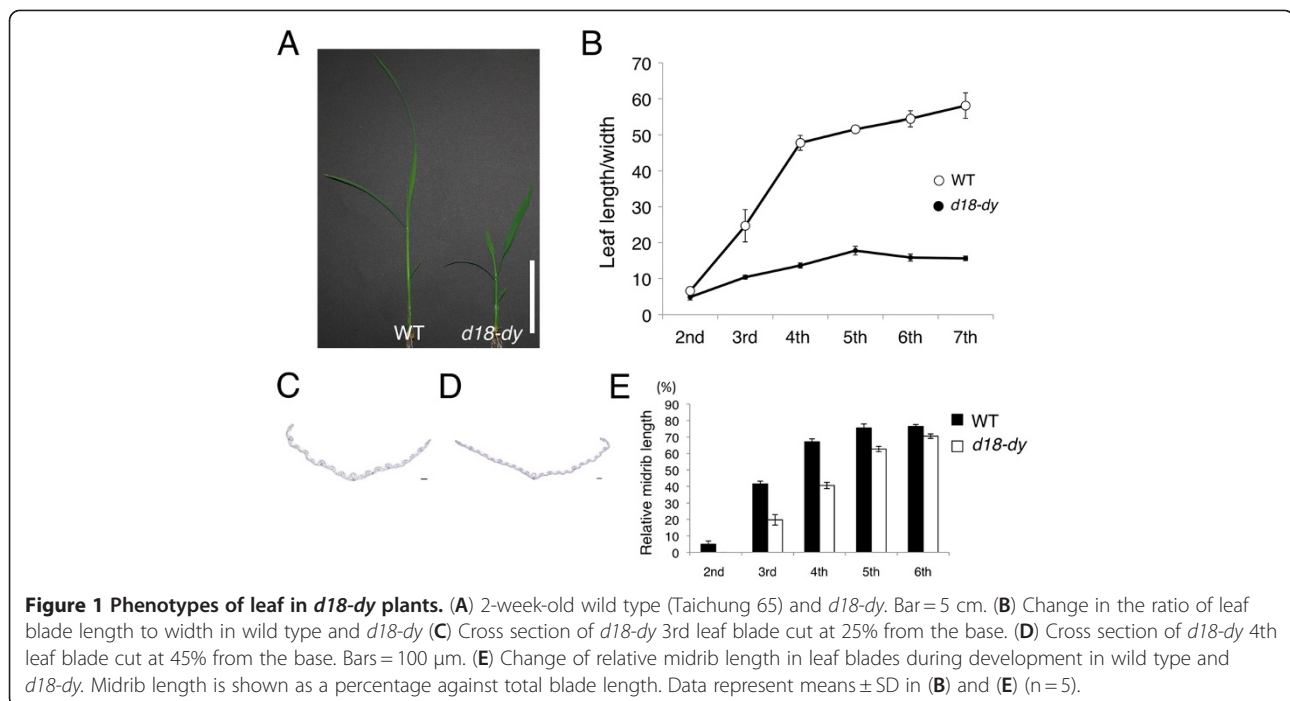

Figure 1 Phenotypes of leaf in *d18-dy* plants. (**A**) 2-week-old wild type (Taichung 65) and *d18-dy*. Bar = 5 cm. (**B**) Change in the ratio of leaf blade length to width in wild type and *d18-dy* (**C**) Cross section of *d18-dy* 3rd leaf blade cut at 25% from the base. (**D**) Cross section of *d18-dy* 4th leaf blade cut at 45% from the base. Bars = 100 μm. (**E**) Change of relative midrib length in leaf blades during development in wild type and *d18-dy*. Midrib length is shown as a percentage against total blade length. Data represent means ± SD in (**B**) and (**E**) (n = 5).

Figure 2 Phenotypes of SAM and stem in *d18-dy* plants. (A)
Wild type and *d18-dy* shoot apices showing temporal change of
SAM size. From left to right, 2nd leaf stage, 3rd leaf stage and 4th
leaf stage. Bar = 50 μm. **(B)** Longitudinal section of 14-day-old wild
type stem. **(H)** Longitudinal section of 20-day-old *d18-dy* stem.
Arrows indicate leaf base nodes. Bars = 100 μm.

Figure 3 Relationship between two miRNAs and GA. (A)
Expression of *miR156* in wild type and *d18-dy* 2nd to 7th leaves. **(B)**
Expression of *miR172* in wild type and *d18-dy* 2nd to 7th leaves. **(C)**
Expression of *miR156* and *miR172* in control and GA$_3$ treated 3-day-
old plants. Each value is the average of three independent real-time
PCR assays. Data in **(A)**, **(B)** and **(C)** represent means ± SD (n = 3).

morphological traits in *d18-dy*, I concluded that GA
promoted juvenile-adult phase change in rice.

GA and two miRNAs independently regulate juvenile-adult phase change

To demonstrate the relationship between GA and two
miRNAs in juvenile-adult phase change, I examined
miR156 and *miR172* expression patterns in wild type and
d18-dy leaves (Figure 3A,B). In both wild type and *d18-dy*
leaves, expression level of *miR156* was the highest in 2nd
leaf, rapidly decreased to approximately one-third in 3rd
leaf (Figure 3A), and was maintained at low level until the
7th leaf (Figure 3A). Wild type and *d18-dy* also showed
similar expression pattern of *miR172*: the expression was
quite low in 2nd leaf, and increased dramatically toward
the 7th leaf (Figure 3B). In conclusion, expression patterns
of the two miRNAs in *d18-dy* were identical to those in
wild type (Figure 3A,B). From normal expression patterns
of two miRNAs and retarded juvenile-adult phase change
in *d18-dy*, I estimated that GA promotes adult phase tran-
sition independently of *miR156* and *miR172*. To confirm
the relationship between GA and two miRNAs, I treated
wild type plants with GA$_3$. There is a report that the ex-
pression level of GA deactivation gene, *GA2ox4* is up-
regulated by GA treatment (Yamaguchi 2008). In GA trea-
ted plants, expression level of *GA2ox4* was higher than in
control plants (Additional file 1: Figure S1). This indicates
that the experimental system of GA treatment is normally
functioning. Subsequently I examined *miR156* and *miR172*
expression levels in control and GA$_3$ treated plants. Both
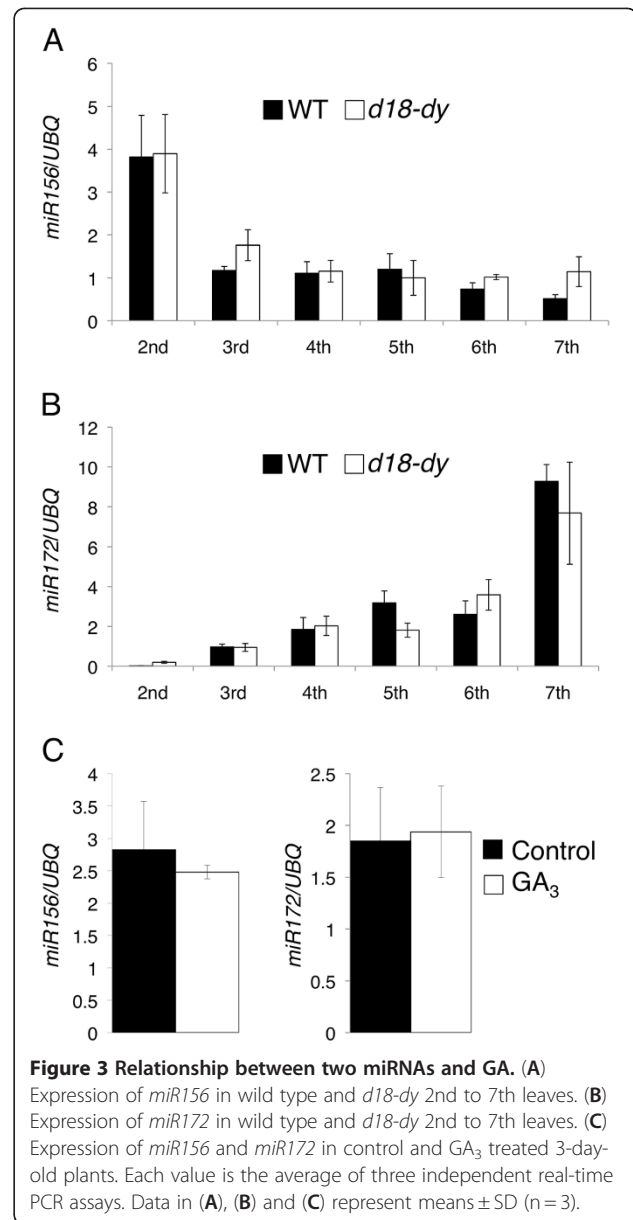
expression levels of two miRNAs were not affected by the
application of GA$_3$ (Figure 3C). Thus I concluded that GA
did not regulate the onset of adult phase upstream of
miR156.

Expression patterns of Os*SPL*

To further confirm the hypothesis that GA functions in-
dependently of *miR156* pathway in juvenile-adult phase
change, I examined the expression levels of Os*SPL13*
and Os*SPL14* that are the ortholog of *Arabidopsis SPL3*
and *SPL9* (Xie et al. 2006). Both Os*SPL13* and Os*SPL14*
contain *miR156* target sites, and Os*SPL14* ortholog,
SPL9 regulates *miR172* expression positively in *Arabi-
dopsis* (Wu et al. 2009). The expression levels of both

OsSPL13 and OsSPL14 in leaves were almost comparable between wild type and *d18-dy* (Figure 4A). Next, I examined OsSPL13 and OsSPL14 expression in GA₃ treated plants. Both OsSPL13 and OsSPL14 expression levels were similar between control and GA₃ treated plants (Figure 4B). These results indicate that GA does not affect the expression of *miR156*-target genes. I again confirmed that GA-related pathway did not act upstream of *miR156*.

Discussion

GA and *miR156* function independently to regulate juvenile-adult phase change

The study of juvenile-adult phase change is difficult because it is accompanied by subtle morphological traits. In addition, the morphological markers for juvenile-adult phase change are different among plant species. However, *miR156* and GA are reported as common juvenile-adult phase change regulator in many flowering plants (Lawson and Poethig 1995; Telfer et al. 1997; Wu et al. 2009; Chuck et al. 2007; Wang et al. 2011; Tanaka et al. 2011). GA-related mutant, *d18-dy* showed prolonged juvenile phase, such as delayed midrib formation,

small leaf size, small SAM size and delayed node-internode differentiation. These results strongly indicate that GA also promotes adult phase transition in rice.

The expression patterns of *miR156* and *miR172* were comparable between wild type and *d18-dy* plants. These results suggest that GA and *miR156* regulate juvenile-adult phase change through independent genetic pathway. Over-expressed *MIR156* line in *Arabidopsis* shows prolonged juvenile phase, however it can enter the reproductive phase (Schwarz et al. 2008). In rice, *MIR156* over-expressed plant also showed dwarfism, but developed to flowering stage (Xie et al. 2012). Similarly, GA-deficient mutants can enter the reproductive phase in many flowering plants (Telfer et al. 1997; Itoh et al. 2004). These phenotypes indicate that *miR156* functions redundantly with GA in the determination of the exact time of juvenile-adult phase change. Double mutant of *MIR156* over-expressed plant and GA deficient mutant might show persistent juvenile phase.

In *d18-dy* 6th leaf stage, the plant was obviously dwarf. This indicates that amount of active GA in *d18-dy* is still low at 6th leaf stage. However, *d18-dy* showed normal midrib formation at this stage (Figure 1E). This suggests
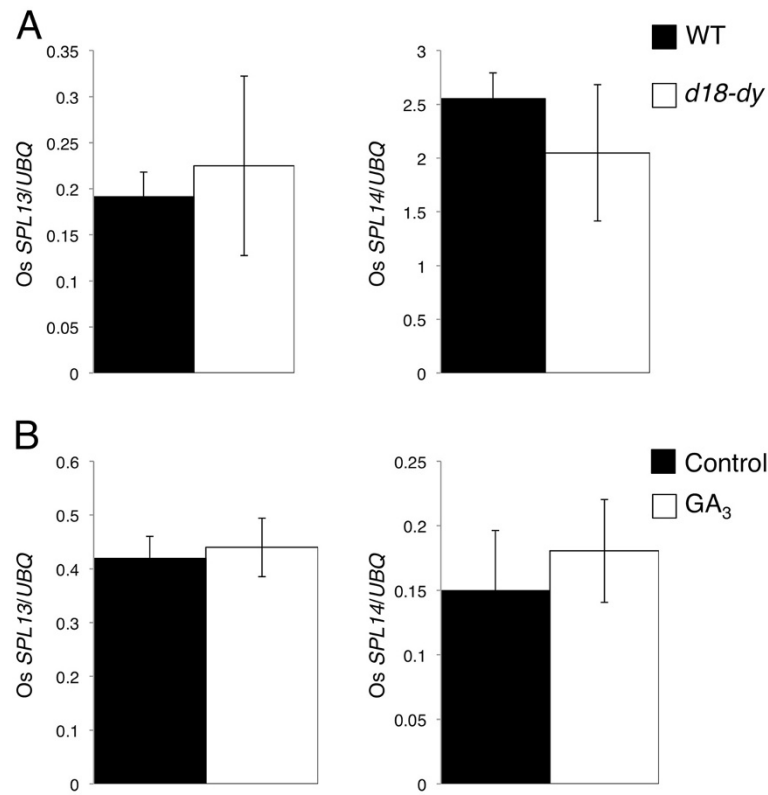
Figure 4 Expression patterns of Os *SPLs* in *d18-dy*. (**A**) Expression of Os *SPL13* and Os *SPL14* in wild type and *d18-dy* 2nd leaves. (**B**) Expression of Os *SPL13* and Os *SPL14* in control and GA₃ treated 3-day-old plants. Each value is the average of three independent real-time PCR assays. Data in (**A**), (**B**) and (**C**) represent means ± SD (n = 3).

that the function of GA is less important for midrib formation after 6th leaf stage than during 2nd-to-5th leaf stages.

Short plastochron is also known as juvenile phase character in rice (Itoh et al. 2005). *MIR156* over-expressed plant had more leaves than wild type (Xie et al. 2012). In contrast, the rate of leaf initiation was comparable between *d18-dy* and wild type. These indicate that *miR156* and GA have different functions in regulation of plastochron.

Conclusions

Long juvenile phase phenotype of *d18-dy* indicated that GA is the adult phase promoter in rice. In higher plants, *miR156* and *miR172* are also known as juvenile-adult phase change regulator. Our study demonstrated that GA does not regulate juvenile-adult phase change via a pathway of *miR156*. Moreover, GA does not regulate Os*SPL*s that are the *miR156*-target genes.

Methods

Plant materials

I used *d18-dy* that is a dwarf mutant defective in GA biosynthetic gene encoding GA3 OXIDASE 2. Mutants and wild type plants were grown in pots under natural field conditions.

Paraffin sectioning

Leaves and shoot apices were fixed with FAA (formalin: acetic acid:50% ethanol, 1:1:18) for 24 h at 4°C. They were dehydrated in a graded ethanol series and embedded in Paraplast plus (McCormick Scientific). Microtome sections (8 μm thick) were stained with Delafield's hematoxylin.

Gene expression profiling

The real-time PCR for *miR156* and *miR172* was performed using TaqMan MicroRNA Assay (Applied Biosystems). Total RNA was extracted using TRIzol reagent (Invitrogen) from wild type 2nd, 3rd, 4th, 5th, 6th and 7th leaves. In addition, RNA was also isolated from *d18-dy* 2nd, 3rd, 4th, 5th, 6th and 7th leaves. To quantify the *miR156* and *miR172* expression, PCR was performed using the TaqMan Fast Universal PCR Master Mix (Applied Biosystem). I used *UBQUITIN* (*UBQ*) as inner control and qPCR was conducted using SYBR green master mix (Applied Biosystem). For quantifying the *UBQ* expression, Real-time PCR was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems).

To observe the Os *SPL13 and* Os *SPL14* expressions, total RNA was isolated from wild type and *d18-dy* 2nd leaves. I used *UBQ* as inner control and qPCR was conducted using SYBR green master mix (Applied Biosystem).

Real-time PCR was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems).

For observing *miR156*, *miR172*, Os *SPL*s and *GA2ox4* expression patterns in GA treated plants, sterilized seeds of wild type were plated on MS medium (Murashige and Skoog 1962) containing 10^{-5} M GA_3 (SIGMA). Plants were grown in a growth chamber under the continuous light at 28°C. Total RNA was extracted using TRIzol reagent (Invitrogen) from 3-day-old wild type seedlings. To quantify the *miR156* and *miR172* expression, PCR was performed using the TaqMan Fast Universal PCR Master Mix (Applied Biosystem). I used *UBQUITIN* (*UBQ*) as inner control and qPCR was conducted using SYBR green master mix (Applied Biosystem). For quantifying the Os *SPL13*, Os *SPL14* and *UBQ* expression, Real-time PCR was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems). For quantifying the *GA2ox4* and *UBQ* expression, Real-time PCR was performed using PrimeScript RT Master Mix (Takara). qPCR was conducted using SYBR Premix Ex Taq ll (Takara). PCR was performed using Thermal Cycler Dice TP800 (Takara). Gene-specific primes for Os *SPL13*, Os *SPL14 GA2ox4* and *UBQ* are listed in Additional file 2: Table S1 online.

Additional files

Additional file 1: Figure S1. Expression patterns of *GA2ox4* in GA_3 treated plants. Expression of *GA2ox4* in control and GA_3 treated 3-day-old plants. Each value is the average of three independent real-time PCR assays. Data represent means ± SD (n = 3).

Additional file 2: Table S2. List of primers for semi-quantitative RT-PCR.

References
Asai K, Satoh N, Sasaki H, Satoh H, Nagato Y (2002) A rice heterochronic mutant, *mori1*, is defective in the juvenile-adult phase change. Development 129:265–273
Chuck G, Cigan AM, Saeteurn K, Hake S (2007) The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. Nat Genet 39:544–549
Itoh H, Tatsumi T, Sakamoto T, Otomo K, Toyomasu T, Kitano H, Ashikari M, Ichihara S, Matsuoka M (2004) A rice semi-dwarf gene, Tan-Ginbozu (D35), encodes the gibberellin biosynthesis enzyme, ent-kaurene oxidase. Plant Mol Biol 54:533–547
Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol 46:23–47

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

Lawson EJ, Poethig RS (1995) Shoot development in plants: time for a change. Trends Genet 11:263–268

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

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Sakamoto T et al (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol 134:1642–1653

Sasaki A et al (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. Nature 416:701–702

Schwarz S, Grande AV, Bujdoso N, Saedler H, Huijser P (2008) The microRNA regulated SBP-box genes *SPL9* and *SPL15* control shoot maturation in *Arabidopsis*. Plant Mol Biol 67:183–195

Smith MR, Willmann MR, Wu G, Berardini TZ, Moller B, Weijers D, Poethig RS (2009) *Cyclophilin 40* is required for microRNA activity in *Arabidopsis*. Proc Natl Acad Sci U S A 106:5424–5429

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

Telfer A, Poethig RS (1998) *HASTY*: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. Development 125:1889–1898

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

Wang JW, Czech B, Weigel D (2009) *miR156*-regulated *SPL* transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell 138:738–749

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

Wu G, Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by *miR156* and its target *SPL3*. Development 133:3539–3547

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

Xie K, Wu C, Xiong L (2006) Genomic organization, differential expression, and interaction of *SQUAMOSA* promoter-binding-like transcription factors and *microRNA156* in rice. Plant Physiol 142:280–293

Xie K, Shen J, Hou X, Yao J, Li X, Xiao J, Xiong L (2012) Gradual increase of *miR156* regulates temporal expression changes of numerous genes during leaf development in rice. Plant Physiol 158:1382–1394

Yamaguchi S (2008) Gibberellin Metabolism and its Regulation. Annu Rev Plant Biol 59:225–251