

## A rice heterochronic mutant, *mori1*, is defective in the juvenile-adult phase change

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### SUMMARY

We have identified five recessive allelic mutations, *mori1-1* to *mori1-5*, which drastically modify the shoot architecture of rice. The most remarkable feature of *mori1* plants is a rapid production of small leaves and short branches. The *mori1* plants are about 5 cm in height even 7 months after sowing. No reproductive growth was attained in *mori1* plants even if inductive short-day treatment was applied. Leaves of *mori1* at any position were very small and the size and shape were comparable to those of the wild-type 2nd leaf. The stem of *mori1* 7 months after sowing did not differentiate node and internode and had randomly oriented vascular bundles, which were characteristic of the basal part of the wild-type stem where 2nd and 3rd leaves were inserted. These structural characteristics indicate that *mori1* maintains the 2nd-leaf stage (juvenile phase) of the

wild type. The short plastochron and high cell division activity in the shoot apical meristem further confirmed the juvenility of *mori1*, corresponding to the 2nd-leaf-differentiation stage in the wild-type embryo. Furthermore, the apparent photosynthetic rate in *mori1* leaves was low as in the wild-type 2nd leaf. Thus, *mori1* is a heterochronic mutation that suppresses the induction of adult phase and the termination of the juvenile phase. Therefore, *MOR1* plays an important role in the juvenile-adult phase change. The importance of heterochronic mutations in modifying shoot architecture is discussed.

Key words: *mori1*, Rice, Heterochrony, Juvenile phase, Shoot apical meristem

### INTRODUCTION

Organismal development comprises several more or less discrete stages, which we can recognize as phase changes. It is plausible that the development is regulated temporally by genetic programs. The important role of heterochronic mutations in the development and evolution has long been suggested. As Gould (Gould, 1977) indicated, heterochronic mutation may drastically modify the developmental program, which could eventually lead to macroevolution. In animals, the significance of heterochrony in evolution is widely accepted (McKinney, 1988; Raff, 1989). Evolutionary processes due to heterochrony have also been suggested in plants (Lord and Hill, 1987). In contrast with mutations of morphogenetic genes that modify the shape of one or a limited number of organs, and will be usually deleterious to the individuals, heterochronic mutations may affect the total body plan without impairing the morphogenetic genes. Thus heterochronic mutation can produce viable individuals with profoundly different architecture.

In recent years, it has been widely accepted that plants undergo phasic development (Lawson and Poethig, 1995; Poethig, 1990). Even though heterochrony may be recognized in the development of individual organs such as leaves (Freeling, 1992; Lu et al., 1996; Villani and Demason, 1999),

the fundamental importance is in the juvenile-adult-reproductive phase changes of the whole plant. Apparently constant plant form during vegetative phase makes it difficult to recognize juvenile to adult phase change, compared to the drastic change from vegetative to reproductive phase. It is long known that many plant species show heteroblasty during the vegetative development (Allsopp, 1967; Goebel, 1900). In many cases, heteroblasty is recognized as heterophylly, and is interpreted as a juvenile-adult phase change. Heteroblasty is obvious in woody species such as English ivy (Geneve, 1991; Hackett, 1985; Zimmerman et al., 1985). Also in herbaceous plants, morphological, anatomical and physiological traits change temporally, reflecting the juvenile-adult phase change. In maize, several traits such as epicuticular wax, epidermal features and aerial roots characterize the juvenile phase (Poethig, 1990). Recently, juvenile-adult phase change has also been discussed in *Arabidopsis* (Telfer et al., 1997). Thus, it is supposed that most higher plants, if not all, have a genetic program for juvenile-adult phase change. To unravel the regulatory mechanism of the phase change, it will be important to identify genes associated with the phase change, and to elucidate how their expressions are regulated.

Several mutants have been obtained in maize that affect the juvenile-adult phase change, such as *Teopod1*, *Teopod2*, *Corngrass* (= *Teopod3*) and *glossy15* (Bassiri et al., 1992;

Dudley and Poethig, 1991; Dudley and Poethig, 1993; Evans et al., 1994; Moose and Sisco, 1994; Poethig, 1988). Most of them are derived from dominant mutations, and are characterized by the ectopic expression of juvenile traits in the adult phase. Another kind of heterochronic mutant is *Lax midrib1-O*, in which each developmental phase is abbreviated (Schichness and Freeling, 1998). An interesting mutant, *embryonic flower (emf)*, is identified in *Arabidopsis*. In the *emf* mutant, reproductive phase is induced immediately after the embryonic phase, skipping the vegetative phase (Sung et al., 1992; Yang et al., 1995). *EMF* can be interpreted as a heterochronic gene suppressing the reproductive program to operate at the early vegetative stage. Although the important role of phase change in plant development is being accepted, comprehensive understanding of the juvenile-adult phase change has not been obtained because of the lack of loss-of-function mutations of heterochronic genes.

In rice, although juvenile phase-specific traits have not been obvious, several phase-dependent mutants have been reported. In *sho* mutants, abnormal phenotypes such as random phyllotaxy, short plastochron and malformed leaves are confined to the early vegetative phase (Tamura et al., 1992; Itoh et al., 2000). Also, most of virescent mutations are associated with the early vegetative phase (Iba et al., 1991; Omura et al., 1977). Recently, an interesting heterochronic mutant has been identified in rice. The *plal* plant produces leaves rapidly in the vegetative phase, and the primary panicle branches are converted into vegetative shoots (Itoh et al., 1998). Since the reproductive phase begins normally in *plal*, this panicle phenotype is regarded as the ectopic expression of vegetative program in the reproductive phase. Thus, the reproductive phase begins independent of the termination of the vegetative phase. In *plal* mutants, the shoot apical meristem (SAM) is enlarged, and cell divisions in the SAM are activated. Therefore, the SAM is presumed to play an important role in the phase change.

In this study, we describe a rice mutant, *mori1*, which exhibits unique phenotypes after germination that have not been reported in other plant species. The phenotypic analysis shows that *mori1* is a heterochronic mutant reiterating early vegetative stage (2nd-leaf stage), and which fails to become adult.

## MATERIALS AND METHODS

### Plant materials

We identified five single recessive mutants of rice (*Oryza sativa* L.) that showed abnormal shoot architecture. Allelism test revealed that these five mutations were allelic. Since they grew like a forest, they were designated *mori1-1*, *mori1-2*, *mori1-3*, *mori1-4* and *mori1-5*, respectively (the Japanese word "mori" has the same connotation of the word "forest"). *mori1-1* and *mori1-2* were derived from the M<sub>2</sub> population of cv. Kinmaze and *mori1-3* to *mori1-5* from that of Taichung 65 mutagenized with N-methyl-N-nitrosourea (Hong et al., 1995).

The *MOR1* locus is mapped between two microsatellite markers on the long arm of chromosome 3: 15.6 cM from OSR31 and 2.5 cM from RM16.

### Plant culture

Seeds set on heterozygous plants for *mori1* were surface-sterilized in

2% sodium hypochlorite for 30 minutes. They were washed with sterilized water and inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 6% sucrose and 1% agar at pH 5.8 in culture boxes 6 cm square and 10 cm high. They were cultured at 28°C in 14 hours light and 10 hours dark. Seven days after sowing, wild-type plants were transplanted to soil, while the mutants were maintained to be cultured on nutrient medium because they soon died when transplanted to soil.

Every week after sowing, we measured plant height, leaf size and the number of leaves in five to ten plants of *mori1* and wild type, respectively. For examining the response to day length, 5-7-month-old *mori1* plants were treated with short day (10L:14D) for 2 months.

### Plastic sectioning

Leaves and shoot apices at various developmental stages were fixed with FAA (formalin:acetic acid:50% ethanol, 1:1:18). They were dehydrated in a graded ethanol series, embedded in Technovit 7100 resin (Heraeus Kulzer, GmbH, Germany), and sectioned at 3 µm thick by a rotary microtome. Sectioned samples were stained with Toluidine Blue-O, and observed with a light microscope (AX-80, Olympus, Tokyo).

### Clearing of shoot apical meristem

For the observation of SAMs, five to ten shoot apices of *mori1* and the wild type were sampled every 7 days. Samples were hand-sectioned to about 1 mm thick, and fixed with FAA. Then they were dehydrated through a graded ethanol series, and transferred to BB4-1/2 clearing fluid (Herr, 1982) for 16 hours. The cleared samples were observed using a microscope (MT-2, Olympus, Tokyo) equipped with Nomarski differential interference contrast optics.

### Shoot regeneration

Mature dry seeds of *mori1* and wild type were surface-sterilized in 2% sodium hypochlorite for 30 minutes, and washed with sterilized water. For inducing calli, they were inoculated on N6 medium (Chu et al., 1975) supplemented with 3% sucrose, 0.2% 2,4-D and 0.2% gerlite at pH 5.8, and cultured at 28°C in the dark. Calli proliferated from the scutellum for 3 weeks in the dark and were then transplanted to regeneration medium (MS medium supplemented with 3% sucrose, 3% sorbitol, 2% 6-benzylaminopurine, 0.1% naphthalene acetic acid, 0.2% casamino acid and 0.4% gerlite at pH 5.8). They were cultured at 28°C in the light.

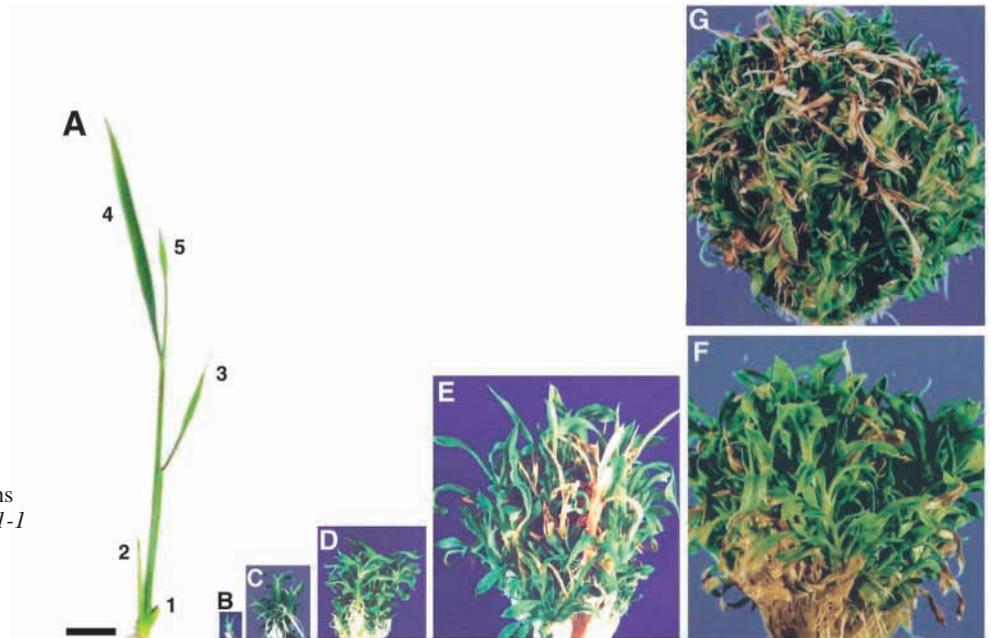
### In situ hybridization

We carried out in situ hybridization probed with digoxigenin-labeled antisense RNA produced from the coding region of rice histone *H4*. Shoot apices were fixed with 3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and embedded in Palaplast Plus (Oxford Labware, MO). Microtome sections (8 µm thick) were applied onto slide glasses treated with Vectabond (Vector Laboratories, CA). Hybridization and immunological detection of the hybridized probe were performed according to the methods of Kouchi and Hata (Kouchi and Hata, 1993).

### Measurement of photosynthesis

Seedlings of *mori1-1* in plant boxes containing the culture medium described above, and those of the wild type (cv. Taichung 65) in pots were grown in a phytotron. Mature 2nd, 3rd and 4th leaves of the wild type were used for measuring photosynthesis. In *mori1*, mature leaves at upper positions of 3-month-old plants were used.

Apparent leaf photosynthesis per unit leaf area was measured when each leaf was fully expanded, using a portable photosynthesis and transpiration measuring system with an infrared gas analyzer (SPB-H4, Analytical Development Co., England). The measurements were conducted on six leaves at each leaf position. A leaf was clamped into the leaf chamber and maintained under controlled conditions of



**Fig. 1.** Development of *moril-1* plant. (A) Wild-type rice plant 10 days after sowing. (B-F) *moril-1* plants at 1 week (B); 1 month (C); 2 months (D); 5 months (E); 7 months (F). (G) Top view of *moril-1* plant shown in F. The *moril* plant invariably produces many small leaves throughout the development. Bar in A, 1 cm (A-G).

temperature, humidity and CO<sub>2</sub> concentration. The measurements were carried out at CO<sub>2</sub> concentration of about 470  $\mu$ l/l, at an irradiance above 1850  $\mu$ mol/m<sup>2</sup>/s PFD under artificial light and at the temperature of 32 $\pm$ 2°C, after pre-illumination for 60 minutes to obtain steady-state readings of photosynthesis.

#### *moril sho1* double mutant

*sho1* is a mutant that shows aberrant shoot architecture in the early vegetative phase but becomes normal in the late vegetative phase (Tamura et al., 1992; Itoh et al., 2000). In the early vegetative phase, the phyllotaxy is irregular, and leaves are narrow except the first three leaves that show normal alternate phyllotaxy. To examine the phenotypes of *moril sho1* double mutant, heterozygous *moril-1* plant was fertilized with pollen from heterozygous *sho1-1* plant. The segregation of phenotypes was examined in the F<sub>2</sub> progenies.

## RESULTS

The five *moril* mutants (*moril-1* to *moril-5*) showed indistinguishable phenotypes. Analysis was mainly carried out on *moril-1* and *moril-2* plants.

#### Growth of *moril* plants

Until 7 days after sowing, *moril* plants grew very slowly compared to the wild type that germinated at 1 or 2 days after sowing, and two or three leaves emerged within 7 days after sowing. However, in *moril*, the first three leaves emerged almost simultaneously 7 days after sowing, and subsequently many leaves and branches were produced quickly (Fig. 1). In spite of the rapid production of leaves and branches, their growth was severely suppressed. In wild-type rice, upper leaves grew larger, whereas *moril* plants invariably produced small leaves. In addition, although the upper four or five internodes of the wild-type stem elongated at around floral induction, internode elongation was not observed in *moril*. As a result, the *moril* plants showed an extreme dwarfism, about 1 cm in height 1 month and about 5 cm 7 months after sowing (Fig.

1C,F). The resulting plant form was semi-spherical due to the large number of short leaves and tillers (Fig. 1F,G).

In these cultural conditions (28°C, 14L:10D), we could not recognize any sign of reproductive development (differentiation of flag leaf or panicle primordia) in *moril* even 1 year after sowing, whereas in the wild type, panicles were observed before 4 months after sowing. Even when the short day treatment (10L:14D) was applied to 5- to 7-month-old *moril* plants for 2 months, reproductive development could not be induced. Thus, it is considered that *moril* is not competent for floral induction even at older stages.

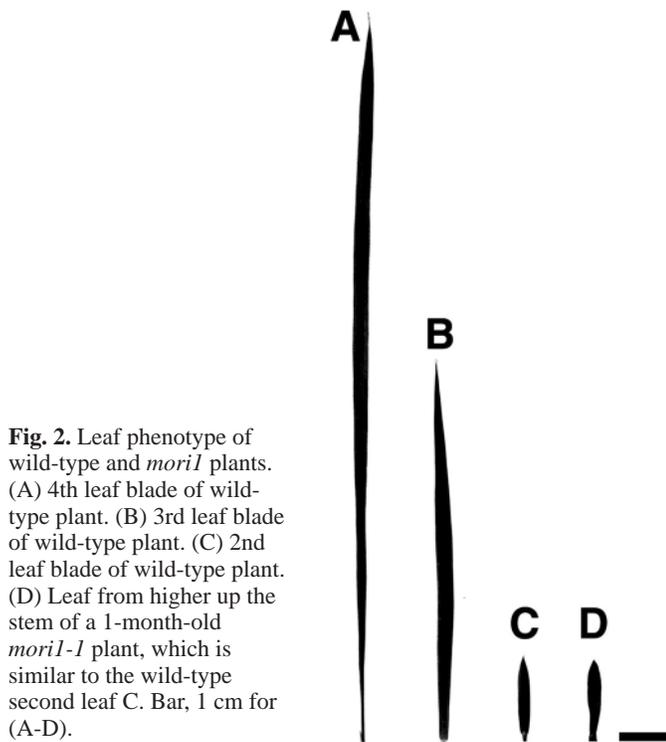
In summary, the external characteristics of *moril* plants are: (1) rapid production of leaves and branches, (2) small leaf size, and (3) inability to induce reproductive phase.

#### Leaf morphology

The shape and size of *moril* leaves were very similar to those of the wild-type second leaf (Fig. 2). Leaf size of *moril* was measured in detail. In the wild type, we can easily specify the main stem and each leaf position on it throughout development. However, since *moril* rapidly differentiated a large number of leaves and tillers, we could not specify the main stem and the leaf position except at the early stages. We examined the first ten leaves and mature leaves in the upper part of the highest stem of 7-month-old plants.

In the wild type, leaves became longer with the elevation of leaf position in both blade and sheath reaching the maximum size at 12th leaf (Fig. 3A-C). In contrast, the leaf length of *moril-1* and *moril-2* did not vary with leaf positions; the blade was about 1.5 cm and the sheath 1 cm (Fig. 2, Fig. 3A,C), which were comparable to those of the wild-type 2nd leaf. Similarly, the blade width of the wild type increased with the leaf number, but *moril* showed nearly constant blade width, again comparable to the size of the wild-type 2nd or 3rd leaf (Fig. 3B).

The ratio of leaf blade length to width in the wild-type plant, representing leaf shape, rapidly increased through 4th leaf and



**Fig. 2.** Leaf phenotype of wild-type and *moril* plants. (A) 4th leaf blade of wild-type plant. (B) 3rd leaf blade of wild-type plant. (C) 2nd leaf blade of wild-type plant. (D) Leaf from higher up the stem of a 1-month-old *moril-1* plant, which is similar to the wild-type second leaf C. Bar, 1 cm for (A-D).

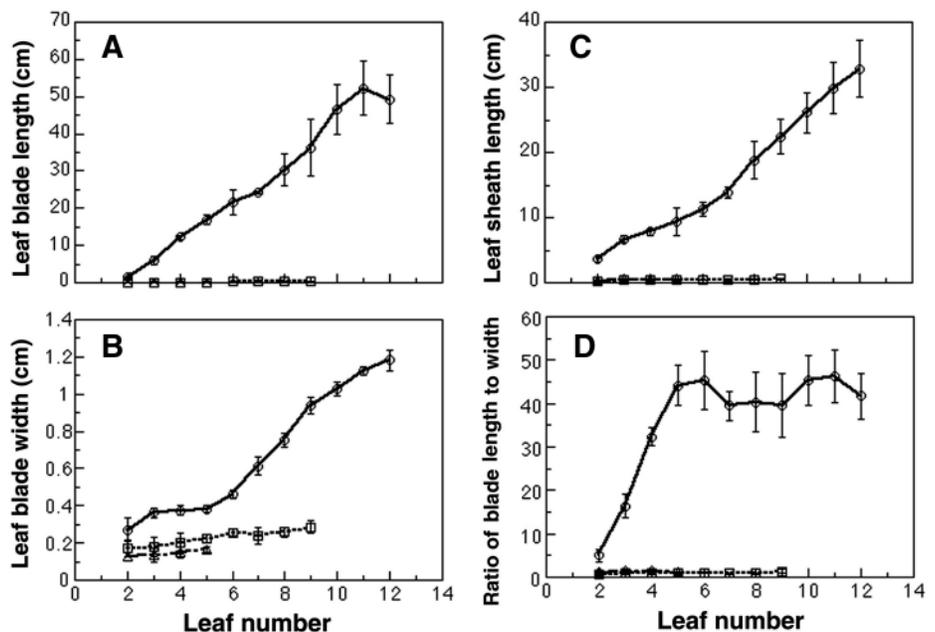
subsequently became nearly constant. However, in *moril-1* and *moril-2*, the ratio was constant to be about 5 irrespective of the leaf position (Fig. 3D), the value being equal to that of the wild-type 2nd leaf.

For anatomical examination of the leaf, we made cross sections of the wild-type leaves and mature upper leaves of *moril-1* and *moril-2*, 42 days after sowing (Fig. 4). A clear difference of leaf structure was detected at the midrib. In the wild type, the midrib was not developed in the 2nd leaf (Fig. 4A,D). The 3rd and 4th leaves showed incomplete formation of midrib, i.e., the midrib was observed in the proximal 1/3 ~ 2/3 of blade length. In leaves higher than the 4th leaf, midrib was formed in more than 90% of blade length (Fig. 4B,E). In contrast, *moril* leaves at 42 days after sowing, presumably higher than 15th leaf, did not exhibit apparent midribs (Fig. 4C,F) as in the wild-type 2nd leaf. These results indicate that *moril* leaves at any position are structurally similar to the wild-type 2nd leaf.

### Stem structure

From the above results, we conclude that *moril* repeats the production of the 2nd

**Fig. 3.** Change in leaf size with position. (A) Leaf blade length. (B) Leaf blade width. (C) Leaf sheath length. (D) Leaf blade to sheath ratio. The *moril-1* (squares) and *moril-2* (triangles) leaves show small and constant sizes compared to the growing leaves in the wild type (circles). Vertical bars indicate s.e.



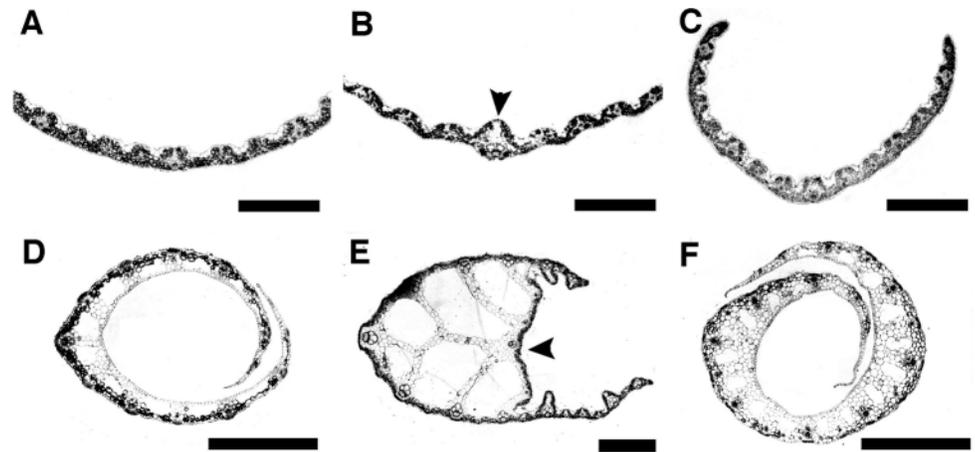
leaf. Then we examined the stem structure, since leaves and stem originate together from the shoot apical meristem. In the wild-type seedlings, the young stems showed no differentiation of node and internode where 2nd and 3rd leaves were inserted, and this state was maintained in the basal region of the stem throughout the development. In the longitudinal section of wild-type stem 1 month after sowing, nodes and internodes were identified in the upper region, but they were not differentiated in the basal region where 2nd and 3rd leaves were inserted (Fig. 5A). In addition, vascular bundles were randomly oriented in the basal region, in contrast with the regular orientation in the adult stem (Fig. 5A).

Interestingly, the *moril* stem even 7 months after sowing did not differentiate node and internode, and showed randomly oriented vascular bundles (Fig. 5B). This architecture of *moril* stem is very similar to that of the wild-type basal stem (compare Fig. 5B with the basal region in 5A). Therefore, *moril* maintains the stem structure of the juvenile phase.

### Rate of leaf initiation (plastochron)

The rapid production of leaves and tillers in *moril* suggests a short plastochron (the elapsed time from the initiation of one leaf primordium to that of the next one). We asked whether the rapid leaf production reflected the juvenility of *moril*. The wild-type plants show a considerable change of plastochron during the life cycle. The first three leaves that were produced in the embryo were estimated to have short plastochrons because the first leaf primordium was differentiated 5 days after pollination, and 2nd and 3rd leaf primordia initiated 6 and 8 days after pollination, respectively. From the examination of embryo sections, the mean plastochron of 2nd and 3rd leaves was estimated to be 1.56 days. After germination, the rate at which leaf primordia were initiated was slowed. Sectioning of shoot apices 1-16 days after sowing revealed that 4th, 5th, 6th and 7th leaf primordia were formed around 4, 7, 11 and 15 days after sowing, respectively, giving the mean plastochron of 5th through 7th leaves to be 3.7 days (Table 1).

The *moril-1* and *moril-2* mutants differentiated 4th, 5th,



**Fig. 4.** Anatomy of wild-type and *moril-1* leaves. (A,D) Wild-type 2nd leaf. (B,E) Wild-type 11th leaf. (C,F) *moril-1* leaf from a higher position of a 42-day-old plant. (A-C) Leaf blade. (D-F) Leaf sheath. A large midrib (arrowhead) develops in the wild-type 11th leaf (B,E), but midrib is not prominent in the wild-type 2nd leaf (A,D) and *moril-1* leaves (C,F). Bars, 500  $\mu$ m.

6th, 7th and 8th leaf primordia about 7, 9, 11, 12 and 14 days after sowing, respectively. Detailed measurement showed that the mean plastochron of 5th through 8th leaf in *moril-1* and *moril-2* was estimated to be 1.85 days in *moril-1* and 1.64 days in *moril-2* (Table 1). These results indicate that *moril* initiates leaves rapidly at a rate comparable to that of the wild-type 2nd and 3rd leaves produced in the embryo. Thus, it is suggested that the rapid leaf production of *moril* after germination results from the maintenance of a short plastochron characteristic to the wild-type juvenile phase.

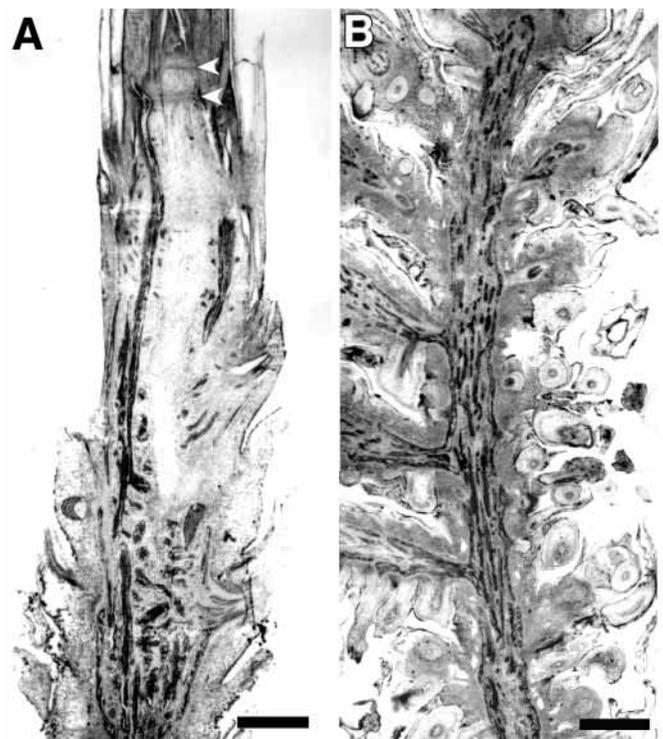
#### Size of shoot apical meristem (SAM)

It is known that the size of the SAM changes during development. We examined the SAM sizes of *moril* and the wild-type every week after sowing (Fig. 6). In the wild type, the SAM became enlarged in both height and width with the developmental progress (Fig. 6A-F). The SAM size at the late vegetative stage was about twice that at the seedling stage. In contrast, SAM size of *moril* did not change significantly throughout development (Fig. 6G-M). The SAM size of *moril* was nearly equal to that of the wild-type mature embryo in which three foliage leaves were formed. This shows that the *moril* maintains the SAM size characteristic to the juvenile phase.

#### Cell division activity in shoot apical meristem

The rapid leaf production of *moril* suggests that cell divisions in the SAM are more active than in the wild-type SAM. Furthermore, since the plastochron of *moril* is comparable to that of the wild-type juvenile phase, the cell division rate in *moril* SAM may be close to that of the embryonic SAM when the 2nd or 3rd leaf primordium is being formed. Here we examined the cell division activity in the SAM by in situ hybridization using histone *H4* as a probe. Histone *H4* is known to be expressed specifically in S-phase of the cell cycle.

In the wild-type SAM 7 days after sowing, hybridization

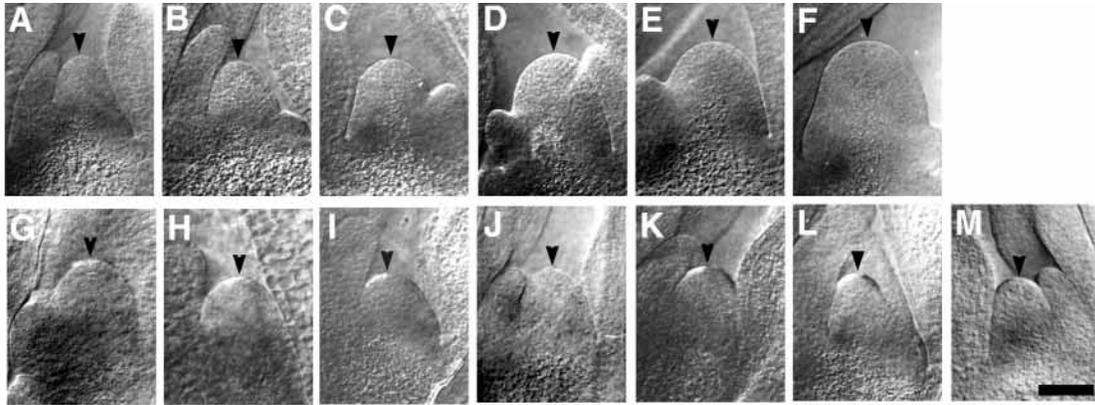


**Fig. 5.** Stem structure of wild-type and *moril-1*. (A) Wild-type stem 1 month after sowing, in which nodes (arrowheads) and internodes are apparent in the upper region, but they are not differentiated, and vascular bundles are randomly oriented in the basal region. (B) *moril-1* stem 7 months after sowing, where vascular bundles are randomly oriented and nodes are not formed in any region. Bars, 1 mm.

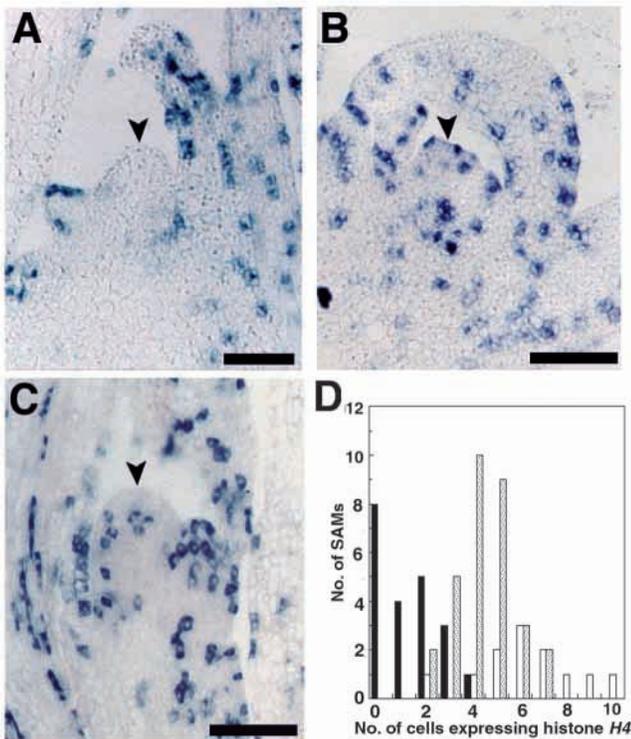
signals of histone *H4* were detected in 0-3 cells per median longitudinal section (Fig. 7A,D). Higher cell division activity, however, was observed in the wild-type embryonic SAM 6-8

**Table 1.** Leaf production in *moril*

	Wild type		<i>moril-1</i>	<i>moril-2</i>
	2nd and 3rd leaves	5th-7th leaves	5th-9th leaves	
Plastochron (days)	1.56	3.70	1.85	1.64
Leaf emergence rate (days)	–	3.72	1.80	1.23

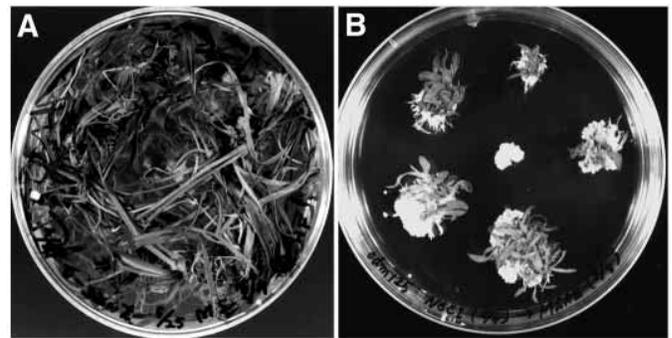


**Fig. 6.** Change of SAM size during development in wild-type and *moril*. (A-F) Wild-type SAM. (G-M) *moril-1* SAM. (A,G) 1 week; (B,H) 2 weeks; (C,I) 3 weeks; (D,J) 4 weeks; (E,K) 5 weeks; (F,L) 6 weeks; (M) 7 months, after sowing. In the wild type, the SAM becomes larger as development progresses, but in *moril-1*, the SAM size is almost constant irrespective of the developmental stage. Arrowheads indicate the SAM. Bar (in M), 50  $\mu$ m (A-M).



**Fig. 7.** Expression of histone *H4* RNA in wild-type and *moril* SAM 1 week after sowing. (A-C) SAM of (A) wild-type seedling 7 days after sowing in which histone *H4* is not expressed; (B) wild-type embryo 6 days after pollination in which several cells express histone *H4*; (C) *moril-1* seedling 7 days after sowing in which several cells express histone *H4*. (D) The number of cells expressing histone *H4* per median longitudinal section of the SAM. In the wild-type SAM 7 days after sowing (solid bar), histone *H4* is expressed in only one or two cells, in contrast to the frequent expression (4-6 cells) in the wild-type embryo 6 days after pollination (dotted bar) and *moril-1* 1 week after sowing (open bar). Bars, 50  $\mu$ m.

days after pollination when 2nd and 3rd leaf primordia were being formed (Fig. 7B). At this stage, signals were detected in 3-6 cells per median, longitudinal section of most SAMs (Fig.



**Fig. 8.** Regeneration of adventitious shoots from scutellum-derived calli. (A) Normal adventitious shoots from wild-type calli. (B) Adventitious shoots with *moril* phenotype regenerated from *moril-1* calli.

7D). In the *moril* SAM seven days after sowing, 4-8 cells showed hybridization signals (Fig. 7C,D). Considering the fact that the *moril* SAM after germination was slightly larger than that of the wild-type embryo 6 or 7 days after pollination, the cell division activity in the SAM is comparable in *moril* and wild-type embryos. Accordingly, it is concluded that *moril* maintains the high cell division activity of the embryonic (juvenile) SAM after germination.

### Phenotype of adventitious shoots

We asked whether the *MOR11* gene functions only in embryo-derived shoots or also in adventitious shoots, which were induced from scutellum-derived calli. All the adventitious shoots regenerated from *moril* calli showed the same phenotypes as the seedlings (Fig. 8). Therefore, *MOR11* gene is considered to function generally in shoot formation.

### Photosynthesis

Since *moril* plants were viable only in nutrition medium containing sucrose, and withered soon when transplanted to soil, a possibility is suggested that *moril* plants are heterotrophic. We therefore measured photosynthetic activities of higher leaves of 3-month-old *moril* plants and the 2nd, 3rd

**Table 2. Photosynthesis in *mori1* leaves**

	Wild type			<i>mori1-1</i> *
	2nd leaf	3rd leaf	4th leaf	
Photosynthetic rate (mmol CO <sub>2</sub> /m <sup>2</sup> /second)	2.91±0.38	18.12±0.81	22.01±1.36	2.61±0.73

Green leaves in the upper positions of 3-month-old *mori1-1* plants were used. Values are mean±s.e.m.

and 4th leaves of the wild type. In the wild type, the apparent photosynthetic rate of the 2nd leaf was extremely low, approx. 1/7 of that of the 4th leaf (Table 2). The 3rd leaf showed a relatively high activity. It is already known that higher leaves than 4th have high photosynthetic rate (Kura-Hotta et al., 1987). Thus, the wild-type plant is heterotrophic through the 2nd-leaf stage. We confirmed that the wild-type embryos separated from the endosperm could germinate but failed to grow in the nutrition medium lacking a carbon source (data not shown).

As for *mori1*, we first examined whether or not sucrose in the culture medium was essential for the growth. When transplanted to the medium lacking sucrose, *mori1* plants became chlorotic and etiolated within 3 to 4 weeks (Fig. 9). Measurement of photosynthesis showed that *mori1* leaves of 3-month-old plants had very low apparent photosynthetic rate as did the wild-type 2nd leaf (Table 2). This low photosynthetic rate of *mori1* leaves would not be sufficient for maintaining the growth of *mori1* plant rapidly producing leaves, and would cause death when transplanted to soil. These results suggest the heterotrophism of *mori1*, and further support that *mori1* reiterates 2nd-leaf differentiation.

#### ***mori1 sho1* double mutant**

*sho1* plants show irregular phyllotaxy and small narrow leaves at the early stage after germination, but become normal at later stages (Tamura et al., 1992; Itoh et al., 2000). However, the first three leaves of *sho1* produced in the embryo are relatively normal with alternate phyllotaxy, and random phyllotaxy is observed after the fourth leaf. We crossed *MOR1/mori1-1* heterozygous plant with *SHO1/sho1-1* plant. Among 124 plants of a F<sub>2</sub> population in which both *mori1* and *sho1* plants were segregated, 67 plants were normal, 32 showed *mori1* phenotype, and 25 were *sho1*-type. This segregation ratio fitted well a 9:4:3 ratio. Thus, the *mori1 sho1* double mutant showed a phenotype indistinguishable from *mori1* plant. This result



**Fig. 9.** Effect of sucrose in culture medium on *mori1* growth. A *mori1* plant cultured in the absence of sucrose (–) for 25 days, becomes chlorotic and etiolated, in contrast to the green plant grown in the presence of sucrose (+).

strongly supports that *mori1* plant remains at the 2nd-leaf stage.

## **DISCUSSION**

### ***mori1* is a unique heterochronic mutation**

This is the first report of a mutant in which juvenile-adult phase change is completely blocked. All the traits of *mori1* examined in this study are equivalent to those of the 2nd-leaf stage of the wild type. Thus, it is concluded that *mori1* plants maintain juvenile phase and fail to become adult. This conclusion is supported by the following facts: (1) *mori1* does not flower even after long periods of cultivation of more than one year and after the short-day treatment; (2) *mori1* leaves at any position are similar to the 2nd leaf of the wild type; (3) the *mori1* stem never differentiates nodes and internodes and shows the same anatomical features as the basal part of the wild-type stem where 2nd and 3rd leaves are inserted; (4) the size and structure of *mori1* SAM is comparable to that of the wild-type SAM of mature embryo; (5) plastochron and cell division activity in *mori1* SAM are comparable to those of the wild-type embryo when 2nd and 3rd leaf primordia are being formed, and (6) *mori1* plants are heterotrophic like the wild-type plants at the 2nd-leaf stage. Accordingly *mori1* is a heterochronic mutant in which the juvenile-adult phase change is blocked.

To date, *mori1*-like mutants have not been reported in other plant species. Several heterochronic mutants associated with juvenile-adult phase change have been identified in maize (Bassiri et al., 1992; Dudley and Poethig, 1991; Dudley and Poethig, 1993; Evans et al., 1994; Moose and Sisco, 1994). They show heterochrony in that juvenile-specific traits are extended into the adult/reproductive phase. Then in these mutants, the juvenile-adult phase change takes place incompletely or is delayed. In contrast, adult traits are completely suppressed in *mori1*. In this respect, *mori1* is a unique mutant in which the developmental progress from juvenile to adult phase is suppressed, and repeats the juvenile program. It is suggested that the onset of an advanced stage (adult phase) is closely correlated with the termination of the former stage (juvenile phase). A similar mutant is obtained in the nematode *Caenorhabditis elegans*, in which a variety of heterochronic mutants have been isolated, and detailed genetic and molecular analyses have been achieved (Ambros and Horvitz, 1984; Ambros and Moss, 1994). One of the *lin-14* alleles of *C. elegans* blocks the advanced stage and causes the repetition of the early stage. This phenotype may be comparable to the coupling of the loss of adult phase and the repetition of juvenile phase in *mori1*. In contrast, in rice *plal* mutants, the reproductive phase starts independently of the

vegetative phase, and the vegetative and reproductive programs are cofunctioning (Itoh et al., 1998). This means that the initiation of reproductive phase does not require the termination of vegetative phase. Accordingly, the mechanism of juvenile-adult phase change appears to be different from that of the vegetative-reproductive phase change.

### Significance of heterochronic mutations in plant development

The shoot architecture of *mori1* is far from that of the wild-type rice. However, the present data shows that the organs of *mori1* are normal at the 2nd-leaf stage. No morphological abnormalities are detected in each organ. This point suggests an important role of heterochronic mutation on the development and morphological evolution. That is, a heterochronic mutation may radically modify the plant architecture without impairing morphogenetic genes.

The *mori1* mutation affects all the traits of shoot such as leaf size, leaf shape, leaf anatomy, stem structure, SAM size and SAM activity, all of which resemble those of the 2nd-leaf stage of the wild type. This implies that *MOR1* plays central role in juvenile-adult phase change. In maize, *glossy15* affects only the leaf epidermis (Evans et al., 1994; Moose and Sisco, 1994). This mutant expresses juvenile leaf epidermis in the adult leaves, and is considered to be specifically associated with leaf epidermal phase change. Recently, phase identity of maize leaves was shown to be determined after initiation (Orkiszewski and Poethig, 2000). Although it is not clear whether or not the SAM regulates the juvenility of leaves in *mori1*, it is supposed that there exist many genes associated with the phase change of specific traits downstream of gene(s) regulating the phase change of the whole plant.

The *Teopod1*, *Teopod2*, *Corngrass* (= *Teopod3*), and *Hsf1-O* are maize heterochronic mutations that affect a large number of traits (Abeldon and Tracy, 1996; Bassiri et al., 1992; Bertrand-Garcia and Freeling, 1991; Dudley and Poethig, 1991; Dudley and Poethig, 1993; Poethig, 1988). The wild-type genes are considered to act in the phase change of many traits. It is significant that they are dominant mutations, indicating that their mutant phenotypes are interpreted as ectopic expression of juvenile traits in the adult/reproductive phase, and juvenile and adult traits are coexisting. However, *mori1* is a loss-of-function mutation, and blocks the juvenile-adult phase change of all the traits. Thus, the *MOR1* functions either in the initiation of adult phase or in the termination of juvenile phase of all traits. Although there is no data supporting either of these two possibilities, the phenotype of *mori1* suggests that there exists a key gene that controls the temporal development of total shoot architecture unifying all the traits.

### Juvenility in grass family

In rice, the first three leaves differentiate in the embryo, although they emerge from the embryo only after germination. As shown in the present data on stem anatomy, midrib development and other traits of the wild type, the first two or three leaves exhibit juvenility. Thus, *mori1* can be regarded as perpetuating the late stage of seed development. Generally, grass embryos are highly developed and complicated, differentiating several leaves and several embryo-specific organs. This is in contrast to the other monocot and dicot

embryos, in which foliage leaves are not present. This indicates that in grasses, early post-germination stage is incorporated into embryo. In fact, maize shows juvenility in the first five leaves that are produced in embryo. Thus, a part or most of juvenile vegetative phase elapses during seed development in grasses (Freeling et al., 1992). In other words, grasses can be regarded as a heterochronic species in that embryogenesis and juvenile phase successively occur during seed development, resulting in the shift of dormant period of embryo from the end of embryogenesis to the end of juvenile phase.

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