

# The *ich1* gene of the mushroom *Coprinus cinereus* is essential for pileus formation in fruiting

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## SUMMARY

The formation of the pileus in homobasidiomycete fungi is essential for sexual reproduction, because the pileus bears the hymenium, a layer of cells that includes the specialised basidia in which nuclear fusion, meiosis and sporulation occur. The developmental mutant *ichijiku* of *Coprinus cinereus* fails to develop a differentiated pileus at the apex of the primordial shaft, which is the basal part of the fruit-body primordia and formed in an early stage of fruit-body differentiation. Genetic analysis indicates that this phenotype is caused by a recessive mutation in a single gene (*ich1*). The *ich1* gene was mapped to chromosome XII using restriction fragment length polymorphism markers and the marker chromosome method, and cloned by

complementation using a chromosome-XII-specific cosmid library. The *ich1* gene encodes a novel protein of 1,353 amino acids. The Ich1 amino-acid sequence contains nuclear targeting signals, suggesting that the Ich1 protein would function in the nucleus. Northern blot analysis indicates that the *ich1* gene is specifically expressed in the pileus of the wild-type fruit-body. No *ich1* mRNA was detected in the *ichijiku* mutant, consistent with loss of the promoter region of *ich1* in the mutant genome. These data demonstrate that the *ich1* gene product is essential for pileus formation.

Key words: Developmental mutant, Ich1, Pileus, Fruit-body morphogenesis, Basidiomycete, *Coprinus*

## INTRODUCTION

The mushroom fruit body of homobasidiomycete fungi is a highly differentiated structure. Fig. 1 illustrates the development of the normal fruit-body of *Coprinus cinereus*. The fruit-bodies are initiated as an aggregation of hyphae, producing hyphal knots of about 0.5 mm or less in diameter. Further growth of the hyphal knots includes an increase in hyphal density and the differentiation of a layer of veil cells, which cover a compact core composed of highly branched short cells (Valk and Marchant, 1978). By the time the primordia have increased to 0.8 mm, the major tissues of a mature fruit body, including the pileus and stipe, are discernible (Fig. 3C), showing that differentiation occurs very rapidly (Matthews and Niederpruem, 1973; Reijnders, 1979; Moore et al., 1979; Moore, 1996). The fruit-body primordium gradually enlarges (Fig. 1A,B) and then enters into a rapid maturation phase in response to certain light conditions (Morimoto and Oda, 1973; Kamada et al., 1978). In the maturation phase, the stipe elongates (Fig. 1C-F) (Gooday, 1985; Kamada, 1994) and the pileus expands to provide an efficient surface to disperse basidiospores which are produced on its undersurface (Fig. 1E-G) (Moore et al., 1979; Rosin et al., 1985).

The fruit-bodies produced by *C. cinereus* are described as angiocarpic (Burnett, 1968; Reijnders, 1986). This means that the pileus develops endogenously within the sub-apical region of the primordial shaft. The primordial shaft is the basal part

of the fruit-body primordium and formed at an early stage of fruit-body differentiation. Hyphal tissue grows laterally from the sub-apical region of the primordial shaft in a centrifugal direction, and gives rise to the annular margin of the pileus (Fig. 2) (Corner, 1934; Burnett, 1968; Matthews and Niederpruem, 1973; Reijnders, 1979; Rosin et al., 1985). The formation of the pileus is essential for sexual reproduction. The pileus bears gills, on both sides of which is the hymenium, a layer of cells that includes the specialised basidia in which nuclear fusion, meiosis and sporulation occur.

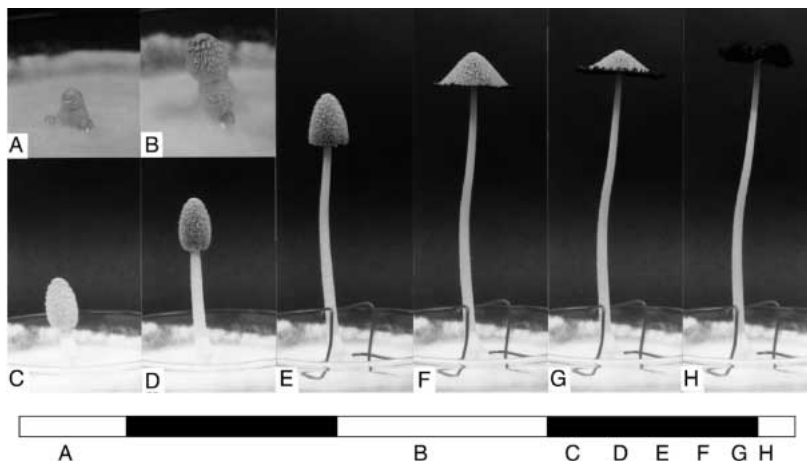
Although the stages in fruit-body development of *C. cinereus* have been well studied at the microscopic level and many of genes involved in fruiting have been identified by mutations (Kimura and Fujio, 1961; Takemaru and Kamada, 1972; Gibbins and Lu, 1982; Kanda and Ishikawa, 1986; Chiu and Moore, 1990), little is known as yet about the molecular mechanisms underlying differentiation and pattern formation. In this report we describe the molecular analysis of a developmental mutant of *C. cinereus*, *ichijiku*, which is blocked at a critical stage in fruit-body development such that it is unable to develop a differentiated pileus (Figs 2C, 3B,D) and cannot produce basidiospores.

## MATERIALS AND METHODS

### *Coprinus* strains and genetic techniques

The *ichijiku* mutant strains KF0#2 (mating type: *A91B91*) and KF0#9

**Fig. 1.** Wild-type fruit-body development in *Coprinus cinereus*. (A) Fruit-body primordium of about 5 mm in height. (B-H) Fruit-body maturation. Meiosis occurs in basidia between the stages shown in B-C; sporulation occurs during C-E, making the pileus black. The bar below the picture indicates a 12-hour light/12-hour dark regime.



(A92B92) were isolated in progeny from a wild-type fruit-body collected in the field. 5302 (A2B2) is a standard monokaryotic strain in our laboratory and was used as a wild-type backcross parent. KF<sub>2</sub>#1 is a wild-type strain that has the same genetic background as those of KF<sub>0</sub>#2 and KF<sub>0</sub>#9. A recipient strain for transformation, L6 (A3B1 *ich1-1 trp1-1,1-6*), was constructed by crossing KF<sub>0</sub>#9 (A92B92 *ich1-1*) to strain #292 (A3B1 *trp1-1,1-6*). A dikaryotic strain 5026+5132 was used to observe wild-type fruiting and to extract total RNAs in various stages, because this strain vigorously produces wild-type fruit-bodies. Standard *C. cinereus* culture conditions were used (Kamada et al., 1984). Crosses and random spore analysis were performed as previously described (Kamada et al., 1984). To test fruiting phenotypes, the germlings isolated were mated with KF<sub>0</sub>#2 (A91B91 *ich1-1*) on slant medium, because KF<sub>0</sub>#2 was compatible with all of the progeny from crosses between 5302 and KF<sub>0</sub>#9, with mutant progeny selected for backcrossing, and with *trp*<sup>+</sup> transformants derived from the recipient strain L6.

#### Bright-field and fluorescence microscopy

Primordia were picked from plate cultures, embedded in ice at -5 to -10°C, and then cryosectioned with an MA101 freezing microtome (Komatsu Solidate Co., Ltd, Tokyo) to give 50 µm thick samples. The sections were observed with a Zeiss microscope equipped with epifluorescence optics. In fluorescence microscopy, a UV-H365 filter set was used.

#### DNA manipulations

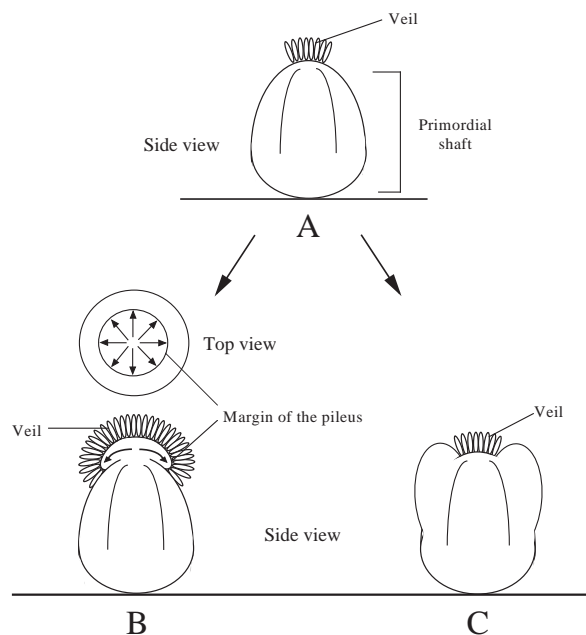
General molecular biological techniques were carried out according to protocols of Sambrook et al. (1989). *C. cinereus* genomic DNAs were isolated from protoplasts prepared as described by Zolan et al. (1992). The pellet of protoplasts was washed once with MM (0.5 M mannitol, 0.05 M maleate, pH 5.5), resuspended in a small quantity of residual MM solution, mixed with 1.5 ml of extraction buffer (5 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 0.25% SDS, 10 µg/ml RNase A) prewarmed at 65°C and incubated at 65°C for 15 minutes. DNAs were purified with 1.5 ml of phenol:chloroform:isoamylalcohol (25:24:1) and subsequently with 1.5 ml of chloroform:isoamylalcohol (24:1), precipitated with NaCl and ethanol, and resuspended in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). Probe-labeling and hybridization was performed using the Gene Images system (Amersham).

Clones carrying a fragment from each chromosome of 5302 were a gift from Dr T. Morinaga of Hiroshima University and used as RFLP markers for chromosomes.

#### CHEF electrophoresis of *Coprinus* chromosomal DNA

Plugs for CHEF (clamped homogeneous electric fields) electrophoresis were prepared as described by Zolan et al. (1992) except that protoplasts were embedded in 0.5% low melting point

agarose and the plugs were treated with NDS (10 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 1% sodium lauroyl sarcosinate) at 37°C for 24 hours (Miao et al., 1991). To separate smaller chromosomes of *C. cinereus*, CHEF electrophoresis was run using 120 ml of 0.9% PFC grade agarose (BioRad) gels at 100 V for 82 hours with a 480 seconds pulse time, in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) at 10-11°C, exchanging the buffer every day.



**Fig. 2.** Schematic diagrams illustrating development of wild-type and *ich1-1* mutant fruit-body primordia. (A) The primordial shaft is formed at an early stage of fruit-body differentiation and becomes the basal part of the fruit-body primordium at later stages, but this stage is actually not apparent because of rapid differentiation of cells. *ichijiku* phenotype suggests the presence of this stage in the developmental sequence. (B) In wild-type, the pileus develops within the sub-apical region of the primordial shaft. Hyphal tissue of the sub-apical region grows in a centrifugal direction, giving rise to the annular margin of the pileus. (C) The *ichijiku* mutant has a defect that blocks the centrifugal growth of hyphal tissue that generates the inverted-bowl-like structure of the pileus. Nevertheless, cells of the primordial shaft in the mutant can gradually elongate and sometimes undergo rapid elongation that causes the *ichijiku* primordia to rupture (Fig. 5A).

**Chromosome-XII-specific cosmid library screening, *ich1* gene cloning and sequencing**

A chromosome-XII-specific cosmid library was constructed using LLC5200 cosmid vector containing the *trp1*<sup>+</sup> gene as a selectable marker in *C. cinereus* (Pukkila and Casselton, 1991), as described by Zolan et al. (1992). The library was composed of 480 clones (96 clones × 5 plates). 12 clones were cultured on a plate of LB/ampicillin solid medium, mixed and subjected to miniprep with FlexiPrep Kit (Pharmacia Biotech). The pooled DNAs (2.0 µg) were used to transform protoplasts (1×10<sup>9</sup> cells/ml, 100 µl) of L6 strain, as described by Binnering et al. (1987). 50 *trp*<sup>+</sup> transformants from each pooled DNAs were cultured on minimum medium for 2 days, to purify the transformed mycelium, and mated with KF<sub>0</sub>#2 on slant medium to test fruiting phenotypes. Three of the 50 *trp*<sup>+</sup> transformants from 1H pooled DNAs produced completely rescued fruit-bodies. Then a single cosmid clone, 1H7, was identified to carry the rescuing activity.

The 1H7 cosmid clone was digested with *Apa*I, *Bgl*II, *Eco*RI, *Xba*I, *Pst*I and *Hind*III, and used to transform L6 strain. When the restriction enzymes used cut the *trp1* gene in LLC5200, the digests were co-transformed with pCc1003 carrying the intact *trp1* gene (Skrzynia et al., 1989). Overlapping clones with 1H7 were identified from the chromosome-XII-specific cosmid library using the 1H7 insert as a probe by dot hybridization and also used to transform L6 strain. Since two *Pst*I fragments were shared only between 1H7 and 5G5 carrying the rescuing activity, these fragments were subcloned into pCc1003 and used to transform L6 strain. A 5.4 kb *Pst*I fragment with the rescuing activity was re-subcloned into pBlueps2, deleted from each side with Nested Deletion Kit (Pharmacia Biotech) and sequenced with a model 373A DNA sequencer (Perkin Elmer) using the ABI PRISM DNA Sequencing Kit (Perkin Elmer). A 14 kb *Eco*RI fragment from 1H7 was subcloned into pUC118. A 4.5 kb *Sal*I fragment and a 3.5 kb *Pst*I fragment from the *Eco*RI fragment were further subcloned into pUC118 and used for sequencing a region downstream of the 5.4 kb *Pst*I fragment.

**Northern analysis**

Total RNAs were isolated from 1.0 g (wet weight) of vegetative mycelia cultured for 7 days in a plate, of primordia picked from a plate culture, and of pileus or stipe tissues, according to a procedure described by Yeager Stassen et al. (1997). 20 µg of total RNA was fractionated by electrophoresis in a 1% agarose formaldehyde gel, transferred to a Hybond-N<sup>+</sup> membrane (Amersham), and fixed by UV crosslinking (Stratagene). The *Sal*I-*Pst*I 2.7 kb fragment from a cloned 5.4 kb *Pst*I genomic fragment was labeled and used to probe the northern blot according to the instructions for the Gene Images (Amersham).

**Isolation of *ich1* cDNA**

Poly(A)<sup>+</sup> RNAs were isolated with mRNA Kit Oligo[dT]30 (BIO 101, Inc.) from total RNA extracted from the pileus at 14:00 on the day of fruit-body maturation. cDNAs were synthesized with the Marathon cDNA Amplification Kit (Clontech). The gene-specific primers (#1, 5'-ATTGCGAGTATGAGCCTATG-3'; #2, 5'-TAACAGCAGCCAT-AACCTTC-3'; #3, 5'-TGCTTTGGACGGTGGTTGATCTATTCCG-3'; #4, 5'-GGTATGCGAAGGAACAATCTGACAACCG-3'; #5, 5'-TATCGTTGCTTTGCCTTGCCATTCCG-3'; #6, 5'-TCTGCTGT-TGTATCTCCTGCTCCTGTAC-3'), whose locations are shown in Fig. 6, were used to amplify *ich1* cDNA with AmpliTaq Gold DNA Polymerase (Perkin Elmer). The PCR products were cloned into pCR2.1 vector using Original TA Cloning Kit (Invitrogen) and sequenced. These primer sets were also used to amplify the *ich1* corresponding region in the mutant genome.

**RESULTS**

**Isolation and genetic analysis of *ichijiku***

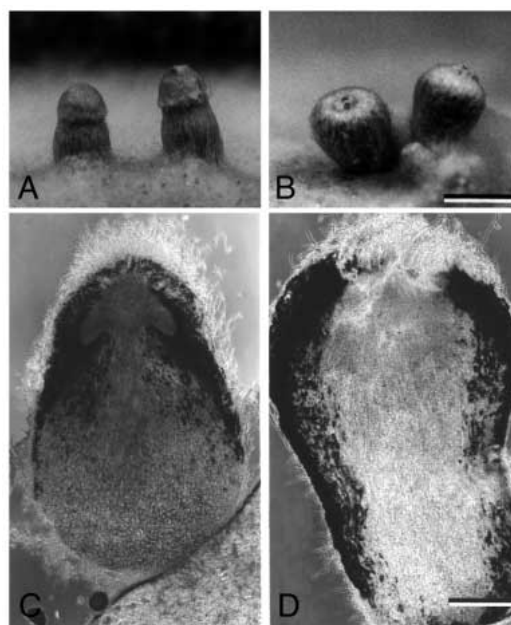
The *ich1* mutant allele, *ich1-1*, was discovered as a

spontaneous mutation in the progeny of a normal fruit-body collected in the field. The progeny of the fruit-body were isolated and mated in all combinations. Some of the mated dikaryons formed an odd shaped fruit-body primordium (Fig. 3B) which never matured to produce basidiospores. Because of the shape of the primordium, we named this mutant '*ichijiku*', which means fig in Japanese and for which the Chinese letters mean a fruit without flowering.

To determine whether *ichijiku* is inherited in a Mendelian fashion, we crossed an *ichijiku* mutant strain, KF<sub>0</sub>#9, to a wild-type strain, 5302, and isolated the F<sub>1</sub> progeny from the cross. The progeny were examined for fruiting phenotypes by crossing with a compatible *ichijiku* mutant strain, KF<sub>0</sub>#2. An *ichijiku* strain was backcrossed four times to 5302, and these progeny were also examined for fruiting phenotypes (Table 1). The wild-type and *ichijiku* primordia segregated in a 1:1 ratio in all generations, except for BC<sub>2</sub>. In addition, the fruiting phenotypes in the F<sub>1</sub> progeny segregated independently of both mating type loci. These genetic analyses demonstrated that the *ichijiku* phenotype is the result of a recessive mutation (*ich1-1*) in a chromosomal gene.

***ichijiku* phenotypes**

Wild-type and *ichijiku* primordia of about 5 mm in height are shown in Fig. 3A and 3B, respectively. In the wild-type, the rudimentary pileus can be clearly recognised on the primordial shaft. As seen in Fig. 3C, even in primordia that are only 1.0 mm in height, the rudimentary pileus is discernible and the downward growing hyphae on the underside of the pileus are



**Fig. 3.** (A,B) Comparison of wild type (A) and *ichijiku* mutant (B) fruit-body primordia. In the *ichijiku* mutant, the rudimentary pileus is missing. However, the surface of the mutant primordia appears to be similar to that in the wild-type. (C,D) Comparison of vertical median sections of a wild-type (C) and an *ichijiku* mutant (D) fruit-body primordium. Differentiation of the rudimentary hymenium can be recognised in the wild-type primordium, but not in the *ichijiku* mutant. The hyphae at the top of the mutant primordium appear to be similar to veil cells in the wild-type. Scale bar: (A,B) 5 mm; (C,D) 0.2 mm.

**Table 1. Genetic analysis of *ichijiku***

Generation	No. of isolates	Mating types	Fruiting			$\chi^2$ (1:1)	P	% Germination
			Wild-type	<i>ichijiku</i>	n.f.			
F <sub>1</sub>	122	A2 B2	18	16	1	0.03	.90-.80	90
		A92B92	13	11	0			
		A2 B92	20	15	1			
		A92B2	8	19	0			
		Total	59	61	2			
BC <sub>1</sub>	41		17	24	0	1.1	.30-.20	67
BC <sub>2</sub>	40		12	26	2	5.1	.05-.02	85
BC <sub>3</sub>	62		30	32	0	0.06	.80-.70	85
BC <sub>4</sub>	63		29	33	1	0.2	.70-.50	93

n.f.: fruit-body primordia were not formed.

*ich1-1* mutant strain, KF<sub>0</sub>#9 (A92B92), was crossed with a wild-type strain, 5302 (A2B2), and the F<sub>1</sub> progeny were examined for fruiting phenotypes and mating types. An *ich1-1* mutant strain in the F<sub>1</sub> progeny was backcrossed four times to 5302 and the progeny in each generation were tested for fruiting phenotypes. The wild-type and *ichijiku* primordia segregated in a 1:1 ratio in all generations except BC<sub>2</sub>. These data indicate that *ichijiku* phenotype is brought about by a single gene mutation.

differentiating into the hymenium. In contrast, *ichijiku* primordia do not form the typical inverted-bowl shaped pileus and hence the future hymenium is not discernible (Fig. 3D).

Both the outer surface of the *ichijiku* primordium (Fig. 3B) and the inner cortical tissue seen in section (Fig. 3D) appear to be similar to those of the primordial shaft in wild-type primordia (Fig. 3A,C). This suggests that the major part of the *ichijiku* primordium is composed of primordial shaft cells.

In the wild type, the upper surface of the pileus is covered in a veil composed of chains of large cylindrical cells (Fig. 3C). These veil cells emit blue or green autofluorescence when UV-irradiated (Fig. 4A). The green fluorescence is emitted from the distal cells of the veil, while the blue fluorescence is emitted from the cells proximal to the tramal tissue of the pileus, especially from the margin of the pileus. In the *ichijiku* mutant, chains of large cylindrical cells were seen to emanate from a small area on the top of the primordium (Figs 3D, 4B). As shown in Fig. 4B, these cylindrical cells emit autofluorescence in the same way as the veil cells of the wild-type, indicating that differentiation of veil cells is restricted to a small area on the top of the *ichijiku* primordium. Taken together, we conclude that the *ichijiku* mutant has a defect that blocks the lateral growth of hyphal tissue that generates the inverted-bowl-like structure of the pileus at the apex of the primordial shaft (Fig. 2).

### Light responses of *ichijiku*

Maturation of fruit-body primordia in *C. cinereus* requires both light and darkness (Borriss, 1934; Morimoto and Oda, 1973; Kamada et al., 1978). In a 12-hour light and 12-hour dark regime, the wild-type primordium enlarges gradually and finally matures within one night. There is rapid stipe elongation, pileus expansion and production of basidiospores (Fig. 1).

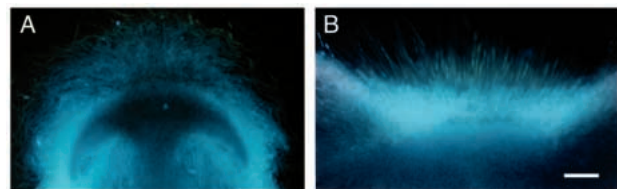
Although most of the mutant primordia arrest growth and de-differentiate into vegetative hyphae, cells of the cortical tissue in *ichijiku* primordia sometimes elongate rapidly during the night and cause the primordia to rupture (Fig. 5A). This rapid cell elongation is reminiscent of what occurs during maturation of the wild type fruit-body, but lack of a pileus means that there is no basidiospore production.

In continuous darkness, wild-type primordia etiolate, producing a so-called 'dark stipe' (Tsuusé, 1969). The *ichijiku* mutant also produces a dark stipe in continuous darkness (Fig. 5B), indicating that it still has a normal response to light/dark conditions despite lacking a differentiated pileus.

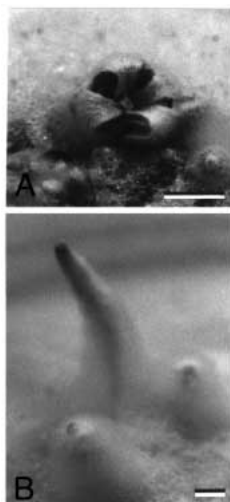
### Cloning of the *ich1* gene

To assign the *ich1* gene to a chromosome, we looked for RFLP markers linked to the *ich1* locus using 32 F<sub>1</sub> progeny from a cross between 5302 and KF<sub>0</sub>#9. We found that an RFLP marker, H8(6), is linked to the *ich1* locus with 18.8% (6/32) recombination. We also found a marker chromosome for *ich1-1* (data not shown), which is observed on CHEF gels in mutant progeny backcrossed repeatedly to a wild-type strain (Zolan et al., 1993, 1994). The RFLP probe hybridised with chromosome XII (approx. 2 Mbp) of 5302 and with the marker chromosome separated by CHEF electrophoresis. Thus, we conclude that the *ich1* wild-type gene is located on chromosome XII of 5302.

We constructed a chromosome-XII-specific cosmid library and screened it for clones that complement the *ichijiku* mutation. Through sib-selection, a cosmid, 1H7, was identified that completely rescued the *ichijiku* mutation, yielding a wild-type fruit-body with basidiospores. Random spore analysis of



**Fig. 4.** Autofluorescence emitted from cylindrical cells at the top of wild-type (A) and *ichijiku* mutant (B) fruit-body primordia, both of which are about 5 mm in height. Veil cells, which cover the surface of the pileus in the wild type, emit green or blue autofluorescence, when UV-irradiated. Cylindrical cells emanating from a small area on the top of *ichijiku* mutant primordium also emit autofluorescence. The autofluorescence, together with the shape of hyphae, provides evidence for differentiation of veil cells in *ichijiku* mutant. Scale bar, 0.3 mm.



**Fig. 5.** Light responses of *ich1-1* mutant. (A) Under a regime of 12-hour light and 12-hour dark, *ich1-1* mutant primordia sometimes rupture within a night without producing basidiospores. (B) Under the continuous darkness, *ich1-1* mutant primordia etiolate, producing so-called 'dark stipe', as a wild-type. Scale bar, 5 mm.

the basidiospores showed 1:1 segregation for fruiting phenotypes, demonstrating that the 1H7 fragment is integrated at a single site in the genome and complements the *ich1-1* mutation. An RFLP, detected using the insert of 1H7 as a probe, exhibited complete linkage between the 1H7 region and the *ich1* locus (recombination: <1/32) (data not shown), strongly suggesting that the 1H7 insert contains the *ich1* wild-type gene and not an extragenic suppressor.

To define the *ich1* region in the 1H7 insert, we looked for restriction enzymes that did not abolish the rescuing activity. When 1H7 was digested with *Pst*I, *Bgl*II or *Eco*RI, the rescuing activity was unaffected. We also identified an overlapping clone, 5G5, that had the rescuing activity. 5G5 and 1H7 shared two *Pst*I fragments and we found that one of these, a 5.4 kb *Pst*I fragment, carried the *ich1* activity (Fig. 6).

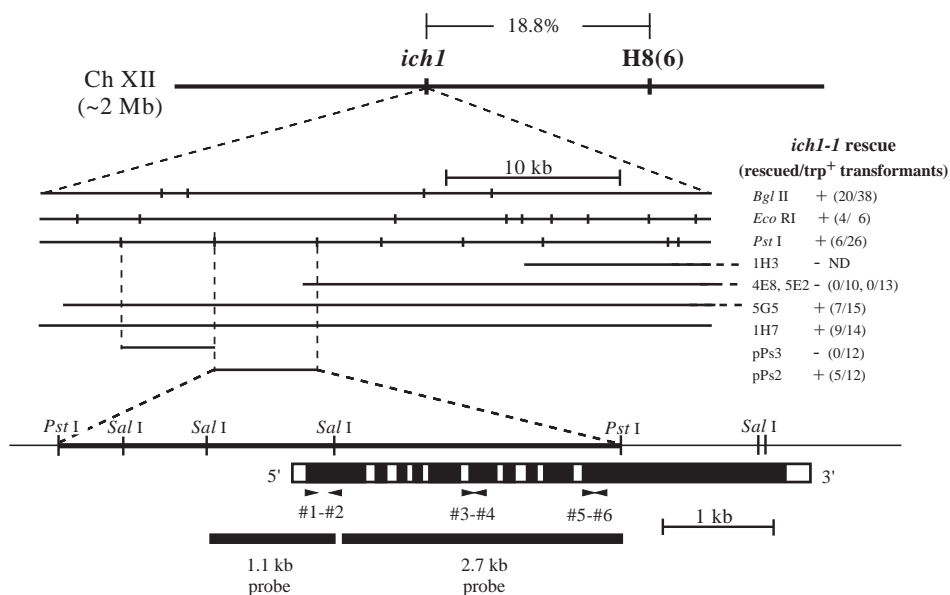
***ich1* encodes a protein that would function in the nucleus**

The 5.4 kb *Pst*I fragment with the rescuing activity was sequenced on both strands. Based on the sequence, we designed gene specific primers and performed PCR amplification of cDNA synthesised from mRNA present in the pileus. The 5' end of the *ich1* transcript was established by

5'-RACE experiments. 3'-RACE experiments revealed that the transcript extends further approx. 2.0 kb downstream of the 5.4 kb *Pst*I fragment (Fig. 6), in good agreement with the results of northern blots (described below).

Comparison of the genomic and the cDNA sequences revealed an open reading frame interrupted by nine introns ranging in size from 60 to 82 bp (Fig. 6), a size consistent with those found in genes of other filamentous fungi (Gurr et al., 1987). The comparison also revealed a few polymorphisms (Fig. 7A), because the cDNA was derived from a dikaryotic strain with a different genetic background from the strain used for analysing the genomic sequence. Based on the genomic sequence analysed, the open reading frame encodes a protein of 1,353 amino acids with a predicted molecular mass of approximately 148 kDa (Fig. 7A). The *ich1* mRNA has a 68 nt 5'- and a 243 nt 3'-untranslated region. A potential polyadenylation signal, AATAA, is found 24 nt upstream of the polyadenylation site. The promoter region of *ich1* does not contain canonical TATA boxes, but three CAAT elements (Fig. 7B).

The rescuing activity of the 5.4 kb *Pst*I fragment indicates that the first 763 amino-acid region is sufficient for *ich1* function and the remainder of the protein (residues 764-1353) is nonessential. To confirm this, we probed northern blots of total RNA from the pileus of the rescued fruit-body with a 2.7 kb *Sal*I-*Pst*I genomic fragment from the *ich1* coding region. *ich1* transcript in the rescued fruit-bodies is ~3.0 kb in size (data not shown), which is shorter than that in the wild-type



**Fig. 6.** Genetic and physical map of the *ich1* genomic region. This diagram illustrates the strategy used to define the *ich1* gene. Digestion of 1H7 with *Bgl*II, *Eco*RI and *Pst*I does not abolish the rescuing activity of 1H7. Overlapping clones with 1H7 are also shown, one of which, 5G5, has the rescuing activity. The 5.4 kb *Pst*I fragment, which is shared only between 1H7 and 5G5, carries the rescuing activity. RACE experiments revealed that the 5.4 kb *Pst*I fragment (pPs2) encodes a truncated *ich1* gene product lacking the C-terminal one-third of the protein. Schematic structure of the *ich1* gene is shown under the physical map. Exons are shown as black boxes, and 5' and 3' untranslated region and introns are shown as open boxes. The two fragments used as probes in Fig. 8 are shown by thick lines. The gene specific primers (#1-#6) used to amplify cDNA and to detect alterations in the mutant genome are shown by arrowheads. Rescue results: +, rescue; -, no rescue; ND, not determined.

## A

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MTFATLRALENIIGEALDDIEQVYHSYRPDTVASDVEDDVGDCYEYFMP IPTPYPAVTRGSSAQLVGGQVBKVTRTSAGSMNVIARAYVSPVPPSPCVPTVHSVDLVCRVRASGVIIPVGAATVIVS 120
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TBHIFPREISPNVVFLNVRISVSLMVDSGVKSVIVRELVREYVHALVGRVPEVLKYVQGTVNGIAVAVFPVVGLVCVSDVEVINKVASAYVMTEAYVLLVSSVSRVVRVRAVNEVPTVLAPVFLAVFGVTABLVGVVDFVGWVLEVCEVDRVKLVGLVRVD 360
GEDGKBDLVRNEVDGNVADVVRGVARDVGSAVAGEDVRVCDVPSVGVSRVRAVSTVHAPVPSVRVPSVNVLSVSDAVQVNSVLVPDVGCVQVIVVPSVHVTRAPVSSIVDIVRSVGNVPNVRFVRLVERVFGVKAMVSGVTGSVWEVAPVGAVIPVNAVFDVWVRSVLVPRG 480
SIIVDVVGGGVIGVSTVMLLVASVAPVSSVSDVGENVRLVKVFPVVIVQVDRVSVVVEMVGEVKAVWVREVKVPEVLLESVGLVARVFVVVHVDFVPTVQVPIVQVNASVIFVLLVRVVLEVDVWVPDVNVYARVKVILVLVHLVRAVARDVDDVQVIVHEVEDVNTVKLVL 600
LADVFPVLVLACKVETVGGVSEVGLVSGVIEVGVVSVVGGVGGVTVQVGEVYVDSVAPVFPVLPVLNVLVGVKASVANVVVWVMDVHTVMVAMPVNSVQVERVTLVRVEVIVVALVTRVSAGVWVRVVVRVVTVQVAPVGVSHVPGVHVIVALVPCVIPVGVVGSVMRVRPV 720
REVKVRVPPVQVGRVQRVDRVVVQEVIVQQVSSVWEVQVGREVEQVLYVQVELVQVAREVKLVQQVNERVQVEVERVLVQVWVHVSHVQVKKVPRVVVRVKSVSSVISVAILVASSVIEVQVTKVKKVLVGLVGVEVKVDVSEVEPSVYVDSVALVGTVEDVHEVVVGATVVVGASVNR 840
YVNTVPVTVPVMVPLVRCVSTVPTVPFVGVSRVMELVASVVVKVDLVVARVLGGVVVRRVYVRVRTVSALVGVSGVMVSSVSSVSSVSSVSSVSSVSSVSSVSSVNVLPVLTVRTVITVTTVTAVPPVEPVPKVKRVPSVLSVMVPLASVYASVESASVQVVEVIDAVHVPPVPGVAPSVST 960
STVSAVQLVKSVQVVSPVSLVFTAVHVKVQVERVESSVPRVKTVIRVRVHVVSLASVLSVRRVIRVMSVSEVPAVAEVVVLDVVVPPVFPVFRVVVQVQLVPTVSEVQVVGVPPVGRVQTVQVYVQVVVHPVRENVHVQLVPTVPEVSPVDVYVPPVTRVFRVESSVBVVEL 1080
RQVDSVVASSVMVVPVSPVSIVSPVMPVSPVSPVSPVKVFVVSMVPPVSPVSPVKVFVVSMVPPVSPVSPVKVFANVMPV[MEVPPVSPVSPVKVFVVSMV]LAMPVPSVSPVSPVPHVMPVVVIPVPDVLVPSVSAVVVBYVRRVHSVLSVRAVPTVLPVIPVTSVSVVYVYP 1200
PTVSALVSRVFNVTVGVVDINVTRANTVPHVPHVGLVSRVPRVCVVSTVTLVPTVRDVHVGHVGTVRVKVPRVKTVGVVLVRLVDVNGVGRASVGVVWDVKVVDEVGLTVQVVVDVVQVSRVWVSDVGNVRREVALLVPLVRRVQVASTVRRVEDVERVLSVFTVPDVI 1320
QPVERVVGRVARRVMSVAVVPTVRVGLVLRVSEVQNVFVSSAKV 1353

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## B

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-544 gcgaagggcagtgcccttgettcactggtcttctctgggttcttctcacttcaaaagcttcacttctacttotctgtgatggagatgatgcgtcaaaa -445
-444 gctaacccttgcgatcctaataatagcctatattgaaagccgatttttcgaaagaccgggaggggtaagggttaagggtattctgtagggttctgatt -345
-344 caagcttgccggtggggtgaataatgggagtggtggtggagtggatga caattggcaattggatacgcaggcgcgagaaagatgggaaatggagaggtaaag -245
-244 taagcaagcttacttacattgacttggacttgacatttggactgacatcttggccactgctg caataatgatgcatcgaaaccgcactcggtgctttgta -145
-144 tttagatgccccttgattttggctttttgatattgggtttgggagtgccgctgttacggcgattggcgcaccgtccctttctgaaaaggttttctgcttg -45
-44 gggatttgcaacttggttggctgttcttctctgcccacgtcgtGTCTATTCCAGCTGAAACTGATACTCTGCCCCGTCTCGTAGTAGCGGTCGGCA 56
57 CCGACAACAGCCATGACGTTCCCGACTCTCCGAGCGTGCATAACATCATCGGCAGGGCGTTGGACGACATCGAGCAAGTCTATCATTCTGATCGACCTG
1 M T F A T L R A L E N I I G E A L D D I E Q V Y E S Y R P D

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**Fig. 7.** (A) The predicted amino acid sequence of Ich1. Open arrowheads indicate the positions of introns. Three putative nuclear targeting signals are double-underlined below the amino acid sequence. One end of the 5.4 kb *Pst*I fragment (see Fig. 6) is indicated by a closed arrowhead and the region downstream of the site is inessential for the rescuing activity. Repetitive sequence in the C-terminal region of Ich1 is indicated by underlines numbered 1 to 6. The putative phosphorylation sites (SPK) by protein kinase C are in bold lettering. Comparison between the genomic and cDNA sequences revealed two polymorphisms. A deletion of 45 nucleotides was found in the repetitive sequence of the C-terminal region in cDNA analysed, and the resulting amino acid deletion is indicated in brackets. An additional codon for glycine (GGA) was found in cDNA in a second position of the glycine repeat region (residues 629–633), which is indicated with a broken underline. (B) The promoter region of *ich1*. Three potential CAAT boxes are underlined. The two CAAT can be considered to constitute a 13 bp imperfect palindrome. The nucleotide sequence data (5600 nt) will appear in the DDJB/EMBL/GenBank nucleotide sequence databases with the accession number AB012131.

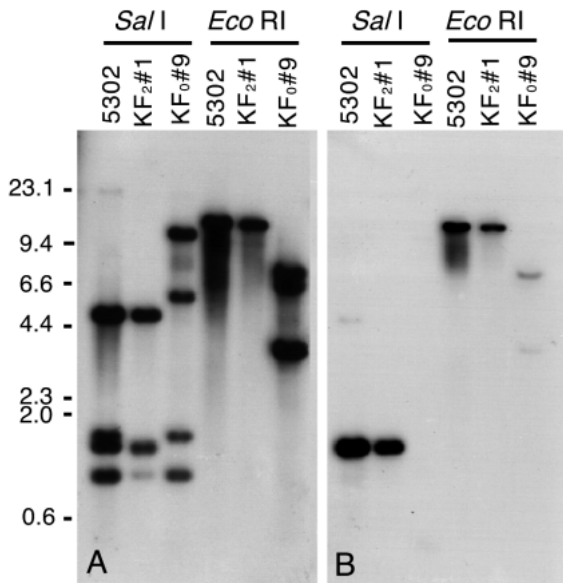
fruit-body (Fig. 9), supporting that the truncated Ich1 protein is sufficient to complement the *ich1-1* mutation.

Searches of the sequence databases with the predicted Ich1 polypeptide sequence failed to identify any proteins with significant similarity. However, the Ich1 protein contains three separate nuclear targeting signals (Fig. 7A) (residues 717–733, 781–785, 922–929), one of which, RRPPEKRPQGRQRDR (717–733), fits a Robbins & Dingwall consensus (Robbins et al. 1991). The C-terminal region (residues 764–1353), which is dispensable for *ich1* function, is rich in proline (15.5%) and serine (14.3%). In addition, the C-terminal region has a repetitive sequence, in which a similar sequence repeats six times and a protein kinase C phosphorylation site (SPK) repeats four times, and a putative microbody targeting signal (AKL) (de Zoysa and Connerton, 1994) (Fig. 7A).

### ***ich1-1* mutant allele**

The causative lesion in the *ichijiku* mutant involves gross alterations in the *ich1* genomic region. Southern analysis using the 5.4 kb *Pst*I fragment as a probe revealed that two wild-type strains, 5302 and KF2#1, present a similar band pattern despite different genetic backgrounds. In contrast, the *ichijiku* mutant strain, KF0#9, which has the same genetic background as KF2#1, showed RFLP that might have arisen from both deletion(s) and insertion(s) in the *ich1* region (Fig. 8A).

To further examine the alterations in the mutant genome, we probed the same blot with two sub-fragments from the 5.4 kb fragment. Southern blots using a 2.7 kb *Sal*I-*Pst*I fragment as a probe (see Fig. 6) showed a single 4.5 kb *Sal*I band in both wild-type strains and only two larger bands, 5.5 kb and 10 kb, in the mutant genome (data not shown, see Fig. 8A). This



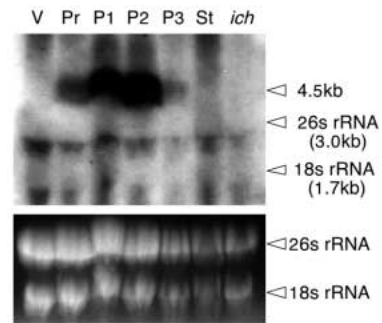
**Fig. 8.** Molecular lesions in the *ichijiku* mutant. Southern analysis of two wild-type strains, 5302 and KF<sub>2</sub>#1, and an *ichijiku* mutant strain (KF<sub>0</sub>#9) using the 5.4 kb *Pst*I fragment containing the promoter and two-thirds of the *ich1* coding region (A), or the 1.1 kb *Sal*I fragment containing the promoter region (B) (see Fig. 6). 5302 and KF<sub>2</sub>#1 have different genetic backgrounds from each other, while KF<sub>0</sub>#9 has the same genetic background as KF<sub>2</sub>#1. These genomic DNAs were digested with *Eco*RI or *Sal*I, and subjected to the Southern analysis. The blot shown in A was reprobed for B, exhibiting faint residual signals in B.

suggests that a large insertion(s) may have occurred within the 2.7 kb *Sal*I-*Pst*I region of *ich1*. The Southern blots were also probed with a 1.1 kb fragment containing the promoter region of *ich1* (see Fig. 6). The 1.1 kb probe did not detect any signal in the KF<sub>0</sub>#9 (Fig. 8B), showing a deletion of the promoter region in the mutant genome. Furthermore, the mutant genome could be amplified by a gene-specific primer set, #5-#6, as the wild-type genome, but not amplified by the primer sets, #1-#2 and #3-#4 (see Fig. 6) (data not shown). These PCR results suggested that the deletion covers the 5' half of the *ich1* coding region.

***ich1* is specifically expressed in the pileus**

To examine whether the expression of *ich1* is developmentally controlled, we probed northern blots of total RNAs isolated from various stages in development with the 2.7 kb *Sal*I-*Pst*I genomic fragment from the *ich1* coding region. We expected that *ich1* should be required specifically for fruit-body development, because dikaryons homozygous for *ich1-1* display apparently normal vegetative hyphae. A single approx. 4.5 kb transcript was detected only during fruiting (Fig. 9). The size of this transcript corresponds to that of the cDNA obtained by the RACE experiments.

Expression of *ich1* is controlled spatiotemporally. The *ich1* transcript is not expressed in vegetative mycelia, but becomes expressed during fruiting and is expressed strongly in the pileus (Fig. 9) but not in the stipe except for faint expression where the stipe is directly in contact with the pileus (data not shown). The abundance of the *ich1* transcript decreases as



**Fig. 9.** Northern blot analysis of the *ich1* expression. About 20 µg of total RNA isolated from different developmental stages were fractionated on a formaldehyde agarose gel and blotted to nylon membrane. The 2.7 kb *Sal*I-*Pst*I fragment from the coding region was used as a probe. The lower panel shows rRNA in EtBr staining gel used for the blot in the upper panel. V, vegetative mycelia; Pr, primordia that are less than 5 mm in height; P1, pileus at 21:00 hours, one day before fruit-body maturation; P2, pileus at 14:00 on the day of maturation; P3, pileus at 21:00 on the day of maturation, when basidiospore formation has been completed; St, stipe at 14:00 on the day of maturation, which is not in contact with the pileus; *ich*, *ichijiku* mutant primordia.

basidiospores are produced. No *ich1* mRNA was detected in *ichijiku* primordia (Fig. 9). This pattern of expression is consistent with our demonstration that the *ich1-1* mutation prevents the development of the pileus and that the mutant gene lacks a promoter region.

**DISCUSSION**

**The *ichijiku* mutant provides insight into developmental sequence in fruiting**

We have described a novel type of developmental mutant, *ichijiku*, which provides insight into the developmental sequence of fruit-body formation of *C. cinereus*. In angiocarpic fruit-bodies, the pileus develops a short distance below the apex of the primordial shaft (Corner, 1934; Burnett, 1968). However, in *C. cinereus*, stages in the developmental sequence that lead to cellular differentiation in fruiting have remained obscure, probably because it occurs rapidly within the small hyphal knot stage (Reijnders, 1979). The *ichijiku* mutant lacks a differentiated pileus regardless of differentiation of the primordial shaft and veil cells, providing evidence that differentiation of the primordial shaft and veil cells precedes that of the pileus. This is consistent with previous observations on other mushrooms (Corner, 1934; Williams et al., 1985).

**Role of *ich1***

Our data demonstrate that *ich1* is specifically expressed in the pileus, and that the *ich1-1* mutation is loss of the *ich1* promoter and results in lack of the pileus. It seems probable that *ich1* function is required for differentiation of the cells that normally express it. The major part of the developed pileus is composed of the hymenium, a layer of basidia. The abundance of the *ich1* transcript in the pileus suggests that the Ich1 protein plays a critical role in differentiation and maturation of basidia.

The predicted Ich1 amino-acid sequence contains nuclear

targeting signals, suggesting that the Ich1 protein functions in the nucleus. One possibility is that Ich1 regulates the expression of other genes required for the development of the pileus. A well-characterised DNA-binding motif is not evident but this does not preclude a role in DNA-binding or interaction with other proteins involved in transcription regulation. Further study will be required to determine which cells of the pileus express *ich1* and where Ich1 functions in the cells.

Based on the rescuing activity of the 5.4 kb *PstI* fragment, the C-terminal half of the Ich1 protein appears to be dispensable for pileus formation despite the presence of some characteristic motifs. We have suggested that Ich1 may be a transcription regulator and it is known that such proteins may be modular and have different functional domains. The C-terminal domains may normally play a role in regulating the activity, stability or even cellular localisation of Ich1. Alternatively, Ich1 may function as a multimer, and the apparent rescue of the mutant phenotype may be due to complementation between the N-terminal fragment from the 5.4 kb *PstI* fragment and a possible C-terminal fragment, which may be initiated at a non-*ich1* promoter in the vicinity of the mutant *ich1-1* gene.

The deduced Ich1 protein is truly encoded in the cloned fragment that can complement the *ich1-1* mutation. Although there is the possibility that the cloned fragment might harbour another gene which would be expressed at low levels and could complement the mutation, it seems unlikely for two reasons. First, the timing of *ich1* transcription is consistent with the temporal profile of the activity. Second, when the *ichijiku* mutant was transformed with the truncated version of *ich1* which was driven by *C. cinereus*  $\beta$ -tubulin promoter, the mutant phenotype was rescued (data not shown).

### Expression of *ich1*

*ich1* is normally expressed only in the pileus, indicating that transcription should be precisely regulated by *cis*- and *trans*-elements. The two CAAT elements in the promoter region of the *ich1* gene could be considered to constitute a 13-bp imperfect palindrome (5'-CAATTGGCAATTG-3') or two CANNTG motifs. The CAAT imperfect palindrome is recognised by C/EBP (Johnson, 1993), which is involved in tissue-specific expression of genes in various tissues (Johnson and Williams, 1994; Sterneck et al., 1997). One or more CANNTG motifs, which are specifically recognised by MyoD, are necessary for expression of the muscle-specific genes (Weintraub et al., 1991). Therefore, the presence of a dyad-symmetric sequence in the promoter region is of intrinsic interest in that it could implicate binding by a protein with a basic region-leucine zipper (bZIP), or a basic region-helix-loop-helix (bHLH) (Ferre-D'Amare et al., 1993; Freemont, 1993). This dyad-symmetric sequence might be involved in controlling precisely tissue-specific expression of *ich1*. Further study will be required to analyse the cis-elements and to identify trans-elements involved in the expression of *ich1*.

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