The roles of homeodomain proteins during the clamp cell formation in a bipolar mushroom, *Pholiota nameko*

二極性担子菌 *Pholiota nameko* のクランプ形成におけるホメオドメイン蛋白質の役割に関する研究

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Contents

Chapter1 General introduction 1

Chapter2 Genomic structure of the *A* maing-type locus in a bipolar basidiomycte, *Pholiota nameko* 13

Chapter 3 DNA-mediated transformation system in a bipolar basidiomycete, *Pholiota nameko* 36

Chapter 4 *A*-mating type gene expression can drive clamp formation in the bipolar mushroom, *Pholiota nameko* 56

Chapter 5 Conclusion and discussions 93

Abstract 99

Acknowledgements 105

Literatures cited 106

List of publications 123

Chapter1

General introduction

Mating is an essential step in the life cycle of sexually reproducing organisms. In the basidiomycete mushrooms, mating compatibility is controlled by one or two sets of multiple allelomorphic genes known as bipolar or tetrapolar mating systems, respectively. In tetrapolar mushrooms, the genomic structure of mating-type loci and their functions to control clamp formation have been well investigated in *Coprinopsis cinerea* and *Schizophyllum commune* (Kües and Casselton 1992; Stankis et al.. 1992; Wendland et al.. 1995; Hiscock et al.. 1996; Shen et al.. 1996; Vaillancourt et al.. 1997; O'Shea et al. 1998; Riquelme et al. 2005; Casselton and Kües 2007). But few studies have been examined the composition of the mating-type loci and their functions during the clamp formation in bipolar mushrooms. So in this research, we would analyze the genomic structure of *A* mating type and the functions of *A* mating type to control the clamp formation in a bipolar mushroom, *Pholiota nameko*. But before it, we would like to make a concise introduction: i) the typical life cycle of homobasidiomycetes; ii) mating type system in the homobasidiomycetes; iii) the composition and roles of mating type loci in tetrapolar mushrooms; iv) research progress of mating type loci in bipolar mushrooms and previous study.

1-1. The mating-type system governs sexual reproduction in homobasidiomycetes. The basidomycota appear to be divided into three major lineages: rusts (Urediniomycetes), smuts (Ustilaginomycetes), and mushroom-like fungi (Hymenomycetes, including homobasidomycetes and jelly fungi). Most of the described basidomycete species are homobasidomycetes (Kirk et al. 2001). An understanding of the typical homobasidiomycetes life cycle is important to understand the roles that mating type genes play in sexual development. So firstly, we would describe the typical life cycle of homobasidiomycetes and this has been well reviewed by Kües (2000).

Single basidiospore germinates to give rise to monokaryotic mycelium, each cell of which contains a single haploid nucleus. Part of the mycelium grows submerged within the medium and aerial hyphae exist and produce abundant oidia, a feature that facilitates the selection of mutants and has led to highly efficient DNA-mediated transformation system. Two compatible monokaryons with different mating type can mate and form dikaryotic mycelium containing two haploid nuclei, one from each mate, in each cell. The dikaryon typically forms clamp connections to maintain the distribution of nuclei. From the encounter of a possible mate to meiosis, a prolonged dikaryotic stage is observed in homobasidiomycetes (Buller 1924; Casselton 1978; Moore 1995; Polak et al. 1997).

Under appropriate environmental stimuli, the formation of fruit body (mushroom) can be induced. The mushroom remains dikaryotic and nuclear fusion and meiosis occur only in the specialized basidia, which are protected within the gills located underneath the mushroom cap. Haploid nuclei migrate into basidiospores. The spores are released as the fruitbody matures (Kües 2000).

In a word, the mating system governs sexual reproduction in homobasidiomycetes. So investigation of the mating-type genes becomes significant for mushroom research.

1-2 Mating system in the homobasidiomycetes. Mushroom fungi are further distinct from other basidiomycete species because there may be numerous (up to hundreds) mating type-alleles at mating-type locus. Among homobasidiomycetes, an estimated 10% are homothallic (non-outcrossing), 25% to 35% are bipolar, 55% to 65% are tetrapolar. Homothallic, bipolar and tetrapolar species interdigitate along the species phylogeny (Boidin 1971; Hopple and Vilgalys 1999).

Homothallic fungi can form fruit bodies and sexual spores without mating. A reason may be found in genes usually activated only upon mating between two different mating types. If those genes are constitutively expressed in a monokaryotic strain, it may lead to fruit body formation without mating. And

there are secondary homothallic species like commercially grown white button mushroom, *Agracus bisporus*, in which only two basidiospores are formed, which may contain two nuclei of different mating type. The germinating basidiospores in this case can form a dikaryon directly.

Most heterothallic homobasidiomycetes such as *Coprinopsis cinerea* (Hiscock et al. 1996; Casselton et al. 2006), *Laccaria bicolor* (Fries and Mueller 1984; Kropp and Fortin 1988; Fowler et al. 2004), and *Schizophyllum commune* (Frankel and Ellingboe 1977) contain tetrapolar systems. In these tetrapolar mushrooms, two mating-type loci, *A* and *B*, which are located on different chromosomes, regulate mating compatible and clamp formation (Raper 1966, 1983; Iwasa et al. 1998; Fowler et al. 2004). Classical recombination analysis identified two closely linked *A* loci, which were termed *Aα* and *Aβ*, in both *S. commune* and *C. cinerea* (Day 1960; Papazian 1951; Raper et al. 1960). Also the *B* mating-type genes are separated into two discrete loci, *Bα* and *Bβ*, in *S. commune* (Parag and Koltin 1971), and three subloci in *C. cinerea* (Riquelme et al. 2005).

The subloci of *A* locus comprises multigenes encoding homeodomain proteins, and the subloci of *B* locus comprises multigenes encoding pheromones and pheromone receptor proteins (Kües and Casselton 1992; Stankis et al. 1992; Wendland et al. 1995; Hiscock et al. 1996; Shen et al. 1996; Vaillancourt et al.

1997; O'Shea et al. 1998; Riquelme et al. 2005; Casselton and Kües 2007; Niculita-Hirzel et al. 2008).

For *C. cinerea*, the estimates of actual numbers of *A* and *B* specificities vary from 164 *As* and 79 *Bs* (Day 1963) to more than 240 of each (Kimura 1952). In *S. commune*, there are estimates of 339 *As* and 64 *Bs* (Raper et al. 1958), so more than 20, 000 different mating types observed in nature. The *L. bicolor* mating system is considered to be tetrapolar with at least 45 *A* and 24 *B* mating type alleles (Raffle et al. 1995).

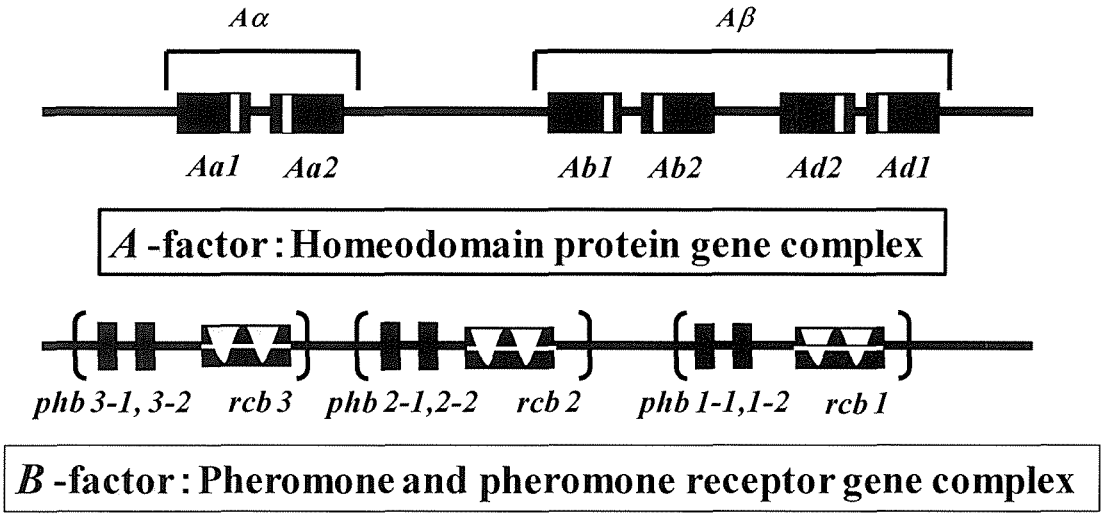


Fig. 1-1 The composition of *A* anf *B* factor in *C. cinerea*. *Phb* and *rcb* showed pheromone and pheromone receptor, respectively.

The mating-type proteins of the *A* locus fall into two distinct subgroups on the basis of the homeodomain sequence, and these have been termed HD1 and HD2 (Kües et al. 1994). Intracellular recognition of sexual compatibility

occurs when an HD1 protein from one mate heterodimerizes with an HD2 protein from the other mate to form a functional regulatory protein (Banham et al. 1995; Kämper et al. 1995; Magae et al. 1995). Heterodimerization between two classes of homeodomain proteins in mushroom brings together potential DNA-binding and activation domains (Asante-Owusu et al. 1996). The heterodimer is assumed to be a transcription factor that binds unique target sites within the promoters of genes whose activity commits cells to a new *A*-regulated developmental pathway.

In bipolar mushrooms, such as *Pholiota nameko* (Ratanatragooldacha et al. 2002) and *Coprinellus disseminatus* (James et al. 2006), only one locus is related to mating incompatibility. The mushroom *P. nameko* (Strophariaceae) has a very similar life cycle to other members of the Agaricales, such as *C. cinerea* (Kües 2000), but in contrast to these tetrapolar species, *P. nameko* is known to carry a bipolar *A* incompatibility factor gene and six types of *A* mating type loci were identified previously (Ratanatragooldacha et al. 2002). The composition of the *A* incompatibility factor gene in the bipolar mushroom *P. nameko* has been recently described (Aimi et al. 2005). These authors sequenced and characterized the *P. nameko* genes encoding the homeodomain protein *hox1* and the pheromone receptor *rcb1*, which are putative homologs of the HD1 protein and Rcb3 protein genes, respectively, of the tetrapolar basidiomycete *C. cinerea*. Restriction fragment length polymorphism (RFLP)

and linkage analyses indicated that the genes are both present as single loci, but on different chromosomes. The *P. nameko hox1* gene was mapped to the *A* mating-type locus in linkage group I; however, *rcb1* was mapped to another linkage group. These results strongly suggested that *hox1* is involved in regulating incompatibility in *P. nameko*, and that *rcb1* is not. A similar phenomenon has been reported for the bipolar mushroom *C. disseminatus* (James et al. 2006). *C. disseminatus* is a common mushroom species that fruits in large troops on stumps, buried wood, tree tip-up mounds, and logs (Buller 1924). In this species, *A* mating type loci is similar to the *A* mating-type locus of *C. cinerea* and encodes two tightly linked pairs of homeodomain transcription factor genes (James et al. 2006). Phernomones and phernomone receptors were also discovered in *C. disseminatus*. But only the homeodomain transcription factor segregated with mating type. In these two species, only the homeodomain protein genes segregate with mating type, negating the hypothesis that a genetic linkage between the *A* and *B* mating-type loci is the reason for bipolar mating behavior (Bakkeren and Kronstad 1994).

1-3 Clamp formation orchestrated by mating type loci. The mating system governs sexual reproduction, including the recognition of mating partners, clamp formation, fruit body formation, meiosis and spore formation (Kertesz-Chaloupková et al. 1998). The fruit body formation, meiosis and spore formation may be the results of integrating intracellular signal induced by

mating-type loci with environmental factors (Kamada et al. 1978; Morimoto and Oda, 1973; Chaloupková et al. 1998; Küe et al. 1998; Murata et al. 1998a and 1998b). In this study, we only focus on the clamp cell formation typically formed in the dikaryon of homobasidiomycetes.

In tetrapolar mushroom, both *A* and *B* mating-type genes are required for the clamp cell formation through a complex sequence of events. First, *B* locus promotes the migration of fertilizing nuclei from each individual into the other. *A* locus then promotes one-to-one pairing between the invading nuclei and the host nuclei in hyphal tip cells. An outgrowth known as a hook cell forms from the side of the tip cell and the nuclei divide synchronously. Septa then form to separate the four daughter nuclei, such that paired nuclei of opposite types exist in the new apical cell and single nuclei exist in each of the hook cell and the new subapical cell. *B* locus permits fusion of the hook cell with the subapical cell via a 'clamp connection', which in turn permits release of the hook cell's nucleus into the subapical cell, producing a dikaryotic subapical cell. The nuclei are always distributed such that each dikaryotic cell contains nuclei derived from each of the original mating individuals (Raper 1983, 1966; Iwasa et al. 1998; Fowler et al. 2004).

If only the *A* alleles differ between mates (common *B*), clamp cells form but unable to fuse with subapical cells, leaving a nucleus trapped in each clamp

cell of the mycelium. If only the *B* alleles are different (common *A*), in *S. commune* a distinctive "flat" phenotype is observed in which aerial growth is lacking, the hyphae branch frequently, and nuclear migration and septal disruption occur continuously (Raper 1966).

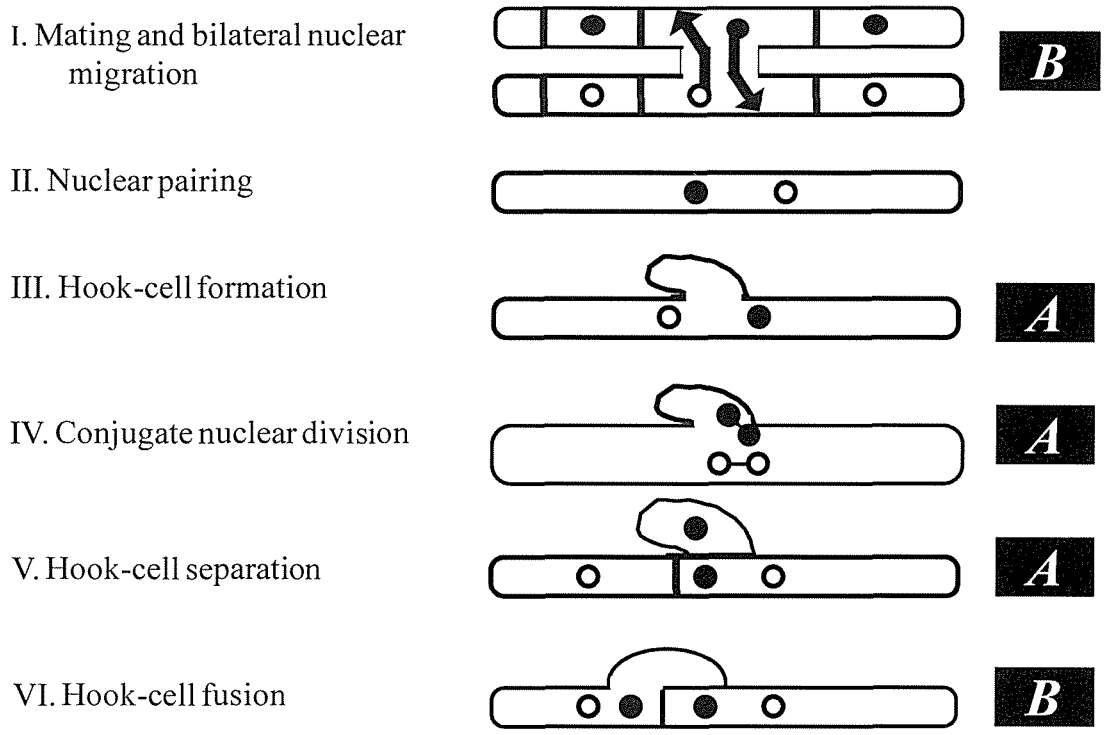


Fig. 1-2 The course of *A* and *B* mating type loci controlling the clamp cell in tetrapolar mushrooms. The *A* and *B* marked indicated the step controlled by them.

In bipolar mushroom, only one locus is related to mating incompatibility. How it control and regulate clamp formation like the tetrapolar mushrooms become magnetic. In *C. disseminatus*, James et al. (2006) transformed both *A* and *B* homologs to a tetrapolar mushroom *C. cinerea*. When transformed into *C. cinerea*, the *C. disseminatus A* and *B* homologs elicited sexual reactions similar

to those elicited by the native mating-type genes. Although mating type in *C. disseminatus* is controlled by the transcription factor genes only, cellular functions appear to be conserved for the products of both gene groups. Therefore, James et al. (2006) suggested that *C. disseminatus* had undergone a loss of mating-type-specific pheromone receptor function. However, in their research, the functions of the *C. disseminatus* mating type were studied in a tetrapolar mushroom, *C. cinerea*, instead of in a homologous bipolar species. In *C. disseminatus*, the functions of *A* and *B* homologs are still unclear. So in *P. nameko*, it is still necessary to focus on the functions of mating type during the clamp formation in this species.

1-4 Purpose of this study. In the previous study, genes encoding the homeodomain protein *hox1* and the pheromone receptor *rcb1* were sequenced. But only *hox1* gene regulates with incompatibility in this mushroom. So in this study, we will focus only on the *A* mating-type locus and investigate its composition and its functions during the clamp formation.

Firstly, although the *hox1* gene has been cloned and sequenced in *P. nameko*, the flank genes were still unknown. The research of Ratanatragooldacha et al. (2002) showed the *A*-factor in *P. nameko* is a bipolar *A* incompatibility factor gene consisting of two functional subunits, *Aα* and *Aβ*, which appear to be located on the same chromosome within 0.3 cM. But we still

can not determine the relationship between *hox1* and these two functional subunits (*Aα* and *Aβ*). If one of the two functional subunits is consistent with the homeodomain protein gene complex, it remains to be determined what kind of genes are components of the two subunits. So we focus on the study of the *A* mating type locus in *P. nameko*. At first we need to know the genomic gene sequence around *hox1* gene and their putative genes. These contents were presented in Chapter 2.

In Chapter 3, to investigate the roles of homeodomain protein genes during clamp cell formation in *P. nameko*, a high efficient transformation system is needed to construct in this species. In the mushrooms, construction of transformation system is not easy due to the unstable and low efficient transformation. So James et al. (2006) tested both the *A* and *B* mating type locus from bipolar mushroom *C. disseminatus* in tetrapolar mushroom *C. cinerea* due to the lack of transformation system in this bipolar species.

Furthermore, using a high efficient transformation system constructed in *P. nameko*, the roles of homeodomain proteins genes during the clamp formation was investigated in Chapter 4. The results of only one homeodomain protein gene introduction, a pair of homeodomain protein genes introdution and over expression of homeoodomain protein genes would be discussed.

Finally, genomic structure and functions of *A* mating type in bipolar mushroom *P. nameko* compared with other bipolar mushrooms and tetrapolar mushrooms, the transformation system construction situation will be concluded and discussed in Chapter 5.

Chapter 2

Genomic structure of the *A* mating-type locus in a bipolar basidiomycete, *Pholiota nameko*

2-1 Abstract

In the bipolar basidiomycete *Pholiota nameko*, the homeodomain protein *A4-hox1*, located at the *A* mating-type locus, is known to regulate mating compatibility. In the present study, we investigated the genomic structure of the *P. nameko A* mating-type locus and its flanking region. A second homeodomain gene (*A4-hox2*) was discovered upstream of *A4-hox1* and the conserved gene order around *A* mating-type locus and their similar transcription direction were found in *P. nameko* and another bipolar mushroom, *Coprinellus disseminatus* and two tetrapolar mushrooms, *Coprinopsis cinerea* and *Laccaria bicolor.* Analysis of the deduced protein sequences of the homeodomain protein genes from two strains of *P. nameko* show that the putative functional domains differ from those of the homeodomain proteins of the tetrapolar mushrooms, *C. cinerea* and *L. bicolor.*

2-2 Introduction

In basidiomycetous mushrooms, mating is controlled by one or two sets of multiple allelomorphic genes known as bipolar or tetrapolar mating system,

respectively (Whitehouse 1949). The mushrooms *Coprinopsis cinerea* (Casselton and Kües 1994; Hiscock et al. 1996), *Flammulina velutipes* (Ashan-Aberg 1960), and *Schizophyllum commune* (Frankel and Ellingboe 1977) are known to carry tetrapolar mating system. In model tetrapolar species *S. commune*, the *A* and *B* factors each comprise two subunits, the α and β subunits, which are encoded on different chromosomes (Day 1960; Frankel and Ellingboe 1977).

The loci encoding the *A* and *B* mating-type locus regulate formation of a dikaryon through a complex sequence of events. First, *B* locus promotes the migration of fertilizing nuclei from each individual into the other. *A* locus then promotes one-to-one pairing between the invading nuclei and the host nuclei in hyphal tip cells. An outgrowth known as a hook cell forms from the side of the tip cell and the nuclei divide synchronously. Septa then form to separate the four daughter nuclei, such that paired nuclei of opposite types exist in the new apical cell and single nuclei exist in each of the hook cell and the new subapical cell. *B* locus permits fusion of the hook cell with the subapical cell via a 'clamp connection', which in turn permits release of the hook cell's nucleus into the subapical cell, producing a dikaryotic subapical cell. The nuclei are always distributed such that each dikaryotic cell contains nuclei derived from each of the original mating individuals (Raper 1983, 1966; Iwasa et al. 1998; Fowler et al. 2004).

The mating-type subloci of *A* and *B* locus in tetrapolar mushrooms such *as C. cinerea*, *S. commune* have all been shown to be multigenic. The *A* subloci comprise multigenes encoding homeodomain proteins, and the *B* subloci comprise multigenes encoding pheromones and pheromone receptor proteins (Kües et al. 1992; Stankis et al. 1992; Wendland et al. 1995; Hiscock et al. 1996; Shen et al. 1996; Vaillancourt et al. 1997; O'Shea et al. 1998; Riquelme et al. 2005; Casselton and Kües 2007; Niculita-Hirzel et al. 2008).

In bipolar fungi, little is known about the makeup of mating type loci and their function to initiating the mating-type specific development. In a land mark study, Bakkeren and Kronstad (1994) demonstrated that the bipolar mating-type locus of *Ustilago hordei* was formed from the fusion of the *A* and *B* mating type loci observed in tetrapolar smut fungi into one nonrecombining mating-type region with two alleles. Although mating type loci of this bipolar fungus has been studied, the research of the constitution of mating type loci in other bipolar species is just initiated.

The mushroom *Pholiota nameko* (Strophariaceae) has a very similar life cycle to other members of the Agaricales, such as *C. cinerea* (Kües 2000), but in contrast to these tetrapolar species, *P. nameko* is known to carry a bipolar *A* incompatibility factor gene and six types of *A* mating type loci were identified previously (Ratanatragooldacha et al. 2002). The composition of the *A*

incompatibility factor gene in the bipolar mushroom *P. nameko* has been recently described (Aimi et al. 2005). These authors sequenced and characterized the *P. nameko* genes encoding the homeodomain protein *hox1* and the pheromone receptor *rcb1*, which are putative homologs of the HD1 protein and Rcb3 protein genes, respectively, of the tetrapolar basidiomycete *C. cinerea*. Restriction fragment length polymorphism (RFLP) and linkage analyses indicated that the genes are both present as single loci, but on different chromosomes. The *P. nameko hox1* gene was mapped to the *A* mating-type locus in linkage group I; however, *rcb1* was mapped to another linkage group. These results strongly suggest that *hox1* is involved in regulating incompatibility in *P. nameko*, and that *rcb1* is not. A similar phenomenon has been reported for the bipolar mushroom *Coprinellus disseminatus* (James et al. 2006). In these two species, only the homeodomain protein genes segregate with mating type, negating the hypothesis that a genetic linkage between the *A* and *B* mating-type loci is the reason for bipolar mating behavior. The mating-type locus of *C. disseminatus* is similar to the *A* mating-type locus of *C. cinerea* in that it encodes two tightly linked pairs of homeodomain transcription factor genes. When transformed into *C. cinerea*, the *C. disseminatus A* and *B* homologs elicited sexual reactions similar to those elicited by the native mating-type genes. Although mating type in *C. disseminatus* is controlled by the transcription factor genes only, cellular functions appear to be conserved for the

products of both gene groups. Therefore, James et al. (2006) suggested that *C. disseminatus* had undergone a loss of mating-type-specific pheromone receptor function.

In *P. nameko*, little is known about which genes are controlled by the homeodomain transcription factor protein; thus, we analyzed the genomic structure of the *P. nameko A* mating-type locus and its flanking region.

2-3 Materials and Methods

2-3-1 Fungal strains used in this study. Auxotrophic *Pholiota nameko* monokaryons, of strains NGW12-163 (*A3*, *Arg4*) and NGW19-6 (*A4*, *pdx1*), were used in this study.

2-3-2 DNA and RNA preparation. Liquid potato dextrose (PD; potato extract with 2% (w/v) glucose) medium was used for fungal culture. Genomic DNA from lyophilized *P. nameko* NGW19-6 and NGW12-163 mycelia was prepared using a Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Tokyo, Japan) according to the manufacturer's instructions.

To prepare total RNA from *P. nameko* NGW19-6 and NGW12-163 mycelia were grown on potato dextrose agar (PDA; potato extract with 2% (w/v) glucose and 1.5% (w/v) agar) at 25°C for one week, after which the ten mycelial agar blocks (5 × 5 × 5 mm), were transferred to 20 ml of liquid PD medium in a

100 ml Erlenmeyer flask and grown at 25°C for one week. Mycelia were then harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. Total RNA was extracted from the frozen powdered mycelia using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

2-3-3 Genomic walking. Genomic walking around *A4-hox1* in the NGW19-6 strain (Aimi et al. 2005) was carried out using cassette amplification via PCR with seven specific primer sets (Table 2-1). These primer sets were designed based on the nucleotide sequences of regions of the DNA fragments amplified by PCR. Template DNAs for cassette PCR were prepared using a Takara LA PCR™ *In Vitro* Cloning Kit (Takara Bio Co., Shiga, Japan) according to the manufacturer's instructions. Genomic DNA from *P. nameko* NGW19-6 was digested with restriction endonucleases shown in Table 2-1, and the fragments were ligated with nucleotide linkers and used as templates for PCR. PCR was carried out in a 100-μl reaction mixture containing 1 × Ex Taq buffer, 100 ng of extracted genomic DNA, 100 pmol of each primer, each dNTP at a concentration of 0.2 mM, and 2.5 U of Ex Taq polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94°C for 1 min, followed by 30 cycles of 30 s at 94°C and 5 min at 68°C. All amplified DNA fragments were subcloned into pT7Blue(R) T-vector (Novagen, Madison, WI, USA) and all plasmids were sequenced (Table 2-1). PCR was performed using a

Takara PCR Thermal Cycler Personal (Takara Bio Co.), and the nucleotide sequences of the amplified regions were determined. To amplify seven segments of the genomic DNA covering the whole *A* mating-type region, seven oligonucleotide primer pairs (Hox up2 2m/nameko up22, Hox-Dn 2m/nameko down 22, hox Up-3m/nameko up 32, hox Dn-3m/nameko down32, hox Dn-4m/Hox down42, hox Dn-5m/-Dn 52, Hox Dn6-m/Hox Dn 62) (Table 2-2) were designed based on the nucleotide sequences of the DNA fragments amplified by the cassette PCR method. The seven amplified DNA fragments were purified using a Microcon-100® filter (Millipore, Bedford, MA, USA) and used as DNA templates for direct sequencing with oligonucleotide primers.

In order to amplify the genomic DNA fragments of *A3-hox1* and *A3-hox2* from *P. nameko* NGW12-163, oligonucleotide primers (MipF and Hox2AmpR2) were designed from the nucleotide sequences of the *A* mating-type gene and its flanking region in *P. nameko* NGW19-6. PCR was carried out in a 100-μl reaction mixture containing 1 × Ex Taq buffer, 100 ng of extracted genomic DNA, 100 pmol of each primer, each dNTP at a final concentration of 0.2 mM, and 2.5 U of Ex Taq polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 5 min at 72°C. The amplified DNA fragments were purified and used as DNA templates for direct sequencing with appropriate oligonucleotide primers.

2-3-4 DNA sequencing and computer analysis of nucleotide and protein sequences. DNA sequencing was carried out using an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator™ Cycle Sequencing kit (version 3.1; Applied Biosystems) according to the manufacturer's instructions. Nucleotide and protein sequence data were analyzed using Genetyx-SV/RC Version 8 (Software Development, Tokyo, Japan). Identification of genes in the chromosomal regions around NGW19-6 A4-hox1 was accomplished using the software Eukaryotic GeneMark.hmm (URL: http://exon.gatech.edu/GeneMark/eukhmm.cgi), *Caenorhabditis elegans* ES-3.0 models (Lomsadze et al. 2005) and BLASTX database searching (Altschul et al. 1997) of the EMBL (European Molecular Biology Laboratory)-EBI (European Bioinformatics Institute) database (URL: http://www.ebi.ac.uk/blast2/) to find open reading frames (ORFs) with significant matches to known genes. We chose a P value of $<10^{-10}$ as the threshold value for homolog assignment. The subcellular localization of the proteins identified was predicted using PSORTII (Nakai and Kanehisa 1992; http://psort.nibb.ac.jp). The existence of coiled-coil regions with a score of 1.3 or above was predicted using the COILS program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_lupas.html; Lupas et al. 1991), which compares a sequence to a database of known parallel two-stranded coiled-coils and derives a similarity score.

The nucleotide sequences of the *A* mating-type locus and its flanking regions from *P. nameko* NGW19-6 and NGW12-163 have been deposited in the DDBJ database under the accession nos. AB435542 and AB435543, respectively.

2-3-5 Reverse transcription-PCR (RT-PCR) procedure. Amplification of full-length cDNA by RT-PCR was performed using a SuperScript™ One-Step High Fidelity kit (Invitrogen, Tokyo, Japan). The reverse transcription reaction and PCR were carried out according to the manufacturer's instructions. The amplified fragments generated using these methods were subcloned and sequenced using appropriate oligonucleotide primers. The primers used for full-length cDNA were designed from genomic DNA and are listed in Supplementary Table 2-3.

2-4 Results and discussion

2-4-1 Structure of the *A* mating-type region in *P. nameko* NGW19-6. In order to investigate the genomic structure of the region surrounding *A4-hox1*, a component of the *A* mating-type locus in *P. nameko* NGW19-6, a 39,882 bp nucleotide region containing *A4-hox1* was amplified and sequenced. A gene map of this region is shown in Fig. 2-1, and position and characterization data for these genes are given in Table 2-4. Fifteen genes with significant similarity to genes in the EMBL database ($P < 10^{-10}$) were identified in this region. First,

another homeodomain protein gene (*A4-hox2*) was found 284 bp upstream of *A4-hox1*, which was transcribed in the opposite direction to the *A4-hox1* gene. *A4-hox2* and *A4-hox1* are located in similar positions to the two classes of mushroom *A* mating-type homeodomain genes in the tetrapolar mushrooms *C. cinerea*, *S. commune* (Casselton and Olesnicky 1998) and *Pleurotus djamor* (James et al. 2004a,b), and the bipolar mushroom *C. disseminatus* (James et al. 2006). In *P. nameko* NGW19-6, this pair of homeodomain genes represents the only genes in this region with similarity to known mating-type genes in other fungi (e.g. *Ustilago maydis* and *P. djamor*), suggesting that these genes function in mating-type determination (Aimi et al. 2005). The other five genes with known function in this region are the mitochondrial intermediate peptidase gene (*mip*), which is conserved in the *A* mating-type region (James et al. 2004b), the methylmalonate-semialdehyde dehydrogenase gene (*mmsd*) (Loftus et al. 2005), the low molecular weight phosphotyrosine protein phosphatase gene (*lmwppp*) (Mondesert et al. 1994), the ammonium transporter protein gene (*amtp*) (Javelle et al. 2002), and glycine dehydrogenase (*glydh*) (James et al. 2006). We also used GenBank similarity searches to find homologues gene in other mushrooms. Surprisingly, eight genes around *A* locus of *P. nameko* (including *β-fg*, *mip*, *up11*, *up10*, *up2*, *up8*, *sec61*, *glydh*) have homologues genes around *A* locus in the bipolar mushroom *C. disseminatus* and the tetrapolar mushrooms *C. cinerea* and *Laccaria bicolor* (Fig. 2-1). Moreover, the order and transcription of these

genes were very similar in these four species. Although four other genes (including *mmsd*, *lmwppp*, *hp1*, *amtp)* in *P. nameko* were not discovered around *A* locus in other three species, they were found upstream of *A* locus both in *C. cinerea* and *L. bicolor*. Also, in *C. cinerea* and *L. bicolor*, *mmsd* and *lmwppp* genes, *hp1* and *amtp* genes link together, respectively, suggesting that these four genes in *P. nameko* may translocate from upstream of the *A* locus to around it. In addition, three genes (including *mip*, *up2* and *glydh*) in *P. namko* have homologues genes in the tetrapolar mushrooms *P. djamor* (James et al. 2004b) (Fig. 2-2). Interestingly, five other genes (including *β-fg, up11, up10, up8, sec61*) in *P. nameko*, which were conserved in *C. disseminatus*, *C. cinerea* and *L. bicolor*, also have homologue sequences around *A* locus of *P. djamor* (Fig. 2-2). According to the position of homologue sequences and homeodomain protein genes between *P. nameko* and *P. djamor*, we may deduce that paracentric inversion once happened in the region of *A* mating type locus in *P. djamor*. Previously, Niculita-Hirzel et al. (2008) compared gene organization of the mating type regions in *Laccaria bicolor* with *C. cinerea*, *P. djamor* and *Phanerochaete chrysosporium,* and concluded that the *A* locus is in a region where the gene order is under strong selection across the Agaricales. The results of our analysis supported their conclusion. Also, conserved gene order around *A* locus discovered between the bipolar mushrooms and tetrapolar mushrooms

agree with the idea that the ancestor of the homobasidiomycetes is accepted as having a tetrapolar mating system (Raper 1996; Raper and Flexer 1971).

2-4-2 Comparative analysis of homeodomain protein sequences. To confirm the transcription and deduced protein sequences of the homeodomain protein genes, we amplified and sequenced the *A* mating-type region of *P. nameko* NGW12-163, which carries the homeodomain protein genes *A3-hox1* and *A3-hox2*, which are dissimilar from the *A4* genes. Furthermore, full-length cDNA fragments of *A4-hox1, A4-hox2, A3-hox1* and *A3-hox2* (the genomic DNA sequence size and expected cDNA size: *A4-hox1,* 2117bp, 1867bp; *A4-hox2* 2002bp, 1727bp; *A3-hox1*, 2147bp, 2006bp; *A3-hox2,* 1867bp, 17006bp) were amplified and sequenced. The full-length cDNA fragments were successfully amplified (Fig. 2-3) and the intron sequences were found to have been excised relative to the genomic DNA sequences, showing that the *A4-hox1, A4-hox2, A3-hox1* and *A3-hox2* genes are transcribed in *P. nameko* cells.

The deduced protein sequences of A3-Hox1 and A4-Hox1 have 28% and 26% amino acid identity, and 43% and 42% amino acid similarity with the whole b1-2 protein sequence of *C. cinerea,* respectively, which is an HD1 type transcription factor (Gieser and May 1994). The deduced protein sequences of A3-Hox2 and A4-Hox2 have 29% amino acid identity, and 42% and 44% amino acid similarity with the whole a2-1 protein sequence of *C. cinerea,*

respectively, which is an HD2 type transcription factor (Kües et al. 1994). Thus, the proteins encoded by *hox1* and *hox2* gene in *P. nameko* correspond to the HD1 and HD2 proteins in *C. cinerea*, respectively.

In order to understand the functions of the homeodomain proteins in this bipolar mushroom and how they have differentiated relative to the homeodomain proteins of tetrapolar mushrooms, we predicted significant functional domains within the protein sequences of the four homeodomain proteins from *P. nameko* NGW12-163 (*A*3) and NGW19-6 (*A*4) using bioinformatics software. The predicted functional domains were then compared with those of the HD1 and HD2 proteins in *C. cinerea*. In Hox1 and Hox2 protein of *P. nameko*, the homeodomain, which identifies the proteins as putative transcription factors, is situated 112 to 149 amino acids from the N terminus. PSORTII analysis (Nakai and Kanehisa 1992) predicted that A3-Hox1 and A4-Hox1 contain two nuclear localization signals (SXKRRR and SXKRKR), and that A3-Hox2 and A4-Hox2 contain two different nuclear localization signals (PSRSKRF and RPRK). Using the algorithm derived by Lupas et al. (1991), three, two and one putative coiled-coil dimerization motifs were predicted for the A3-Hox1, A4-Hox1 and A4-Hox2 proteins, respectively, and no coiled-coil motif was predicted for the A3-Hox2 protein. In A3-Hox1 and A4-Hox1 proteins, positively charged domains with isoelectric points (pI) of 4.15 and 4.07, respectively, were found. On the other hand, the A3-Hox2 and

A4-Hox2 proteins had negatively charged domains with isoelectric points (pI) of 10.02 and 10.51, respectively (Fig. 2-4).

The differences in the potential functional domains of the HD1- and HD2-type proteins in *P. nameko* and the HD1 and HD2 proteins in *C. cinerea* and *L. bicolor* are summarized in Fig. 2-5. Analysis of the HD1 protein b1-2 in *C. cinerea* revealed two potential bipartite nuclear localization signals (NLS; KRRR and KRKR), but no corresponding sequences in the HD2 protein a2-1 and b2-1 (Kües et al. 1994). In *L. bicolor*, also just HD1 protein has two predicted nuclear localization signals (Niculita-Hirzel et al. 2008). However, two potential NLS were found in both the HD1- and HD2-type proteins of *P. nameko*. In *C. cinerea* and *L. bicolor*, heterodimerization may regulate entry of the active transcription factor complex of HD1 and HD2 into the nucleus, because of the absence of NLS in the HD2 protein (Spit et al. 1998, Niculita-Hirzel et al. 2008). However, in *P. nameko* both HD1- and HD2-type proteins have NLS, indicating that these proteins can enter the nucleus without heterodimerization in the cytoplasm

Both HD1 and HD2 in *C. cinerea* have two predicted coiled coil domains (CCDs) in the N-terminal region and one at the downstream homeodomain; furthermore, HD1 also has a predicted CCD in the C-terminal region. Also, in *L. bicolor* both HD1 and HD2 have a predicted CCD in N-terminal region and one

at the downstream homeodomain. The CCDs in the N- and C-terminal regions are predicted to be conserved in A3-Hox1 in *P. nameko*, but the CCD in the downstream homeodomain is absent. A4-Hox1 has only one predicted CCD at the N-terminus, A3-Hox2 has no CCDs, and A4-Hox2 has only one CCD at the N-terminus. The CCD in the C-terminal region in Hox1 was predicted to be conserved in *P. nameko*. In *C. cinerea* the predicted C-terminal helix of HD1 protein was active as a potential activation domain in a yeast cell and may also have a role in transcriptional repression (Asante-Owusu et al. 1996).

The HD1 and HD2 CCDs in N-terminal was proved to have a functional importance for heterodimization and protein discrimination in *C. cinerea* (Banham et al. 1995). However, the CCDs at the N-terminus are not conserved in *P. nameko*. Negatively charged domains were found in the HD1 proteins of *C. cinerea* and in the HD2-type proteins of *P. nameko*; and, conversely, positively charged domains were found in the HD2 proteins of *C. cinerea* and in the HD1-type proteins of *P. nameko*.

Two bipolar mushrooms, *C. disseminatus* and *P. nameko*, appear to utilize only homeodomain proteins rather than pheromone receptors to determine mating type. However, there is no molecular evidence showing that only the homeodomain proteins control clamp cell formation in bipolar

mushrooms. Therefore, in future studies, we will investigate the function of the homeodomain proteins *in vivo* using a molecular technique.

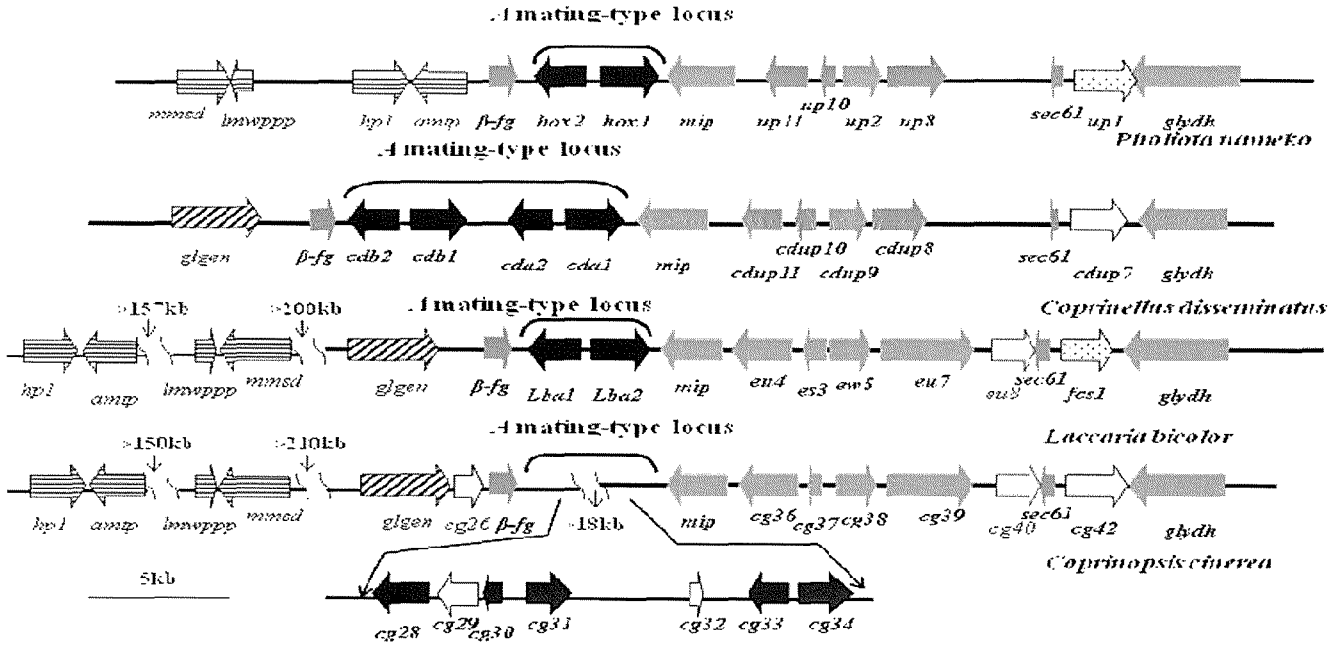


Fig. 2-1 – Comparisons of the genomic structure of the bipolar mushroom *A* mating-type locus and its flanking region among various species. Homeodomain protein genes are shown as black boxes (■). Gray shading (▒) indicate gene sequences that are conserved around the *A* locus among the bipolar mushrooms *P. nameko* and *Coprinellus disseminatus* (James et al. 2006) and the tetrapolar mushrooms *Coprinopsis cinerea* and *Laccaria bicolor* (Niculita-Hirzel et al. 2008) . A stippled pattern (⬚) indicates gene sequences that are conserved between *P. nameko* and *L. bicolor*. White boxes (□) indicate genes that are not conserved among the four species. Diatropic stripes (▤) indicate the gene sequences in *P. nameko* that are found upstream of the *A* locus in *L. bicolor* and *C. cinerea*. Arrows indicate the putative direction of transcription. Date stem from *C. disseminatus* (positions 14970-51817 from GenBank accession no. DQ056143, James et al., 2006), *L. bicolor* (positions 796041-796041, 956657-959763, 1162065-1191442 from contig 1, JAZZ annotation v.10, http://genome.jgipsf.org/Lacbi1/Lacbi1.home.html, Niculita-Hirzel et al. 2008), *C. cinerea* (positions 27679-31768, 182560-185711, 401850-443535 from GenBank accession no. NW_001884709).

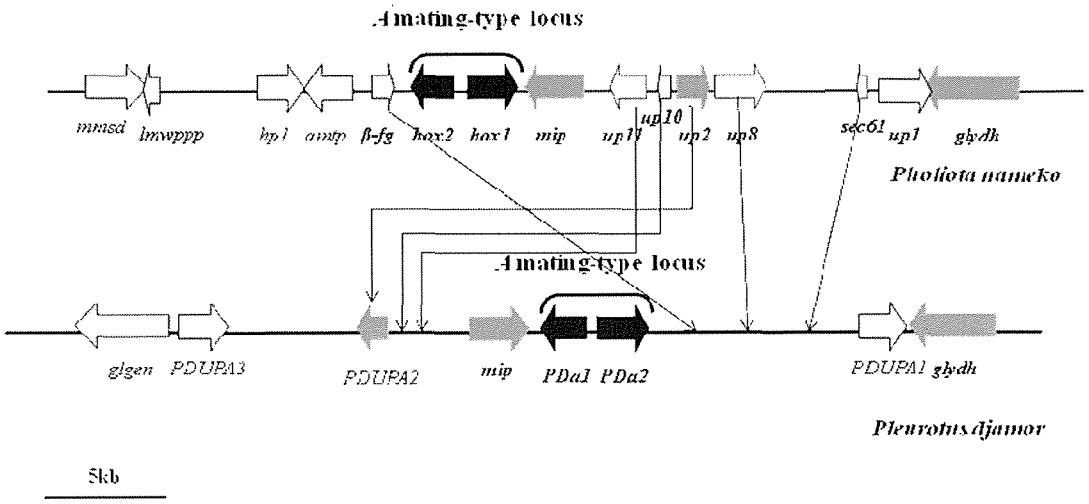


Fig. 2-2 Comparisons of the genomic structure of the bipolar mushroom *A* mating-type locus and its flanking region between *P. nameko* and *Pleurotus djamor* (positions 20102-55643, from GenBank accession no. AY462111, James et al. 2004b). Gray shading (▒) indicate gene sequences that are conserved around the *A* locus between two species. White boxes (□) indicate genes that exist in *P. nameko* are not found in *P. djamor*. Arrows point the DNA sequence position of *P. djamor* which displays high DNA sequence identity with the gene sequence in *P. nameko*.

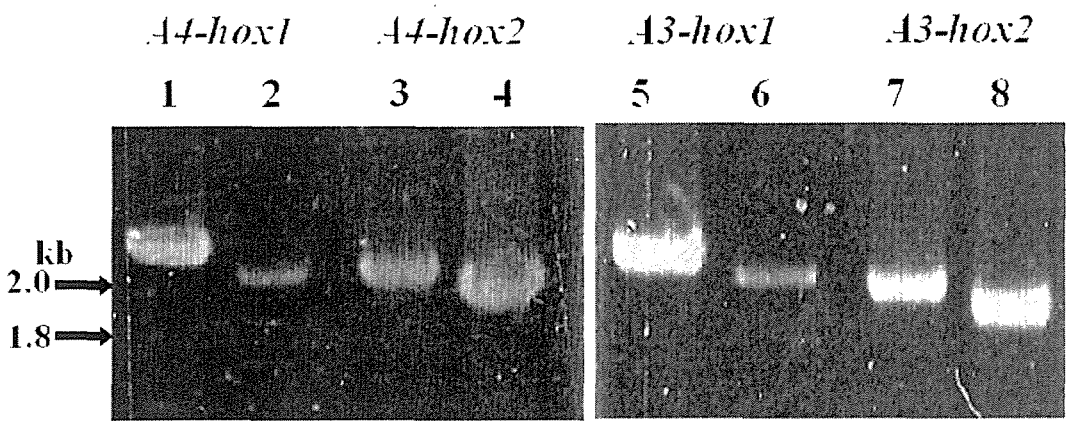


Fig. 2-3 Expression of genes encoding the homeodomain proteins from *Pholiota nameko* NGW12-163 and NGW19-6. Lanes 1 and 2, *A4-hox1* from NGW19-6; lanes 3 and 4, *A4-hox2* from NGW19-6; lanes 5 and 6, *A3-hox1* from NGW12-163; lanes 7 and 8, *A3-hox2* from NGW12-163. Lanes 1, 3, 5 and 7, genomic DNA; lanes 2, 4, 6, and 8, cDNA. The size marker corresponds to a 100 bp DNA ladder.

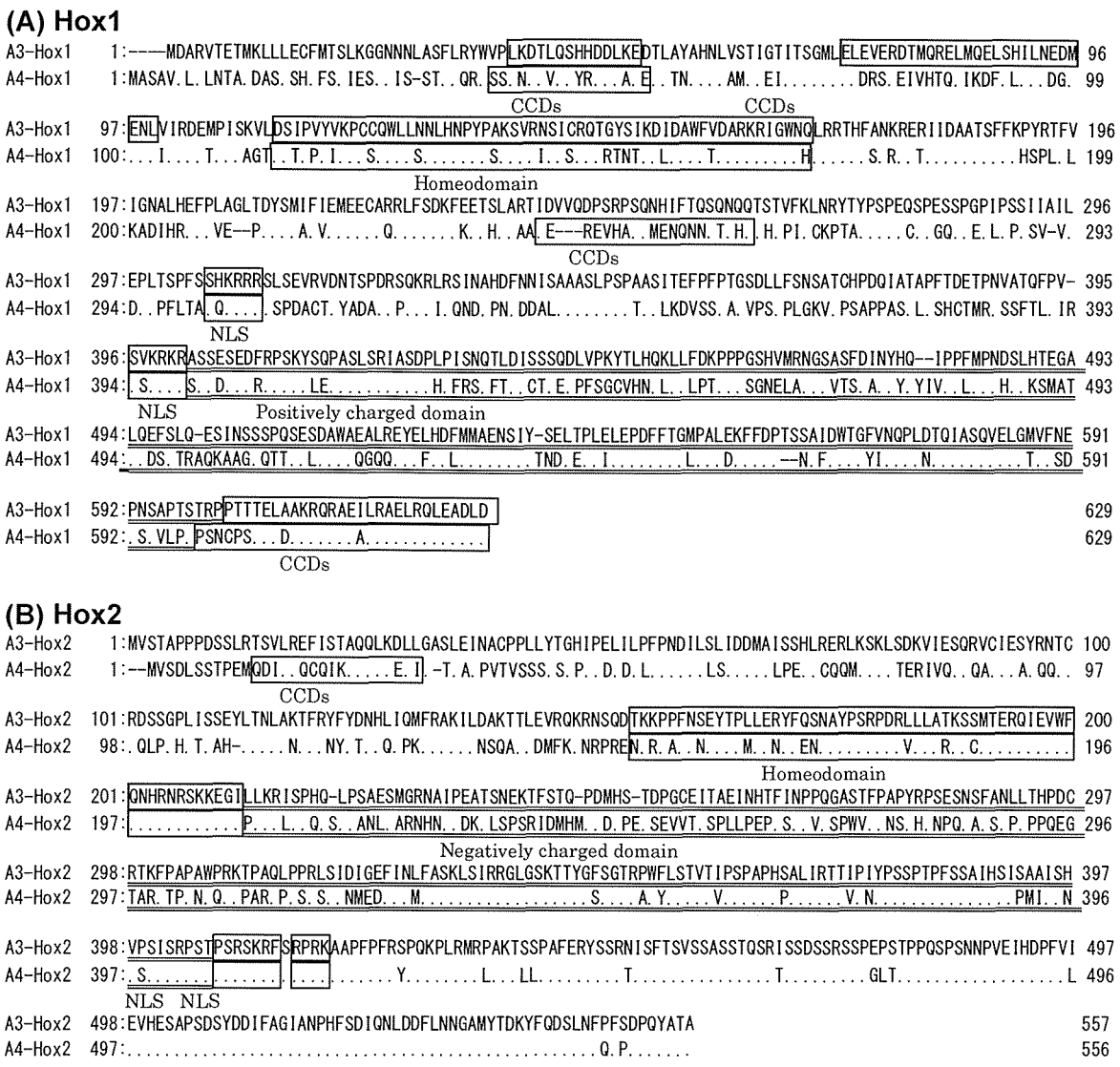


Fig. 2-4 Comparison of the deduced amino acid sequences of the homeodomain proteins of *P.* nameko. (A) Alignment of A3-Hox1 and A4-Hox1. (B) Alignment of A3-Hox2 and A4-Hox2. Amino acid residues that are identical are indicated by dots. The inferred motifs are indicated by boxes: homeodomains, nuclear localization signals (NLS) and predicted coiled-coil domains (CCDs) with a score of 1.3 or above (as determined using COILS; Lupas et al. 1991). Positively charged domains and negatively charged domains are indicated with by double underlines.

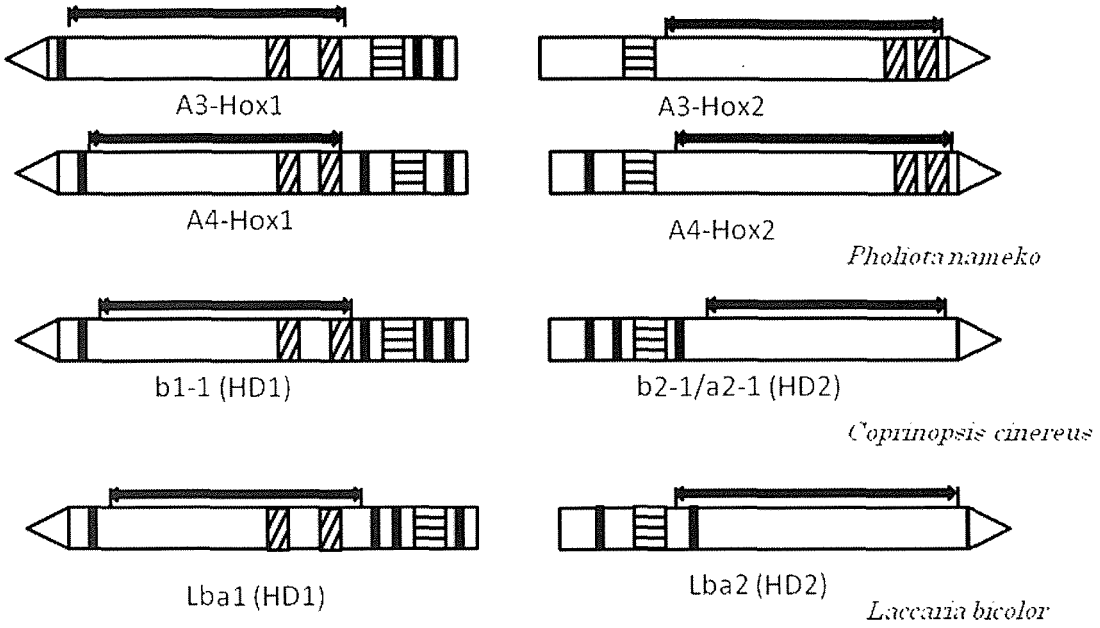


Fig. 2-5 Comparison of the predicted functional domains of *P. nameko* Hox proteins with HD1 and HD2 proteins of *C. cinerea* and *L.bicolor*. The predicted functional domains of HD1 and HD2 proteins of *C. cinerea* were modified from Kües et al. (1994), and those of *L.bicolor* were modified from Niculita-Hirzel et al. (2008). Black boxes (■), diagonal stripes (▨), diatropic stripes (▤) and double arrow (⟷) indicate predicted helical regions, nuclear localization signals (NLS), homeodomains and activation domains, respectively.

Table 2-1 Genomic DNA fragments amplified in this study.

Primer name	Primer sequence	Restriction enzyme	Fragment size (bp)	Clone name	Reference
HOX1-Up	5'-CTTGTCTGCCTGGATATGCTGA-3'	*Bam*HI	1,600	pHU1	Aimi et al., 2005
HOX1-Up3	5'-CCTGGATATGCTGATACGAACAGA-3'				
HOX1-1Dn1	5'-AGACTTGGACGCTTGGTTCATC-3'	*Sal*I	2,500	pHD1	
HOX1-1Dn2	5'-CAGGCAGACAAGGACAAATACC-3'				
nameko up 21	5'-GTCGACCAGATCGACTTCTTCTAG-3'	*Bgl*II	2,562	pHU2	This study
nameko up 22	5'-CGAAAAAACCTTTTCAACGCAGCC-3'				
nameko up 31	5'-ATTACCTTGTCGCCAGAGACGAAGCTTAGTG-3'	*Eco*RI	2,934	pHU3	
nameko up 32	5'-GAGTTGTACTGCTGACCTCCTGTGACTTCT-3'				
nameko down 21	5'-TGAGTGAACACGTTTTGCCAGACT-3'	*Xho*I	2,674	pHD2	
nameko down 22	5'-TGGAATGGCAGGGATCGCTGTGGT-3'				
nameko down 31	5'-ATTCGTACGCTTGATTTGCAGCTTCCACCCA-3'	*Bam*HI	2,872	pHD3	
nameko down32	5'-GTCCAAAATTGACGTTCTCGAATATTGTCG-3'				
Hox down 41	5'-CAGCGCGAAAATACTGTTCAGAGA-3'	*Xba*I	2,925	pHD4	
Hox down 42	5'-GCAAAAGAGACCAGTTTGGATGGA-3'				
Hox-Dn 51	5'-CGGTCTCAGAAGTGAAGGCTGTT-3'	*Xho*I	2,950	pHD5	
Hox-Dn 52	5'-CAGGAGGAACTCAGGTTAATGGTTG-3'				
Hox Dn 61	5'-CGGAATATCTCGCAAAGACTCAAA -3'	*Xba*I	1,358	pHD6	
Hox Dn 62	5'-GCGAGTCAACTGATTACCGTGAAG-3'				

Table 2-2 Oligonucleotide primers used for amplification of a genomic DNA fragment from *P. nameko* NGW19-6 and NGW12-163 genomic DNA.

Primer	Sequence	Fragment name
Hox up2 2m nameko up22	5'-TCCTTCCTCAACCTTGCAAAGCAG-3' 5'-CGAAAAAACCTTTTCAACGCAGCC-3'	HU2 of NGW19-6
hox Up-3m nameko up 32	5'-ATCCGACGAAAACACATCTGCTGACT-3' 5'-GAGTTGTACTGCTGACCTCCTGTGACTTCT-3'	HU3 of NGW19-6
Hox-Dn 2m nameko down 22	5'-ATGGGAGCAGACGGATTGCCGATT-3' 5'-TGGAATGGCAGGGATCGCTGTGGT-3'	HD2 of NGW19-6
hox Dn-3m nameko down32	5'-AGGTTGGGCCAGGATAATATTGGT-3' 5'-GTCCAAAATTGACGTTCTCGAATATTGTCG-3'	HD3 of NGW19-6
hox Dn-4m Hox down42	5'-GCTATGGATATAATCGAAGAGGACG-3' 5'-GCAAAAGAGACCAGTTTGGATGGA-3'	HD4 of NGW19-6
hox Dn-5m Hox-Dn 52	5'-GCTCACCGAGACACGTTTCTCAAG-3' 5'-CAGGAGGAACTCAGGTTAATGGTTG-3'	HD5 of NGW19-6
Hox Dn6-m Hox Dn 62	5'-TAGAAAAGTGGAGACTGCAACCCT-3' 5'-GCGAGTCAACTGATTACCGTGAAG-3'	HD6 of NGW19-6
MipF Hox2 AmpR2	5'-GCAGAGCTAGCCAAATTACACGA-3' 5'-GCTGTAGAGGATTTCTGGGATGAGGGGAGTC-3'	DNA fragment coding *A3-hox1* and *A3-hox2* from NGW12-163 strain

Table 2-3 – Oligonucleotide primers used for amplification of the genomic and full-length cDNA fragments.

Primer	Sequence	Gene
A3-hox1FNcoI A3-hox1RBamHI	CCATGGACGCACGAGTAACAGAAA GGATCCAAAATTTTCAATCAAGGTC	*A3-hox1* from NGW12-163 strain
A3hox2FEcoRI A3hox2RBamHI	GAATTCGCCATGGTATCCGATCTG GGATCCAGCGACGAAAAGCATTAT	*A3-hox2* from NGW12-163 strain
A4hox1FNdeI A4hox1RBamHI	CATATGGCCTCCGCCGTGGACCTCAGA GGATCCAGAAGATGGCAGATCAAT	*A4-hox1* from NGW19-6 strain
A4hox2FNcoI A4hox2RSmaI	ATTACAACCATGGTGTCGACCGCA CCCGGGAATAGCAACAGAAAAGCAT	*A4-hox2* from NGW19-6 strain

Table 2-4 Gene homologs identified in the *Pholiota nameko A* mating-type locus and its flanking region.

Gene	P-value	Position	Homologue	Possible function	Reference
mmsd	1.78×10^{-81}	1734-4309	Putative Methylmalonate - semialdehyde dehydrogenase, [*Cryptococcus neoformans*] (AAW45534)	methylmalonate-semialdehyde dehydrogenase (acylating) activity, oxidoreductase activity	Loftus et al. 2005
lmwppp	3.80×10^{-12}	4945-4213	Low molecular weight phosphotyrosine protein phosphatase [*Schizosaccharomyces pombe*] (P41893)	dephosphorylation	Mondesert et al. 1995
hp1	3.54×10^{-27}	8875-10852	Hypothetical protein [*C. neoformans*] (Q5K9H3)	Prenylcysteine lyase	Loftus et al. 2005
amtp	1.56×10^{-94}	13,022-11,037	Ammonium transporter [*Hebeloma cylindrosporum*] (AAK82417)	ammonium transporter activity	Javelle et al. .2001
β-fg	1.63×10^{-19}	13,677-14,656	Beta-flanking protein [*C. disseminatus*] (AAZ14920)	unknown	James et al. 2006
hox2	5.29×10^{-18}	17,032-15,187	Homeodomain *A* mating-type protein [*C. disseminatus*] (AAZ20167)	transcription factor activity	
hox1	3.56×10^{-21}	17,316-19,420	Homeodomain *A* mating-type protein [*C. disseminatus*] (AAZ20163)	transcription factor activity	Aimi et al. 2005; James et al. 2006
mip	0	22,201-19,725	Mitochondrial intermediate peptidase [*C. disseminatus*] (AAO61501)	hydrolase activity ,mitochondrial intermediate peptidase activity, zinc ion binding	
up11	2.61×10^{-51}	24,740-23,254	Hypothetical protein UP11 [*C. disseminatus*] (AAZ14914)	unknown	James et al. 2006
up10	2.42×10^{-16}	25675-25120	Hypothetical protein UP10 [*C. disseminatus*] (AAZ14913)	unknown	
up2	3.38×10^{-53}	25,876-27,253	Hypothetical protein UPA2 [*P. djamor*] (AAS46739)	unknown	James et al. 2004b
up8	3.56×10^{-21}	27,610-29,701	Hypothetical protein UP8 [*C. disseminatus*] (AAZ14911)	unknown	James et al. 2006
sec61	1.79×10^{-11}	33,868-33,459	secE/sec61-γ [*C. disseminatus*] (AAZ14910)	protein translocase activity	
up1	6.10×10^{-28}	34,093-36,288	Hypothetical protein [*Laccaria bicolor* S238N-H82](XM_001873354)	unknown	James et al. 2006
glydh	0	39,882-36,001	Glycine dehydrogenase [*C. disseminatus*] (AAZ14908)	glycine dehydrogenase (decarboxylating) activity, lyase activity	

P value indicates the probability of the match being due to chance in EMBL similarity searches. In the 'Homologue' column, the species from which the lowest P value hit was obtained is given in brackets, followed by the DDBJ/EMBL/GenBank accession number in parentheses

Chapter 3

DNA-mediated transformation system in a bipolar basidiomycete, *Pholiota nameko*

3-1 Abstract

We cloned a gene for the succinate dehydrogenase iron-sulfur protein subunit (*sip*) from a bipolar mushroom, *P. nameko*, and introduced a point mutation that confers carboxin resistance into it. Using the homologous selective marker gene and a heterologous drug selective marker gene, hygromycin B phosphotransferase gene (*hph*), respectively, we successfully constructed two DNA-mediated transformation systems in *P. nameko*. Both of these two transformation systems have high transformation efficiency; efficiency of carboxin resistant transformation was about 88.8 transformants per μg pMBsip2 DNA using 5×10^6 protoplasts in regeneration plates containing 1.0 μg/ ml carboxin; efficiency of hygromycin B resistant transformation was about 122.4 transformants per μg pMBhph1 DNA using 5×10^6 protoplasts in regeneration plates containing 150 μg/ ml hygromycin B. The results of southern hybridization demonstrated that the introduced sequence (mutant *sip* or *hph*) was integrated into the chromosomal DNA with one or more copy numbers in these transformants.

3-2 Introduction

Pholita namkeo, a wood-decaying homobasidiomycete, is one of the main cultivation mushrooms in China and Japan. It produces an economically important edible mushroom called "Nameko". Due to recent advances in bottle cultivation technology in Japan, this mushroom is now available in Japanese domestic markets throughout the year. However, monokaryotic hyphal cells from dikaryotic mycelia are produced in the commercial spawns of this mushroom, which can lead to very poor production or a nearly total lack of fruiting when using inferior strains (Babasaki et al. 2003). It is possible that this phenomenon is related to the mating system of this fungus.

P. nameko belongs to bipolar mushroom and carries a bipolar *A* mating-type locus (Ratanatragooldacha et al. 2002). The composition of the *A* mating-type gene in the bipolar mushroom *P. nameko* has been recently described (Aimi et al. 2005). In the paper, the genes encoding the homeodomain protein *A4-hox1* and the pheromone receptor *A4-rcb1* were characterized, which are putative homologs of the HD1 and Rcb3 protein genes of the tetrapolar basidiomycete *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys and Moncalvo, respectively (Badrane and May 1999; Halsall et al. 2000). Following that, the genomic structure of the *P. nameko A* mating-type locus were analyzed and a single pair of homeodomain protein genes was found in the region (Yi et al.

2009a). In the next step, the functions of the *P. nameko A* mating type locus will be investigated *in vivo* in this species. In order to perform it, firstly, a transformation system is needed to construct in this species. But up to now, there is no transformation system was reported about this fungus. DNA-mediated transformation is a powerful tool for molecular analysis of fungi mating genes. Besides auxotrophic selective marker (Binninger et al. 1987), a dominant drug resistance marker is a very effective tool for constructing a DNA-mediated transformation system in fungi. As a selectable marker for dominant antibiotic resistance, the hygromycin B phosphotransferase gene (*hph*) from *Escherichia coli* (Migula) Castellani and Chalmers often fuses to homologous promoter before transforming, and has been used successfully in transforming at least seven basidiomycetes, two plant pathogenic smut fungi, *Ustilago maydis* (DC.) Corola (Wang et al. 1988) and *Ustilago violacea* (Pers.) Rouss. (Bej and Perlin, 1989), an ectomycorrhizal fungus, *Laccaria laccata* (Scop. : Fr.) Berk. et Br (Barrett et al. 1990) and four edible fungi, *Pleurotus ostreatus* (Jacq. : Fr.) Kummer (Ming et al. 1992; Li et al. 2006), *Agaricus bisporus* (Large) Sing (Rhee et al. 1996), *Lentinus edodes* (Berk.) Pegler (Hirano et al. 2000), and *Ganoderma lucidum* (Curt. Fr.) Karst (Li et al. 2004). Recently, a homologous drug resistant marker that encodes a mutant iron-sulfur protein (Ip) subunit of succinate dehydrogenase and confers dominant resistance

to a systemic fungicide, carboxin was developed in *P. ostreatus* (Honda et al. 2000) and *L. edodes* (Irie et al. 2003).

To investigate if the *A* mating-type locus in *P. nameko* are functionally redundant and if the pheromone genes also play the role during the dikaryon formation in this bipolar species, in this study we constructed two transformation systems in *P. nameko* using the heterologous and homologous selective marker, respectively.

3-3 Materials and methods

3-3-1 Fungal strain used in this study. Auxotrophic mutant monokaryons of *P. nameko* NGW19-6 (*A4*, *pdx1*) was used in this study.

3-3-2 DNA preparation. To collect carboxin resistant (Cbx^r) transformants, five mycelial agar blocks (5 × 5 × 5 mm) from MYG plate were transferred to 10 ml of liquid MYG medium containing 2.0 μg/ml carboxin in a 100 ml Erlenmeyer flask and grown at 25°C for two weeks. To collect mycelium of hygromycin resistant (Hyg^r) transformants, ten mycelia agar blocks (5 × 5 × 5 mm) from MYG plate were transferred to 5 ml of liquid MYG medium containing 200 μg/ml hygromycin in a 100 ml Erlenmeyer flask and grown at 25°C for three weeks. Mycelia were then harvested by filtration, frozen in liquid

nitrogen, and ground to a fine powder in a mortar and pestle. Genomic DNA was extracted according to the method described by Dellaporta et al. (1983).

3-3-3 Amplification of succinate dehydrogenase Ip subunit gene (*sip*) of *P. nameko*. All amplified DNA fragments were subcloned into the pT7Blue (R) T-vector (Novagen, Madison, WI, USA), and then were sequenced. Primers used were shown in Table 3-1. Initially, degenerate PCR of Ip subunit of succinate dehydrogenase from NGW19-6 strain of *P. nameko* was accomplished using the primers SDIpF2 and SDIpR. The SDIpF2 and SDIpR primers were designed based on the amino acid sequences IKIKNEI and YWWNQDE, respectively, which were conserved in the Ip subunit of *P. ostreatus*, *L. edodes*, *A. bisporus* and *U. maydis* (Keon et al. 1991; Irie et al. 1998; De Groot et al. 1999; Irie et al. 2003). The PCR reaction was carried out in 100 μl volumes containing (100 ng of extracted genomic DNA, 100 pmol of each primer, dNTP at 0.2 mM concentration, 1 × PCR buffer and 2.5 U of Ex Taq polymerase Takara Bio Co. Shiga, Japan). Thermal cycling parameters were an initial denaturation step at 95℃ for 3 min followed by 30 cycles of denaturing at 95 s, annealing at 50℃ for 2 min, extension at 72℃ for 30 s, and a 10 min final extension at 72℃. An approximately 500 bp DNA fragment was obtained. To obtain complete sequence of the Ip subunit gene, two primers sets (IPup1/IPup2, IPdn1/IPdn2), which were designed based on the partial sequence acquired,

were employed for cassette PCR amplification. Template DNA for cassette PCR was prepared with a TaKaRa LA PCR™ *In vitro* Cloning Kit (Takara Bio Co.) according to the manufacturer's instructions. Genomic DNA from *P. nameko* was digested with restriction endnuclease, ligated with nucleotide linker and used as templates for PCR. We cloned and sequenced an approximately 1.6-kbp PCR product from IPup2 to a *Bam*HI site containing the 5'-untranslated region and a 1.9-kbp product from IPdn2 to *Sal*I site including the 3'-untranslated region.

To amplify the whole genomic clone of the Ip subunit gene, oligonucleotide primers Ip-d1F and Ip-d1R were designed based on the sequence of DNA fragments obtained by the cassette PCR method. The thermocycling parameters used were: initial denaturation at 95℃ for 4 min, followed by 30 cycles of 95℃ for 30 s, 64℃ for 30 sec, 72℃ for 3.5 min, and a final 10 min extension at 72℃. The amplified DNA fragment was purified with QIAquick PCR Purification Kit (QIAGEN, Tokyo, Japan) subcloned to the pT7Blue (R) T-vector (Novagen) and constructed plasmid named pMBsip1. The purified fragment was used as a DNA template for direct sequencing with oligonucleotide primers (Table 3-1).

3-3-4 Site directed mutagenesis and construction of the vector plasmid. A point mutation (CAT to CTT) that causes an amino-acid substitution (His240 to

Leu) was introduced into the Ip subunit gene of *P. nameko* NGW19-6 strain. Firstly, 2,262 bp DNA fragments containing the Ip subunit promoter and coding sequence were amplified using primers, Ip-d1F and Ip-cbxF (5'-TTGAAGATAGTAAGACAACGGTACA-3', where A identifies the base substitution). A 969 bp DNA fragment including the Ip subunit coding and terminator sequence was amplified using primers, Ip-d1R and Ip-cbxR (5'-ATGTACCGTTGTCTTAACTATCTTC-3', where T identifies the base substitution). The template DNA used for the two fragments amplification was the genomic DNA from lyophilized mycelium of *P. nameko* strain NGW19-6. The amplification conditions of the former fragment were as follows: initial denaturation at 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 s, 60℃ for 30 s, 72℃ for 1.5 min, and a final 10 min extension at 72℃. The amplification conditions of the latter fragment were as follow: initial denaturation at 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 s, 59℃ for 30 s, 72℃ for 2.5 min, and a final 10 min extension at 72℃. DNA fragments were separated on a 0.8% agarose gel and purified. Subsequently, the two fragments were combined by PCR to form one 3.2-kb fragment using Ip-d1F and Ip-d1R primers and the two purified fragments as templates. The amplification was initiated with 3 min at 94℃ denaturation, followed by 30 amplification cycles (94℃ for 30 s, 64℃ for 30 s, and 72℃ for 3.5 min). PCR product was subcloned to the pT7Blue (R) T-

vector and sequenced to confirm the base substitution. The constructed plasmid was designated as pMBsip2 (see Fig. 3-1).

3-3-5 The construction of plasmid vector containing *hph* gene. To express *hph* gene efficiently in *P. nameko*, this gene was needed to combine with promoter and terminator of this species. In this experiment, we used the promoter and terminator of Ip subunit gene. Firstly, the *hph* gene was amplified using pER8-*Xho*I and pER8-*Bgl*II primers and plasmid vector (pER8, Zuo et al. 2000) as a template, which is a transformation vector for plant. The plasmid DNA was kindly provided by Dr. Hiroyuki Kminaka (Tottori University, Japan). The amplification conditions were as follows: initial denaturation at 94℃ for 4 min, followed by 35 cycles of 94℃ for 30 s, 68℃ for 1 min, and a final 10 min extension at 72℃. The amplified fragment was digested with *Xho*I and *Bgl*II. Secondly, the fragment containing pT7Blue (R) T-vector (Novagen), Ip subunit promoter and terminator, was amplified using Ip-pro-*Xho*I and Ip-ter- *Bgl*II primers and pMBsip1 as a template. The amplification conditions were as follows: initial denaturation at 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 s, 58℃ for 1 min, 72℃ for 5 min, and a final 10 min extension at 72℃.The PCR product was also digested with *Xho*I and *Bgl*II. Then, the two digested fragments above were ligated and formed plasmid named pMBhph1(see Fig. 3-1).

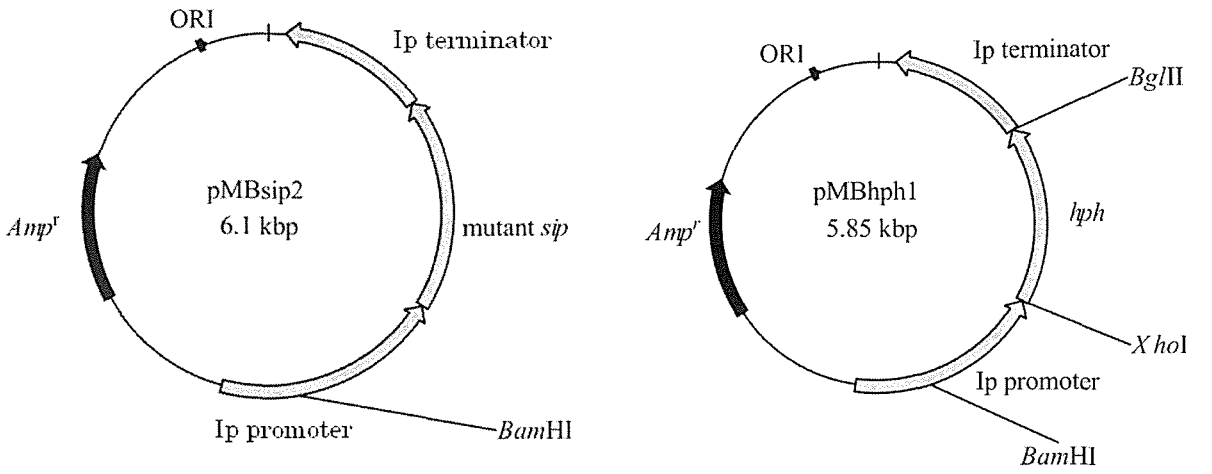


Fig. 3-1 Physical map of the plasmid pMBsip2 and pMBhph1. *Bgl*II, *Xho*I, *Bam*HI were the enzyme cutting site. The arrows indicate the transcription direction.

3-3-6 Transformation method. DNA mediated transformation was carried out essentially according to the method described by Binniger et al. (1987) and Honda et al. (2000). To collect oidia of *P.nameko*, NGW19-6 strain was inoculated on a MYG plate (2% glucose, 0.5% yeast extract, 0.5% malt extract, pH 5.6) and incubated at 25℃ for 2 weeks. Five agar blocks (2 mm × 2 mm) from the plate were inoculated to a 100 ml Erlenmeyer flask containing 10 ml MYG liquid medium at 25℃ for 2 weeks without shaking (if incubating for more than two weeks, the germination rate of oidia will be low). The culture broth and mycelia mats from five flasks were transferred to a 50 ml polypropylene conical tube and shook by hand vigorously. The culture broth was filtered through a stainless net and a 3G-1 glass filter later, and oidia were collected by centrifugation (945 × *g* for 10 min). The harvested oidia were inoculated into 100 ml MYG liquid medium in a Sakaguchi flask and incubated

at 25℃ for 18-24 h with shaking (the collected oidia concentration should not be too high, otherwise, the germination rate of oidia will be low). The germinated oidia was collected by centrifugation (4℃, 945 × *g* for 10 min), and washed with sterilized distilled water and MM buffer (0.55 M mannitol, 50 mM maleic acid, pH 5.5). The germinated oidia was suspended in 3 ml MM buffer containing 2% lywallzyme (Guangdong Institute of Microbiology, Guangdong, China), and was incubated at 30℃ for 3 h with gently shaking at 30min intervals to release protoplasts into suspension. Protoplasts were filtered through a 3G-1 glass filter to a 15 ml polypropylene conical tube and centrifuged (4℃, 340 × *g* for 5 min) and washed with MMC buffer (0.55 M mannitol, 50 mM maleic acid, 50 mM $CaCl_2$, pH 5.5). About 5×10^6 protoplasts were suspended in 50 μl MMC buffer and added 20 μg plasmid (pMBsip2 or pMBhph1) and 12.5 μl PEG buffer (25% PEG4000, 10 mM Tris-HCl, 25 mM $CaCl_2$, pH 7.5). The mixture was placed on ice for 10 min and then 500 μl PEG buffer was added. After keeping it at room temperature for 5 min, 1 ml MMC buffer and 1 ml SMYM medium (1% sucrose, 1% malt extraction, 0.4% yeast extraction, 0.55 M mannitol, pH 5.6) without agarose was joined in. Subsequently, protoplasts were incubated at 25℃ for 18~24 h, then mixed with 3 ml SMYM medium containing 0.7% agar and selection drug (carboxin concentration was set for three concentrations, 0.5 μg/ml, 1.0 μg/ml, 2.0 μg/ml; hygromycin B for four concentration, 150 μg/ml, 200 μg/ml, 300 μg/ml, 400 μg/ml). The mixture

was poured onto a SMYM agar medium (1% sucrose, 1% malt extraction, 0.4% yeast extraction, 0.55 M mannitol, 1.5% agar, pH 5.6) containing selective drug with corresponding concentration. The plate was incubated at 25℃ for 5~7 days. Appeared colonies were subcultured individually onto fresh MYG plates containing selective drug (the Cbx^r transformation was 2 μg/ml carboxin and Hyg^r transformation 200 μg/ml hygromycin B). Synchronous transformation experiments using pMBsip2 and pMBhph1 were done for three times. And three repeats were set for each concentration. In each time of experiments, a control without joining plasmid was set for every drug concentration.

The number of Cbx^r and Hyg^r regeneration colonies in the plates was calculated at the third day and a week later after the colonies appeared, respectively.

3-3-7 Southern hybridization. Southern hybridization analysis of the transformations was done to analyze the integration of the transforming DNA. The genomic DNA (0.3 - 0.5 μg) of Cbx^r and Hyg^r transformants were digested for 5 h at 37°C in a 500 μl reaction containing 20 units of *Bam*HI in the buffer supplied by the manufacturer. The digested fragments were concentrated, and then were separated by 1.0 % agrose gels and blotted onto nylon membrane (Hybond-N+, Amersham Biosciences, London, UK). DNA hybridization probes were labeled with DIG-High Prime DNA Labeling and detected by Detection

Kit (Roche Diagnostics, Tokyo, Japan). We used nested PCR to make probe. About Cbx^r transformants, we amplified part sequence of pMBsip1 using primers Ip-d4R and Ip-d2F. To make probe of Hyg^r transformants hybridization, we amplified part sequence of pMBhph1 with primers Hyg-F3 and Hyg-R3.

3-4 Results and discussion

3-4-1Nucleotide and deduced amino acid sequence of *sip*. In order to reveal genomic structure around *sip* which is succinate dehydrogenase Ip subunit in *P. nameko*, total 3,206 bp of nucleotide sequence around *sip* was amplified and determined. The respective sequence appears in DDBJ/EMBL/GenBank databases under the accession number AB455529. The coding region (from ATG to stop codon) is 1162 bp. The gene encoded a protein of 271 amino acids. The locations of the exons and introns of the gene were deduced from the interruptions in the amino acid sequence that was homologous to that of succinate dehydrogenase Ip subunit of *L. edodes* (Ire et al. 2003), as well as from the sites of consensus sequences of conserved 5' and 3' splice sites of introns. All of the introns started with GT and ended with AG. The coding region was split into seven exons by six introns. The deduced amino acid sequences of the DNA fragment were analyzed for homology with a protein database using BLASTX (Altschul et al. 1997) on the World Wide Web at http://www.ddbj.nig.ac.jp/E-mail/homology-j.html. The deduced protein

sequence of homeodomain in the *sip* has 84% amino acid identity and 91% similarity with the Sdc-ip protein of *L. edodes* (AB092822) and 77% identity and 87% similarity with Sdi protein of *P. ostreatus* (AB007361). We next examined the predicted protein sorting signals. The program PSORTII (Nakai and Kanehisa 1992) predicted that the Sip protein was mitochondrial inner membrane protein (60.9% probability) and the cleavage sites for mitochondrial sequence was between Ser26 and Gln27.

3-4-2 The carboxin resistant transformation. In carboxin resistance transformation, three carboxin concentrations (0.5 μg/ml, 1.0 μg/ml and 2.0 μg/ml) were set for the regeneration plates. In these three kinds of plates, the transformation efficiency was different and no transformant was detected in the control (TE buffer) (Table 3-2). Among them, the regeneration plate containing 1.0 μg/ml carboxin has higher transformant number than other two ones and its average transformation efficiency is 88.8 Cbx^r colonies per μg plasmid DNA using 4.5×10^6 viable protoplasts. We randomly chose the host strain and six transformants picked from 1.0 μg/ml carboxin regeneration plate for southern hybridization analysis (Fig. 3-2).

The band of endogenous *sip* sequence of the host strain was between 2.0 – 3.5 kbp (Fig. 3-2). Several hybridization signals of various in sizes were observed in the transformants except for hybridization signal of wild type gene.

Some hybridization signals were lighter comparing to other bands in the same lanes. For example, transformants Cp1-3 and Cp1-6 have an additional band located just below the host band. The two kinds of bands referred above may be the background. The band of endogenous *sip* sequence of the host strain was between 2.0 – 3.5 kbp. For the transformants, several bands of various sizes were observed in addition to the host band. Some bands were lighter comparing to other bands in the same lanes. Transformants Cp1-3 and Cp1-6 have an additional band located just below the host band. The two kinds of bands referred above may be the background. So the transforming band number of the transformants were following: Cp1-1, 2 bands; Cp1-2, 2 ~ 3 bands; Cp1-3, 2 bands; Cp1-4, 1 band; Cp1-5, 3 bands; Cp1-6, 2 bands. These results demonstrated that the introduced sequence (mutant *sip*) was integrated into the chromosomal DNA with one or more copy numbers in these transformants.

3-4-3 The hygromycin B resistance transformation. In the pMBhphI transformation, four hygromycin concentrations (150 μg/ml, 200 μg/ml, 300 μg/ml, 400 μg/ml) were set for the regeneration plate. More than half of the appeared colonies observed in the early day would not grow up to big ones in the regeneration plates, and after picking them to the MYG plates containing 200 μg/ml hygromycin they would not grow. This part of colonies maybe have no *hph* inserting the genome and express temporarily with the rudimental *hph* in the cell. So we counted the resistant transformants one week later after the

appearance of colonies. Along with the increasing of hygromycin B concentration of the regeneration plates, the number of appeared transformant decreased (Table 3-2). And no transformant was detected in the control (TE buffer). The average transformation efficiency of 150 μg/ml plates was about 122.4 transformants per μg plasmid DNA using 4.5×10^6 viable protoplasts. In the regeneration plates containing 400 μg/ml hygromycin, the transformants colonies were sparse and small, suggesting this hygromycin B concentration is too high for *P. nameko*.

Compared with the carboxin resistant colonies, the hygromycin B resistant transformants grow slower in the regeneration plates and MYG plates containing drug reagent, and have little aerial mycelium.

We chose the host strains and seven transformants picked from 150 μg/ml hygromycin B regeneration plate for PCR amplification of part *hph* with primers Hyg-F3 and Hyg-R3 (Fig. 3-3B) and southern hybridization analysis (Fig. 3-3A). In these seven transformants, part sequences of *hph* was all obtained (Fig. 3-3A). About the host strain, there is no band in the PCR amplification and southern hybridization result. The band number of the seven transformants in the results of southern hybridization were following: Hyg1-1, 1 band; Hyg1-2, 2 bands; Hyg1-3, 1 band; Hyg1-4, 1 band; Hyg1-5, 2 bands; Hyg1-6, 2 bands; Hyg1-7, 1 band. These results demonstrated that the

introduced sequence (*hph*) was integrated into the chromosomal DNA with one or more copy numbers in these transformants.

In other mushrooms reported before, such as *P. ostreatus* and *L. edodes*, the transformation efficiency is very low using normal polyethylene glycol-mediated transformation. So researchers are tried to improve the transformation efficiency, like using chemical substances (heparin, ATA, and spermidine) to improve the transformation efficiency (Li et al. 2006) or single-strand carrier DNA (Irie et al. 2001) in *P. ostreatus*. However, in this research, with normal polyethylene glycol-mediated transformation, we constructed two high efficient transformation methods in *P. nameko* which is enough for further molecular analysis of cloned genes. This may be relating to the species itself. Reports have showed that it is very difficult to obtain protoplasts without any wall remnants (Bacon et al. 1969; Darling et al. 1969). The protoplasts of *P. nameko* have more wall remnants than other mushrooms, which can lead to high efficient regeneration. It is pity that the regeneration efficiency of the protoplasts of *P. nameko* obtained in this research was unable to know because of the protoplasts mixing with ungerminated oidia which may germinate later.

Cloning mating type locus in non-model species lacks the advantage of directly testing the functions of cloned genes by transformation of DNA into a mating-compatible host strain. But in this research we successfully constructed

two transformation systems, carboxin resistant and hygromycin B resistant transformation, in non-model fungus, *P. nameko*. So in the future, we will use these two types of transformation system in *P. nameko* to investigate the functions of the *A* mating type locus in this species.

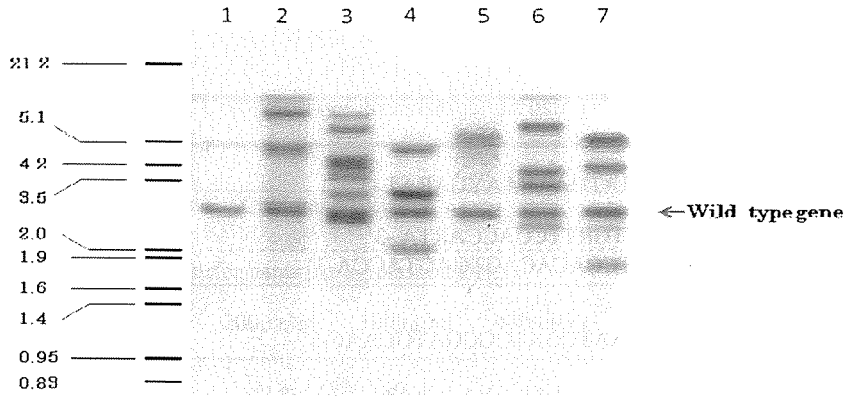


Fig. 3-2 Southern hybridization analysis of carboxin resistant transformants and the host strain. *Bam*HI-digested DNA extracted from wild type (*lanes* 1) and transformants (lanes 2-7) was probed with the part of sip sequence labeled with DIG. The arrow indicates the wild type band which exists in wild type strain NGW19-6. Samples are: lane1, NGW19-6; lane 2, Cp1-1; lane 3, Cp1-2; lane 4, Cp1-3; lane 5, Cp1-4; lane 6, Cp1-5; lane 7, Cp1-6.

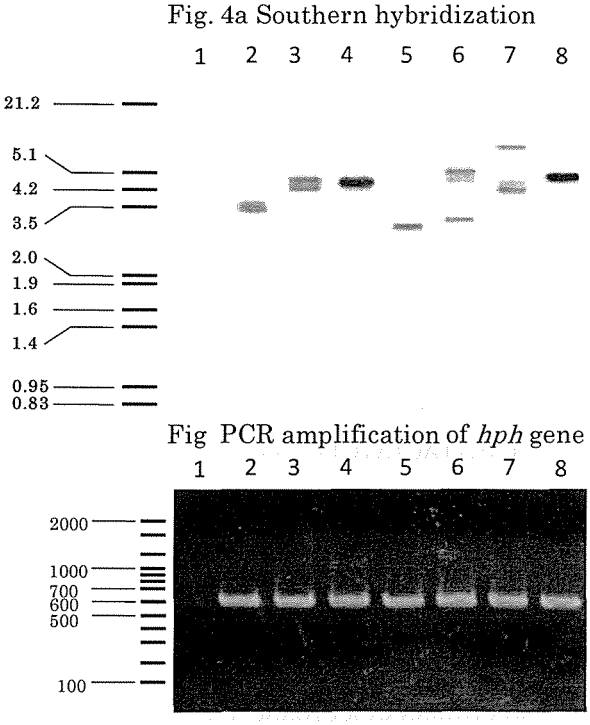


Fig. 3-3 Part A, Southern hybridization analysis of hygromycin B resistant transformants and the host strain.. *Bam*HI-digested DNA extracted from wild type (lane 1) and transformants (lanes 2-8) was probed with the part of pMBhph1 sequence labeled with DIG. Part B, The PCR amplification of part *hph* gene in the host strain and hyg^R transformants. Samples are: lane1, NGW19-6; lane 2, Hy1-1; lane 3, Hy1-2; lane 4, Hy1-3; lane 5, Hy1-4; lane 6, Hy1-5; lane 7, Hy1-6; lane 8, Hy1-7.

Table 3-1 Primers used for identification of *sip* and *hph* sequences.

Primer	Sequence	Remark
SDIpF2	5'- ATHAARATHAARAAYGARAT -3'	Used for initial amplification
SDIpR	5'- TCRTCYTGRTTCCACCARTA -3'	
IpUp2	5'-CCCTCTTCCACACAGTGAGTACCA-3'	Used for cassette PCR
IpUp1	5'-CTGCAGAATCGATCGCGATGCAAC-3'	
IpDn2	5'-GATCGATTCTGCAGAGGCAAGCGA-3'	
IpDn1	5'-GATTTTCGCGTTCTTGGTTGCATC-3'	
CaPrC1	5'-TCGTTAGAACGCGTAATACGACTCA-3'	Used for cassette PCR
CaPrC2	5'-CGTAATACGACTCACTATAGGGAGA-3'	
Ip-d1F	5'-CTTACAAACACTGCCGCCA-3'	Used for amplification of complete *sip* and the flanking region
Ip-d1R	5'-TCGACGCAGATGGCACT-3'	
Ip-d2F	5'-ACTCTGGTCGACGTCA-3'	Used for direct sequencing
Ip-d2R	5'-GTCCCACAACCATTCT-3'	
Ip-d3F	5'-ACTTTCCGCAATCCTC-3'	
Ip-d3R	5'-TTTACGACGGTCTTCTG-3'	
Ip-d4F	5'-CAGAACTCTAGCCCACC-3'	
Ip-d4R	5'-GTCGCGAGAATGGAT-3'	
Ip-d5F	5'-ACGTTGCATGTTGGAC-3'	
Ip-d5R	5'-TTGACAAGGCAGAACC-3'	
Ip-cbxR	5'-ATGTACCGTTGTCTTACTATCTTCA-3'	Used for site direct mutagenesis
Ip-cbxF	5'-TTGAAGATAGTAAGACAACGGTACA-3'	
Ip-pro-XhoI	5'-TGGCGGCCTCGAGTGGCAGTGAGGAGGA-3'	Used for amplification of promoter and terminator reagion of *sip* including pT7 blue T-vector
Ip-ter-BglII	5'-ACCGACTAAGATCTCAGTCTTCTC-3'	
pER8-XhoI	5'-CGGATCCTCGAGAATGAAAAAGCCTGAACTCACCGCGACG-3'	
pER8-BglII	5'-TTGAAAGATCTCTATTCCTTTGCCCTCGGACGAGTGCTGG-3'	
Hyg-F3	5'-GGGAATTCAGCGAGAGCCTG-3'	Used for amplification of *hph* gene
Hyg-R3	5'-CGAAATTGCCGTCAACCAAGC-3'	

Table 3-2 Transformation efficiency of Cbx^r and Hyg^r transformation using *P. nameko* strain NGW19-6

	Protoplasts number	Cbx^r transformation (transformants per μg plasmid DNA)				Hyg^r transformation (transformants per μg plasmid DNA)		
		150μg/ml	200μg/ml	300μg/ml	400μg/ml	0.5μg/ml	1.0μg/ml	2.0μg/m
Experiment 1	4.12×10^6	86.9	73.8	54.4	34.2	44.5	69.5	46.1
Experiment 2	4.45×10^6	153.4	133.6	60.6	41.2	95.8	125.3	79.0
Experiment 3	4.03×10^6	126.9	105.9	69.9	27.0	49.9	71.7	40.6
Average value		122.4	104.4	61.6	34.1	63.4	88.8	55.2

Chapter 4

A-mating type gene expression can drive clamp formation in the bipolar mushroom, *Pholiota nameko*

4-1 Abstract

In the bipolar basidiomycete *P. nameko*, a pair of homeodomain protein genes located at the *A* mating-type locus regulates mating compatibility. In the present study, we used a DNA-mediated transformation system in *P. nameko* to investigate the homeodomain proteins that control the clamp-cell formation. When a single homeodomain protein gene (*A3-hox1* or *A3-hox2*) from the *A3* monokaryon strain was transformed into the *A4* monokaryon strain, the transformants produced many pseudo-clamps but very few clamps. When two homeodomain protein genes (*A3-hox1* and *A3-hox2*) were transformed either separately or together into the *A4* monokaryon, the ratio of clamps to the clamp-like cells in the transformants was significantly increased to approximately 50%. We, therefore, concluded that the gene dosage of homeodomain protein genes is important for clamp-cell formation. When the *sip* promoter was connected to the coding region of *A3-hox1* and *A3-hox2* and the fused fragments were introduced into NGW19-6 (*A4*), the transformants achieved more than 85% clamp formation and exhibited two nuclei per cell, similar to the dikaryon (NGW12-163 × NGW19-6). The results of real-time RT-PCR confirmed that

sip promoter activity is greater than that of the native promoter of homeodomain protein genes in *P. nameko.* So, we concluded that nearly 100% clamp formation requires high expression levels of homeodomain protein genes and that only *A* mating-type genes control the clamp-cell formation. Also, we deduced that another pair of homeodomain protein genes may exist and be required to yield the nearly 100% clamp formation that occurs in wild-type *P. nameko.*

4-2 Introduction

In basidiomycetous mushrooms, mating compatibility is controlled by one or two sets of multiple allelomorphic genes known as bipolar or tetrapolar mating systems, respectively (Wendland et al. 1995). In tetrapolar mushrooms, such as *Coprinopsis cinerea* (Hiscock et al. 1996; Casselton et al. 2006), *Laccaria bicolor* (Fries et al. 1993; Kropp and Fortin 1988; Fowler et al. 2004), and *Schizophyllum commune* (Frankel and Ellingboe 1977), the mating-type loci *A* and *B*, which are located on different chromosomes, regulate mating and clamp formation (Raper 1966, 1983; Iwasa et al. 1998; Fowler et al. 2004). The *A* locus comprises multigenes encoding homeodomain proteins, and the *B* locus comprises multigenes encoding pheromones and pheromone receptor proteins (Kües and Casselton 1992; Stankis et al. 1992; Wendland et al. 1995; Hiscock et al. 1996; Shen et al. 1996; Vaillancourt et al. 1997; O'Shea et al. 1998;

Riquelme et al. 2005; Casselton and Kües 2007; Niculita-Hirzel et al. 2008). On the basis of the homeodomain sequence, the mating-type proteins of the *A* locus are divided into two subgroups, HD1 and HD2 (Kües and Casselton 1992; Kües et al. 1994). When an HD1 protein from one mate heterodimerizes with an HD2 protein from the other mate to form a functional regulatory protein, sexual compatibility is intracellularly recognized, and the *A* developmental pathway is initiated (Banham et al. 1995; Kamper et al. 1995; Magae et al. 1995).

Few studies have examined the composition and function of mating-type loci in bipolar basidiomycetes. In a landmark study, Bakkeren and Kronstad (1994) discovered that in bipolar fungus, *Ustilago hordei*, the *A* and *B* mating-type loci were fused into one nonrecombining mating-type region with two alleles. However, subsequent studies revealed that although both the *A* and *B* mating-type homologs are found in bipolar mushrooms, they are present on different chromosomes, and only the *A* mating-type homologs are related to mating compatibility (Aimi et al. 2005; James et al. 2006).

Although *Pholiota nameko* (Strophariaceae) has a very similar life cycle to other members of the order Agaricales, such as the tetrapolar mushroom *C. cinerea*, it has a bipolar *A* incompatibility factor and at least six different mating types (Ratanatragooldacha et al. 2002). Ratanatragooldacha et al. (2002) concluded that the bipolar *A* locus of *P. nameko* contains two functional

subunits, *Aα* and *Aβ*, which appear to be located 0.3 centi-Morgan (cM) apart from each other on the same chromosome. Aimi et al. (2005) sequenced and characterized the *P. nameko* genes encoding the homeodomain protein, *hox1*, and the pheromone receptor, *rcb1*, which are putative homologues of the HD1 protein and putative pheromone receptor protein genes in the tetrapolar basidiomycete *C. cinerea*, respectively. RFLP and linkage analyses indicated that these two genes are present on different linkage groups and that only *hox1* is involved in regulating mating incompatibility in *P. nameko*. A second homeodomain gene (*A4-hox2*) was discovered upstream of *A4-hox1*, and only two homeodomain protein genes exist in this *Aα* sublocus (Yi et al. 2009a) Similarly, the bipolar mushroom *Coprinellus disseminatus* (James et al. 2006) contains two unlinked mating-type homologs (*A* and *B*), and only the homeodomain protein genes segregate with mating type. And, the *A* factor of *C. disseminatus* encodes two tightly linked pairs of homeodomain transcription factors similar to the *A* mating-type locus of *C. cinerea*. Due to the lack of a DNA-mediated transformation system in *C. disseminatus*, the *C. disseminatus A* and *B* homologues were transformed into *C. cinerea*, and sexual reactions similar to those of the homologous mating-type genes were elicited. Thus, the functions of the *C. disseminatus* mating type were studied in a tetrapolar mushroom, *C. cinerea*, instead of in a homologous bipolar species. In a previous study of *P. nameko*, we successfully constructed a DNA-mediated

transformation system using a homologous selective marker (a carboxin-resistance mutant gene of the succinate dehydrogenase iron-sulfur protein subunit) and a heterologous drug selective marker (hygromycin B phosphotransferase gene) (Yi et al. 2009b). In the present study, we examined the functions of the *P. nameko A* mating-type locus during clamp cell formation *in vivo* using our transformation system.

4-3 Materials and methods

4-3-1 Fungal strains. Monokaryons of *P. nameko* were obtained by monospore isolation from the fruit bodies of various wild strains (Masuda et al. 1995). Auxotrophic mutant monokaryons of *P. nameko* NGW19-6 (*A4*, *pdx1*) and NGW 12-163 (*A3*, *Arg4*) were derived from wild monokaryotic strains NGW19 (*A4*) and NGW12 (*A3*), respectively.

4-3-2 Mycelium preparation, DNA and RNA extraction. To collect mycelium of auxotrophic mutant strain NGW19-6 and NGW 12-163, five mycelial agar blocks ($5 \times 5 \times 5$ mm^3) cut from an MYG plate (glucose 2%, malt extract 0.5%, yeast extract 0.5%, agar 1.5%, pH 5.6) were transferred to 5 ml of liquid MYG medium (glucose 2%, malt extract 0.5%, yeast extract 0.5%, pH 5.6) in a 100-ml Erlenmeyer flask. To collect mycelium of co-transformants, the MYG plates and liquid medium contained 2.0 μg/ml carboxin or 150 μg/ml hygromycin B (in the case of two-step transformations, both drug reagents were

mixed). The mycelium were grown at 25°C without shaking for 2 weeks and then harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Genomic DNA was extracted from the frozen mycelium according to the method described by Dellaporta et al. (Dellaporta et al. 1983).

To prepare total RNA from NGW19-6, NGW12-163 and the transformants, the mycelium was grown on PDA [potato extraction with 2% (w/v) glucose, 1.5% agar] at 25°C for two weeks, after which the mycelium, along with 3 square agar blocks (5 × 5 × 5 mm^3), was transferred to a piece of sterilized cellophane (40 × 40 mm^2) on an MYG plate and grown at 25°C for a week. To isolate total RNA, the mycelium was scraped from the cellophane, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. An RNeasy Mini kit (Qiagen, Tokyo, Japan) was used to extract RNA from the powder, and the integrity of total RNA was examined by separation on a 1.0% agarose gel. A 1:10 dilution of stock total RNA was used for real-time RT-PCR.

4-3-3 Amplification of *A3-hox1* and *A3-hox2* genes. To introduce homeodomain protein genes to the NGW19-6 strain, *A3-hox1* or *A3-hox2* DNA fragments from the NGW12-163 strain were amplified. The *A3-hox1* gene was amplified with MipF and 163mipR6 (see Fig. 4-1), and the *A3-hox2* gene was amplified with Hox2-A3-R1 and 163mipF6. The *A3-hox1* gene amplification

conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 3.5 min, and then a final extension at 72°C for 10 min. The amplification conditions for *A3-hox2* consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2.5 min, and then a final extension at 72°C for 10 min.

To introduce the DNA fragment containing *A3-hox1* and *A3-hox2* into NGW19-6, the genomic DNA fragments of both *A3-hox1* and *A3-hox2* from *P. nameko* NGW12-163 were amplified using primers MipF and Hox2-A3-R1. PCR was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 5 min at 72°C. The PCR product was subcloned into the pT7Blue (R) T-vector (Novagen, Darmstadt, Germany) to create pMBhox12.

4-3-4 Co-transformation method. The DNA-mediated transformation method was performed with pMBsip2 or pMBhph1, as described in our previous study (Yi et al. 2009b). pMBsip2 carries a carboxin-resistance gene, and pMBhph1 carries a hygromycin B-resistance gene. The homeodomain protein gene and the selective plasmid were introduced together into NGW19-6. For each transformation, 5×10^6 protoplasts, 5-10 μg of plasmid DNA, and 10-15 μg of amplified DNA containing the homeodomain protein gene were used.

After the colonies appeared on the regeneration plate, they were individually subcultured onto fresh MYG plates containing 2 μg/ml of carboxin and/or 200 μg/ml of hygromycin B, as appropriate. After a 7- to 10-day incubation at 25°C, the mycelia edges of the colonies were microscopically examined for clamp-cell formations.

To introduce two separate homeodomain protein genes, *A3-hox1* and *A3-hox2*, into NGW19-6, a two-step transformation was performed. In the first step, the *A3-hox2* gene and pMBsip2 were transformed into the NGW19-6 strain. Then, carboxin-resistant transformants expressing *A3-hox2* were identified (Hox2-1, Hox2-2), and one strain (Hox2-1) was used as the host strain for the second co-transformation with *A3-hox1* and pMBhph1.

4-3-5 DAPI and Fluorescent Brightener 28 staining and microscopic observation. Autoclaved slide glass was dipped into 1.0% agar medium and then placed in a sterilized plate. The mycelium was put on the glass-containing agar, incubated for 5 - 7 days, and then stained for 20 min with a solution of 50 μg/ml DAPI (4', 6-diamino-2-phenylindole) (Merck, Darmstadt, Germany), which stains nuclei, and 20 μg/ml Fluorescent Brightener 28 (Sigma-Aldrich, Saint Louis, MO, USA), which stains the cell wall. The stained slides were studied with a Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan).

4-3-6 Construction of two plasmid vectors for overexpression of the *A3-hox1* and *A3-hox2* genes. To determine if the high expression level of homeodomain protein genes may increase the ratio of clamps in *P. nameko*, we connected the *sip* (iron-sulfur protein subunit of succinate dehydrogenase) promoter (Yi et al. 2009b), which is expressed continually in the citrate cycle (TCA), to *A3-hox1* and *A3-hox2*. First, the *A3-hox1* and *A3-hox2* gene fragments were amplified using A3-hox1-Eco52I/A3-hox1-sacII and *A3-hox2*-Eco52I/*A3-hox2*-SacII, respectively, and NGW12-163 mycelium DNA as the template. Thermal cycling parameters were as follows: initial denaturation at 94°C for 4 min; followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 2 min; and a final extension at 72°C for 10 min. The amplified fragments were digested with *Eco*R52I and *Sac*II. Second, the fragment containing pT7Blue (R) T-vector (Novagen), *sip* promoter and terminator, was amplified using Ip-pro-Eco52I and Ip-ter-SacII primers and pMBsip1 (Yi et al. 2009b) as a template. The amplification conditions were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 5 min, and a final 10-min extension at 72°C. The PCR product was also digested with *Eco*R52I and *Sac*II. Then, the two kinds of digested fragments were ligated to form plasmids named pMBsiphox1 and pMBsiphox2 (see Fig. 4-2). The identity of these plasmids was confirmed by sequencing.

4-3-7 Southern hybridization. Southern hybridization analysis of the transformants was performed to analyze the integration of the transforming DNA. Genomic DNA (0.3 - 0.5 μg) from NGW19-6, NGW12-163 and the co-transformants was digested for 5 h at 37°C in a 500-μl reaction mixture containing 20 units of restriction enzymes in the buffer supplied by the manufacturer (Toyobo, Osaka, Japan). The digested fragments were concentrated by ethanol precipitation and then electrophoretically separated in a 1.0% agarose gel and blotted onto a nylon membrane (Hybond-N+; Amersham Biosciences, London, UK). DNA hybridization probes were labeled and detected using Dig-High Prime DNA Labeling and Detection kits (Roche Diagnostics, Tokyo, Japan). We used nested PCR to label the probe. To detect the *A3-hox1* gene in the transformants, we amplified a partial *A3-hox1* sequence using primers Hox1-A3-3RACE1 and 163mipF4. To detect the *A3-hox2* in the transformants, we amplified a partial *A3-hox2* sequence with primers 163mipF-d5 and 163mip-d7 (Table 4-1). Also, these two probes were used for transformants from the pMBhox12 transformation.

4-3-8 Real-time PCR assay. We used the actin gene as the housekeeping gene. A partial actin gene in *P. nameko* was cloned by degenerate PCR primers, ActindpF and ActindpR. Primers for *A3-hox1*, *A3-hox2*, *A4-hox1*, *A4-hox2* and actin were designed according to their cDNA sequences using GENETYX 9.0 (Genetyx, Tokyo, Japan). The primers were designed according to the principles

of primer design, and 3 - 6 bp of the 3' site were designed to cross the intron in the primer spanning the intron. All primers were tested to ensure amplification of single bands with no primer-dimers. Plasmid extraction was performed according to the method modified by Birnboim (1983). Four 10-fold dilutions of plasmid were performed to construct standard curves. Real-time PCR was conducted using RNA-direct™ SYBR Green® Realtime PCR Master Mix (Toyobo, Osaka, Japan) and Linegene (BioFlux, Hangzhou, China). Each reaction was run twice. The cycling parameters were 90°C for 30 s to activate thermostable DNA polymerase, 61°C for 20 min to reverse transcription, 95°C for 30 s pre-denaturation, and then 35 cycles of 95°C for 15 s, 60°C for 15 s, and 74°C for 30 s. Melting curves were determined according to the manufacturer's instructions. After real-time RT-PCR, samples were also run on a 1.5% agarose gel to confirm amplification specificity. Data analysis was performed according to the manipulation's instructions.

4-3-9 Plasmid construction for yeast two hybrids. To synthesize the cDNA of *A3-hox1*, *A3-hox2*, *A4-hox1* and *A4-hox2*, we isolated total RNA from a dikaryon strain (NGW19-6 × NGW12-163) using an RNeasy Mini kit (Qiagen, Tokyo, Japan). We used 100 ng of total RNA as a template for first-strand cDNA synthesis in the presence of Oligo (dT) (First-strand cDNA Synthesis kit, Takara Shigama, Japan). Primers A3-hox1FNcoI and A3-hox1RBamHI, A3-hox2FEcoRI and A3-hox2RBamHI, A4-hox1FNdeI and A4hox1RBamHI, and

A4-hox2FNcoI and A4-hox2RSmaI were used to amplify the near full-length *A3-hox1*, *A3-hox2*, *A4-hox1*, and *A4-hox2* cDNA, respectively. The amplified cDNA fragments of *A3-hox1*, *A3-hox2*, *A4-hox1*, and *A4-hox2* were digested with *Nco*I and *Bam*HI, *Eco*RI and *Bam*HI, *Nde*I and *Bam*HI, *Nde*I and *Bam*HI, and *Nco*I and *Sma*I, respectively. Then, each fragment was cloned into plasmid pGADT7 (Clontech, Madison, WI, USA) to generate an in-frame fusion with the C-terminus of the GAL4 DNA-binding domain and the fragments of *A3-hox2* and *A4-hox2* were cloned into plasmid pGBKT7 (Clontech) to generate an in-frame fusion with the C-terminus of the GAL4 activation domain. Each construction was sequenced across the ligation junction, and no deviations from the expected sequences were found.

4-3-10 Yeast transformation and α-galactosidase assay. The Matchmaker two-hybrid system 3 (Clontech) and the lithium acetate (LiAc)-mediated method was used for the yeast transformation (Ito et al. 1983). The transformation was performed according to the manufacturer's manual (Clontech). We performed a two-step transformation. First, we introduced pGBKT7-A4-hox2 or pGBKT7-A3-hox2 into the Y187 strain (Trp^-, Leu^-) (Harper et al. 1993) and selected the transformants on SD agar plates (0.67% yeast nitrogen base w/o amino acid, 2% agar, appropriate dropout supplement, 2% glucose) lacking L-tryptophan. Then, each plasmid (pGADT7-A4-hox1, pGADT7-A4-hox2, pGADT7-A3-hox1, and pGADT7-A3-hox2) was

introduced into the transformant with pGBKT7-A4-hox2 or pGBKT7-A3-hox2, as appropriate. The transformants were selected on SD agar plates lacking L-tryptophan and L-leucine. pGADT7-T and pGBKT7-Lam were used as negative controls. The α-galactosidase assay was performed according to the manufacturer's instructions (Clontech). One unit of α-galactosidase was defined as the amount of enzyme that hydrolyzes 1 μmol *p*-nitrophenyl-α-D-galactoside to *p*-nitrophenol and D-galactose in 1 min at 30°C in acetate buffer (pH 4.5) (Lazo et al. 1978).

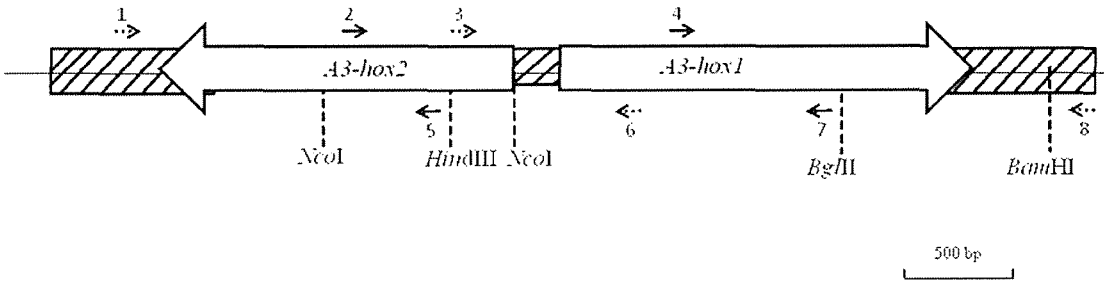


Fig. 4-1. Map of *A3-hox1* and *A3-hox2*. The position of primers used for DNA amplification, the Southern hybridization probe, and the cutting sites of restriction enzymes. The dashed arrows show the primer position used for the amplification of DNA fragments, and the solid arrows indicate the primer position used for making the Southern hybridization probe. The primer names are as follows: 1, Hox2-A3-R1; 2, 163mipF-d5; 3, 163mipR6; 4, Hox1-A3-3RACE1; 5, 163mipR-d7; 6, 163mipF6; 7, 163mipF4; 8, MipF.

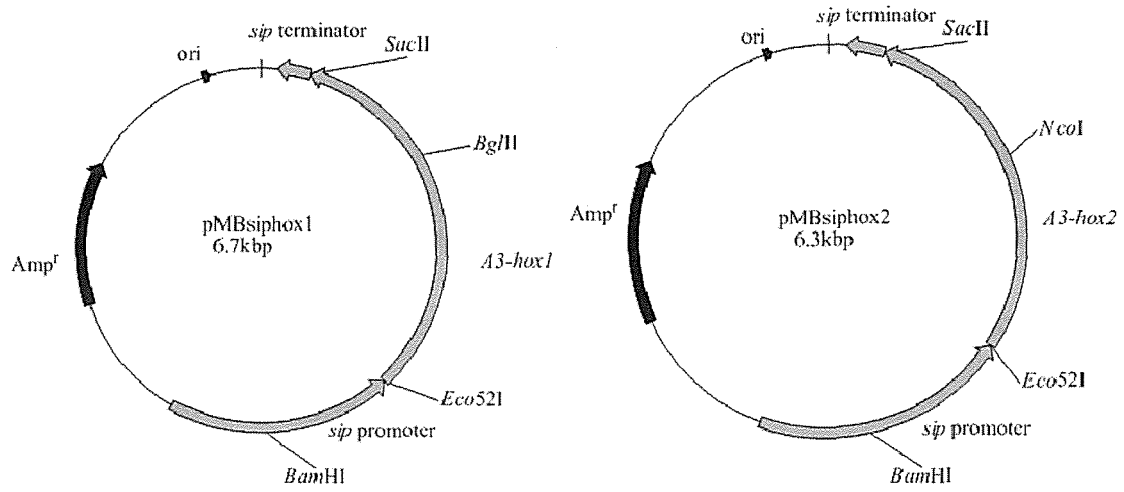


Fig. 4-2 The physical map of plasmid pMBsiphox1 and pMBsiphox2. The *Nco*I, *Bam*HI, *Eco*52I, *Sac*II, *Bgl*II recognition sites are shown. Arrows indicate direction of transcription.

4-3 Results

4-4-1 A single introduced *hox* gene is insufficient to induce true clamps in high frequency. To confirm that the introduction of a single compatible homeodomain protein gene is sufficient for clamp cell formation, *A3-hox1* DNA fragments or *A3-hox2* DNA fragments were co-transformed into the *A4* strain NGW19-6, using pMBsip2 as a carboxin-resistant selective marker. The *A3-hox1* DNA fragments contained an approximately 260-bp partial *A3-hox2* DNA fragment, the 206-bp spacing fragment between *A3-hox1* and *A3-hox2*, and the *A3-hox1* coding and terminator region. The *A3-hox2* DNA fragments contained an approximately 500-bp partial *A3-hox1* DNA fragment, the 206-bp spacing fragment between *A3-hox1* and *A3-hox2*, and the *A3-hox2* coding and terminator region (Fig. 4-1).

We collected carboxin-resistant regenerated colonies and microscopically examined their hook-cell fusion after growth on MYG plates. Clamp-like cells were present in 7 of 146 carboxin-resistant transformants from the transformation with *A3-hox1* and 16 of 111 carboxin-resistant transformants from the transformation with *A3-hox2*. However, all of the transformants with the clamp-like cells contained mostly pseudo-clamps with only rare clamps (see Fig. 4-3A and 3B). Partial pseudo-clamp data are shown in Table 4-2. Hox1-1 and Hox1-2 containing introduced *A3-hox1* had a ratio of clamps to total clamp-

like cells of less than 1%. Hox2-1 and Hox2-1 strain containing introduced *A3-hox2* had a ratio of fused hook-cell to total clamp-like cells of 4%. DAPI and Fluorescent Brightener 28 staining showed that the nuclei were trapped within the hook cell (Fig. 4-3C).

Using PCR amplification, we detected the band of the entire *A3-hox1* DNA fragment in all the *A3-hox1* transformants with mostly pseudo-clamps. The partial data was shown in Fig. 4-4A (Hox1-1 and Hox1-2 strain, lane 4 and 5). In Southern hybridization with a partial *A3-hox1* DNA fragment as the probe, hybridization bands were detected in all the *A3-hox1* transformants with mostly pseudo-clamps (data not shown). No entire, but partial *A3-hox1* DNA fragment was detected in most of the transformants with no clamps (see Fig. 4-4C). All of the *A3-hox2* transformants with mostly pseudo-clamps shared the similar results for PCR (Fig. 4-4A, Hox2-1 and Hox2-2 strain, lane 9 and 10) and Southern hybridization (data not shown). These results confirm that the *A3-hox1* or *A3-hox2* gene was ectopically integrated into the chromosomes of transformants with clamp-like cells.

4-4-2 Two separated, introduced *hox* gene increases the frequency of clamps. Because transformation with a single compatible homeodomain protein gene was not sufficient for clamp cell formation, we examined if a pair of homeodomain protein genes was needed for clamp cell formation. We

selected Hox2-1 as the host strain for the second transformation and introduced *A3-hox1* into it using pMBhph1. The Hox2-1 strain is a single homeodomain protein gene transformant expressing *A3-hox2*. About 200 colonies that were resistant to carboxin and hygromycin B were collected and grown on new MYG plates containing both antibiotics, and the fusion of hook cells were assessed by microscopy. Among these 200 colonies, 21 colonies seem to contain increased clamps, implying that they might receive a copy of *A3-hox1* gene. The ratio of clamps to total clamp-like cells was calculated in these colonies. These colonies contained increased ratios of clamps (around 50%), and partial clamps data is shown in Table 4-2. DAPI and Fluorescent Brightener 28 staining confirmed that some nuclei trapped in the hook cells and that some hooks were fused without nuclei (Fig. 4-3D).

Using PCR amplification, we detected *A4-hox1*, *A4-hox2*, *A3-hox1 and A3-hox2* in the genome of all the co-transformants with increased ratios of clamps, and partial data was shown in Fig. 4-4B (Hox2-hox1-1, Hox2-hox1-2, and Hox2-hox1-3 strain, lane d, e and f). Southern hybridization analysis also confirmed that the *A3-hox2* gene was still present in the chromosomes of these transformants with the same detective band as the host strain Hox2-1 and that *A3-hox1* was ectopically integrated into the chromosomes of the co-transformants (data not shown).

4-4-3 Two introduced combined *hox* gene also increase the frequency of fused hook cell. By successively introducing a pair of homeodomain protein genes to *A4 strain* NGW19-6, a significant increase in true clamps was found in the transformants. So, we wondered if the same phenomenon occurs after transformation with *A3-hox1* and *A3-hox2* gene fragments that are linked together like the native genes. Using pMBsip2, we co-transformed pMBhox12, which was obtained by connecting the fragment of *A3-hox1* and *A3-hox2* gene to pT7Blue (R) T-vector, into the *A4* strain NGW19-6 along with marker plasmid pMBsip2. We collected approximately 120 regeneration colonies and placed them on new MYG plates that contained 2 μg/ml of carboxin. Eight transformants with clamp-like cells were found among these carboxin-resistant colonies, and partial clamp cell formation data is shown in Table 4-2. The ratio of clamps to total clamp-like cells (approximately 50%) and the mycelium configuration in these transformants (Hox1,2-1, Hox1,2-2, Hox1,2-3 strain) are similar to those of transformants that were successively transformed with *A3-hox1* and *A3-hox2* (see Table 4-2 and Fig. 4-3E).

PCR amplification indicated that DNA fragments containing *A3-hox1* and *A3-hox2* exist in almost transformants with clamps, and partial data was shown in Fig. 4-4A (Hox1,2-1, Hox1,2-2, Hox1,2-3 strain, lane 24, 25 and 26). Southern hybridization confirmed that both *A3-hox1* and *A3-hox2* were ectopically integrated into the chromosomal DNA (data not shown).

4-4-4 Greater expression of the *hox* genes drive the real clamp formation.

When *A3-hox1* or *A3-hox2* was introduced into *A4* strain NGW19-6, clamps were only rarely detected in co-the transformants. When *A3-hox1* and *A3-hox2*, either separately or together, were introduced into NGW19-6, approximately 50% clamp cell formation was detected in the transformants expressing two *hox* gene. So the following experiments are to determine the effect of greater expression of the *hox* genes on true clamp cell formation. We connected the code region of *A3-hox1* and *A3-hox2* to *sip* promoter, and constructed pMBsiphox1 and pMBsiphox2, respectively (see Fig. 4-2). Using a carboxin-resistant selective marker, we introduced pMBsiphox1 or pMBsiphox2 into *A4* strain NGW19-6. In each transformation, around 150 regenerated colonies were collected and grown on MYG plates containing 2.0 μg/ml carboxin, and then the clamp-like cell formation was examined microscopically. In the transformation of pMBsiphox1, there were 23 colonies containing clamp-like cells. The ratios of clamps to clamp-like cells in these co-transformants were calculated, and representative data is shown in Table 4-2. The representative colonies Shox1-1 and Shox1-2 with introduced pMBsiphox1 exhibited greater than 85% real clamps among the clamp-like cells (see Table 4-2). Nuclei and cell-wall staining of the mycelium of these two transformants confirmed that the majority of clamp-like cells were not pseudoclamps and that most cells contained two nuclei (see Fig. 4-3F and Table 4-3). The

transformation of pMBsiphox2 yielded 30 carboxin-resistant transformants with clamp-like cells. In these co-transformants, the ratio of real clamps among the clamp-like cells and the nuclei number per cell is similar with the co-transformants with pMBsiphox1 (see Table 4-2).

Amplification with primers Ip-d1R and A3-hox1-sacII, which correspond to the near 5'-end of the *sip* promoter and the 3'-end of the *A3-hox1* gene, respectively, yielded a band of the expected size (around 3.5 kbp) in almost co-transformants, and partial strains Shox1-1 and Shox1-2 were shown in Fig. 4-4A (lane 14 and 15). No bands were amplified from the genomic DNA of host strains NGW19-6 and NGW12-163 (see Fig. 4-4A, lane 11 and 12). In Southern hybridization, the partial *A3-hox1* gene sequence was used as the probe. *Bam*HI and *Bgl*II, located at the *sip* promoter and *A3-hox1* gene, respectively, were used to cut the genomic DNA. A band of the expected sized (around 2.3 kbp) including the partial *sip* promoter and *A3-hox1* gene was detected in Shox1-1 and Shox1-2 strain, and a band with a different size (around 4.7 kbp) was detected in the *A3* strain NGW12-163 (Data not shown). Also, other bands exist in Shox1-2 strain, which may result from a different type of ectopic integration (Data not shown). Similar results were obtained for Shox2-1 and Shox2-2 strain introduced with pMBsiphox2 (see Fig. 4-4A, lane 19 and 20). These results suggest that the fused DNA fragment containing the *sip* promoter and *A3-hox1* or *A3-hox2* was ectopically integrated into the chromosome of NGW19-6.

4-4-5 Different growth condition was observed in different kinds of transformants. In the transformation introduced with single *A3-hox1*, the co-transformants had different mycelium configuration than other carboxin-resistant transformants with no clamp-like cells. The *A3-hox1* co-transformants had procumbent mycelium, uneven colony borders, slower mycelium growth (about 0.13 cm/day in the MYG plate without carboxin) than NGW19-6 (about 0.26 cm/day), and brown deposits around the inoculum. The *A3-hox2* co-transformants had relatively abundant aerial mycelium, uneven colony borders and slower mycelium growth (about 0.14 cm/day). The carboxin-resistant transformants with no clamp-like cells showed abundant mycelium, smooth colony borders and faster growth similar to the host strain NGW19-6.

In the transformants with two combined or separated introduced *hox* gene, when grown on MYG plates without carboxin and hygromycin B, these co-transformants showed abundant aerial mycelium, faster growth (around 0.17 cm/day) than transformants containing a single introduced homeodomain protein gene, and a radiating mycelium configuration similar to the wild-type dikaryon (NGW19-6 × NGW12-163).

In the transformants with greater expression of introduced *hox* gene, most of the colonies also had abundant aerial mycelium, a moderate growth rate (around 0.18 cm/day on MYG plates without carboxin) and a radiating

mycelium configuration similar to that of wild-type dikaryon (NGW19-6 × NGW12-163).

4-4-6 Different expression amount of four *hox* gene in different kinds of transformants. When *A3-hox1* or *A3-hox2* was introduced into *A4* strain NGW19-6, clamps were only rarely detected in the co-transformants (representative strains, Hox1-1, Hox1-2, Hox2-1 and Hox2-2). When *A3-hox1* and *A3-hox2*, either separately or together, were introduced into NGW19-6, approximately 50% clamp cell formation was detected in all of the co-transformants expressing two *hox* gene (representative strains, Hox2-hox1-1, Hox2-hox1-2, Hox1-hox1-3, Hox1,2-2 and Hox1,2-3). When *A3-hox1* or *A3-hox2* connected to the *sip* promoter was used for transformation, there was greater than 85% real clamp among clamp-like cells in the co-transformants (representive strains, Shox1-1, Shox1-2, Shox2-1 and Shox2-2). So, we considered the possibility that hook-cell fusion is affected by the expression level of homeodomain protein genes. Therefore, the rationale for the following experiments of real-time RT-PCR is to measure the expression amount of *hox* gene, which may directly affect the clamp cell formation, in these different kinds of transformants. The level of transcription was determined in triplicate for all transformants.

The quantities of *A3-hox1* and *A3-hox2* transcription in host strains NGW12-163 and NGW19-6 were used as reference values and set at 100%. In Hox1-1 and Hox1-2 strain, which contain only *A3-hox1,* the relative values of *A3-hox1* (0.94 and 1.09) were close to that of NGW12-163 (see Fig. 4-5). In Hox2-1 and Hox2-2 strain, which contain only *A3-hox2*, the relative values of *A3-hox2* were 0.95 and 0.60 (mean). In Hox1-1, Hox1-2, Hox2-1 and Hox2-2 strain, the relative values of *A4-hox1* were around 0.80, and the values of *A4-hox2* ranged from 0.3-0.6. In the transformants with successively introduced *A3-hox2* and *A3-hox1*, Hox2-hox1-1, Hox2-hox1-2, Hox2-hox1-3 strain, the transcription levels of *A3-hox1* were different (range, 0.5 - 1.2). Their relative values of *A3-hox2* were less than 0.4, which is different than the value in Hox2-1 strain. This phenomenon is very interesting. It seems that after introducing *A3-hox1* into Hox2-1 strain, the expression of *A3-hox2* was suppressed from 0.95 to less than 0.4. In Hox1,2-2 and Hox1,2-3 strain, which contain both *A3-hox2* and *A3-hox1*, the mean of the relative value of *A3-hox1* was near that of *A3* strain NGW12-163 and the relative values of *A3-hox2* were 0.68 and 0.33, respectively. Compared to the transformants that contain only a single homeodomain protein gene (Hox1-1, Hox1-2, Hox2-1 and Hox2-2), the quantity of *A4-hox1* transcription in Hox2-hox1-1, Hox2-hox1-2, Hox2-hox1-3, Hox1,2-2 and Hox1,2-3 strain are increased (range, 0.8 - 1.6), while their transcription of *A4-hox2*, with the exception of Hox2-hox1-3, was also

increased (range, 0.7 - 1.0). These results suggest that the gene dosage of homeodomain protein genes also affects the expression amount of four homeodomain protein genes (*A4-hox1*, *A4-hox2*, *A3-hox1* and *A3-hox2*). In the wild-type dikaryon (NGW19-6 × NGW12-163), except for the relative value of *A3-hox1* (>1.2), the transcription level of the other three homeodomain protein genes was around 0.9. Compared to the transformants containing both *A3-hox1* and *A3-hox2*, the wild-type dikaryon (NGW19-6 × NGW12-163) had a greater expression level of *A3-hox2* and a lower expression level of *A4-hox1*.

In the transformants Shox1-1 and Shox1-2 with introduced pMBsiphox1, the relative values of *A3-hox1* (1.5) were greater than those of Hox1-1 and Hox1-2 strain. In the transformants Shox2-1 and Shox2-2 with introduced pMBsiphox2, the transcription quantity of *A3-hox2* (1.4) was greater than that of Hox2-1 and Hox2-2 strain. From these results, we conclude that the promoter activity of *sip* is higher than the activity of the native promoter of homeodomain protein genes in *P. nameko*. Meanwhile, in Shox1-1, Shox1-2, Shox2-1 and Shox2-2 strain, the transcription amount of *A4-hox1* is increased (1.4), and the relative values of *A4-hox2* were near 1.0.

4-4-7 Weak proteins interaction was detected between Hox proteins from different *A* mating type. When *A3-hox1* or *A3-hox2* was introduced into *A4* strain NGW19-6, clamps were only rarely detected in the co-transformants. The

possible reason may be relate to the interaction intensity of Hox proteins from different *A* mating type in *P. nameko*. So we examined the Hox proteins interaction in this species using Yeast Two-hybrid System. We examined the α-galactosidase activity in yeast transformants with different plasmids containing *hox* gene (see Table 4-4). No α-galactosidase activity was detected in negative controls (GADT7-T/GBKT7-Lam). GAD and GBK fusions with the same homeodomain gene (GAD-A4-hox2/ GBK-A4-hox2, GAD-A3-hox2/ GBK-A3-hox2) did not activate transcription. This result indicates that homeodomain protein genes from the same *A* mating type do not undergo a protein-protein interaction. Non-self combinations (GAD-A4-hox2/GBK-A3-hox1, GAD-A3-hox2/ GBK-A4-hox1) had activated *MEL 1*, indicating that A4-Hox2 and A3-Hox1, A3-Hox2 and A4-Hox1 interact; but, the values were very low, about 0.033 and 0.023 mU/ml × cell, respectively, suggesting that their interaction intensity is very weak. The GAD-A4-hox2/GBK-A4-hox1 combination also had no α-galactosidase activity. A very low level of α-galactosidase activity, which may be only background activity, was detected between GAD-A3-hox2 and GAD-A3-hox1.

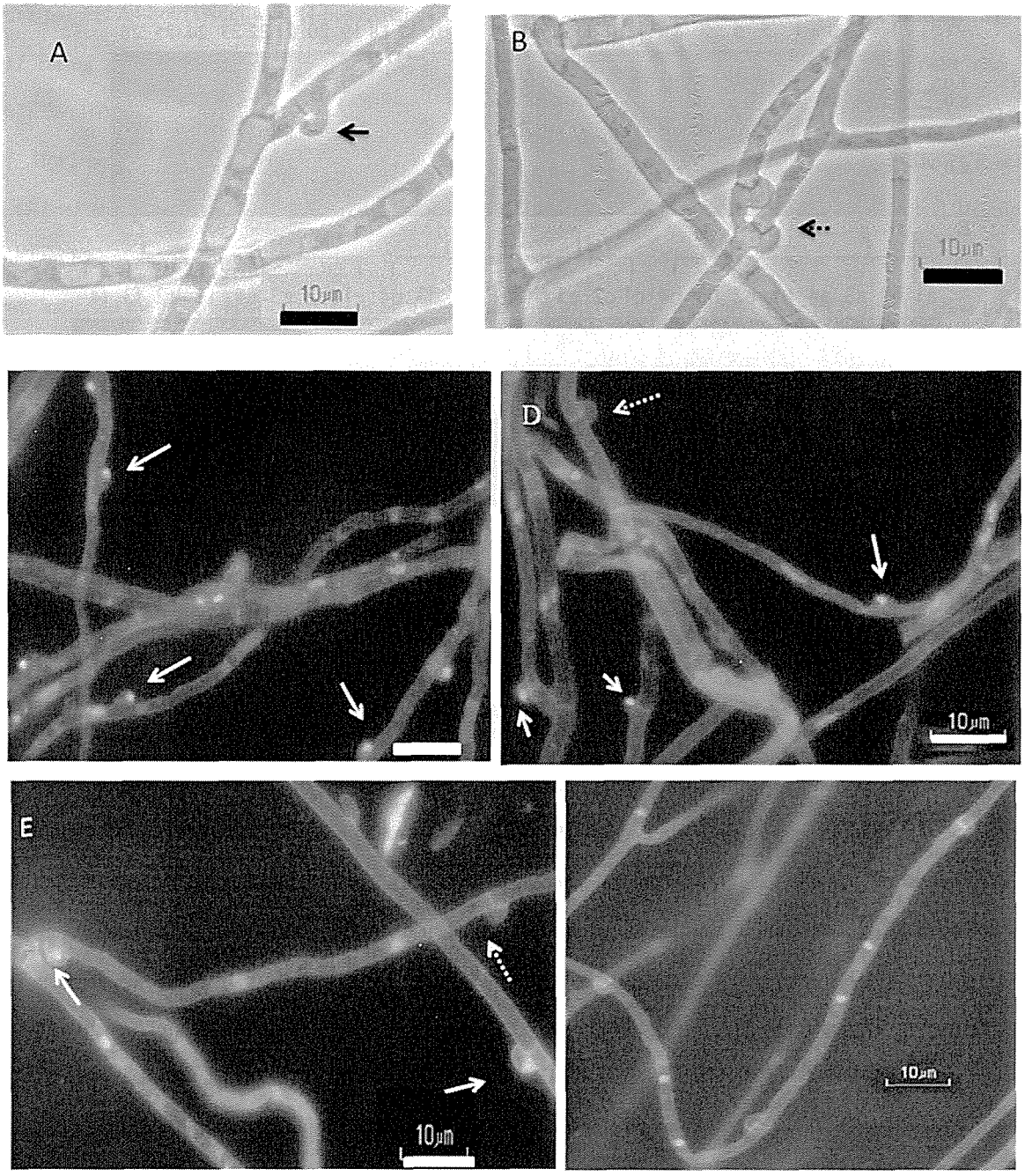


Fig. 4-3 The configuration of clamps and pseudo-clamps, and DAPI and Fluorescent Brightener 28 staining of nuclei and cell walls in the co-transformants. Panel A, Pseudo-clamps in Hox2-1. Panel B, Clamps in Hox2-1. Panel C, Pseudo-clamps with staining of nuclei and cell walls in Hox2-1. Panel B, Pseudo-clamps and Clamps in Hox2-hox1-1. Panel C, Pseudo-clamps and clamps in Hox1,2-1. Panel D. Clamps in Shox1-1. The solid and dashed arrows indicate the pseudo-clamps and the fused hook cell, respectively. *Bars* = 10 μm.

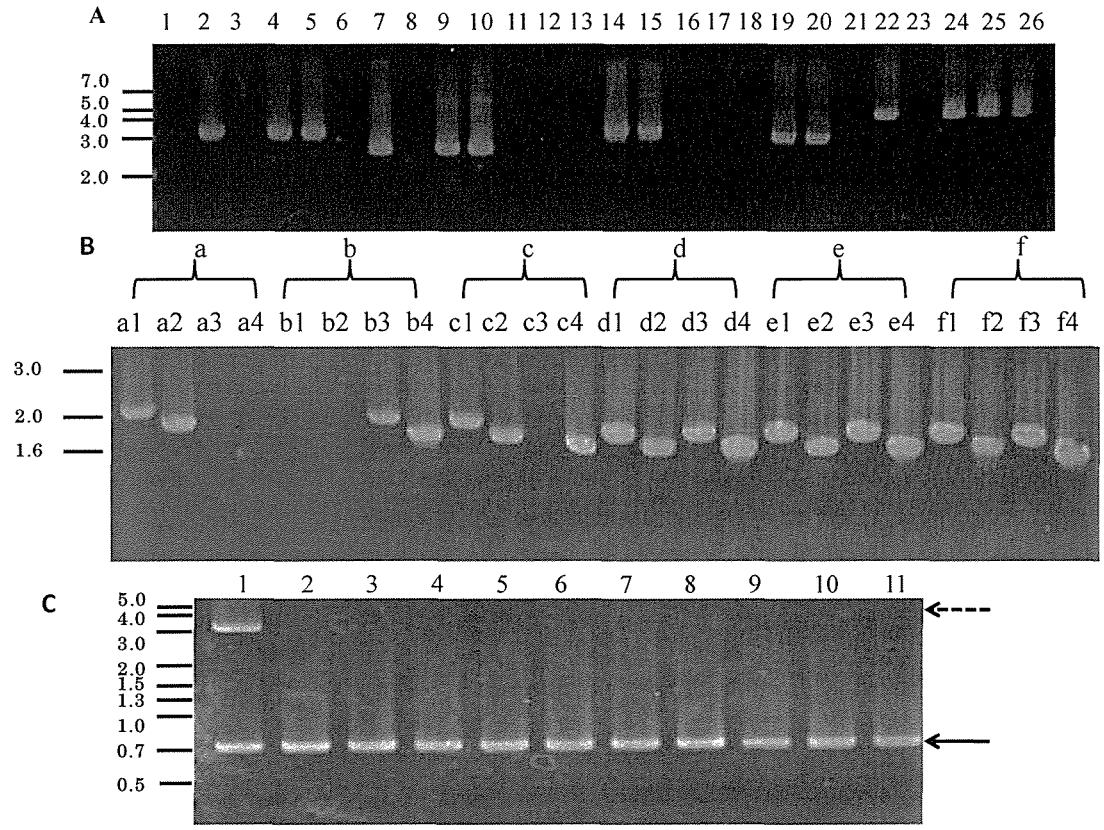
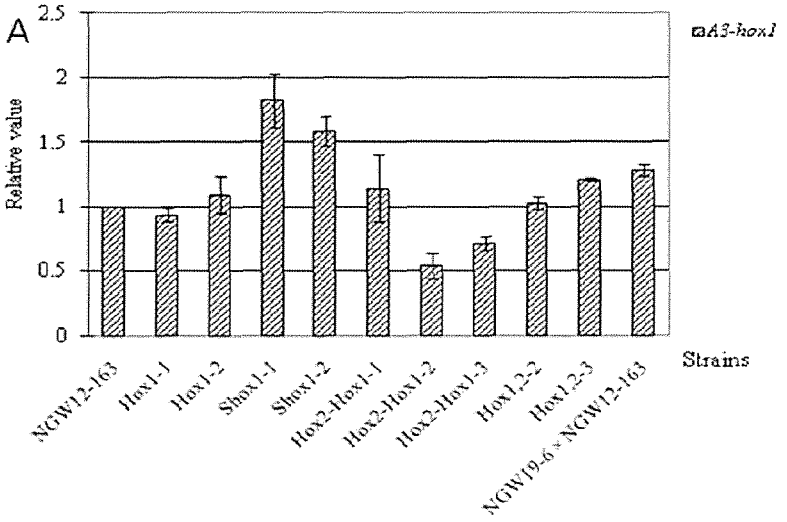
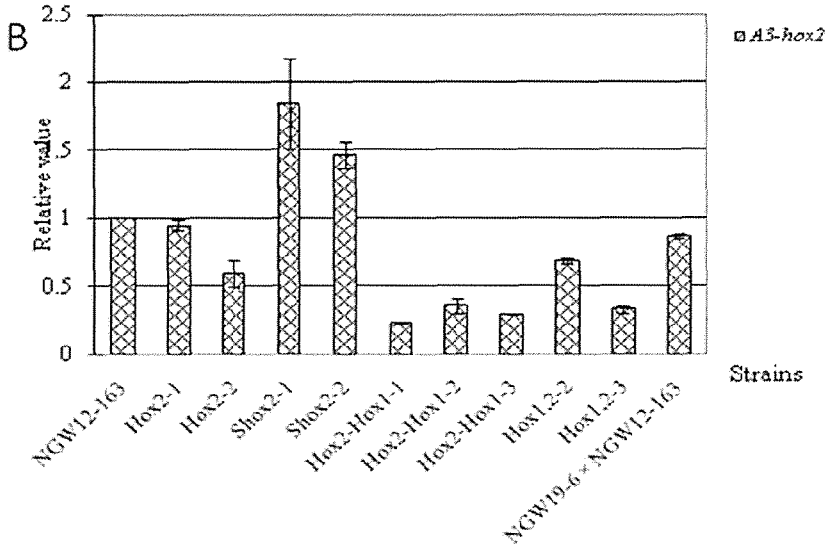


Fig. 4-4 Polymerase chain reaction (PCR) of the host strains, *A3-hox1* and *A3-hox2* transformants. The position and size in kilobase pair (kbp) are indicated on the left. Panel A, PCR amplification of the host strains and transformants. Lane 1 to 5 was PCR results of host strains and *A3-hox1* transformants using primers MipF and 163mipR6. Lane 1 NGW19-6 (*A4*); lane 2 NGW12-163 (*A3*); lane 3 control (transformants with no clamps); lane 4 Hox1-1 (transformants with pseudoclamps); lane 5 Hox1-2 (transformants with pseudoclamps). Lane 6 to 10 was PCR results of host strains and *A3-hox2* transformants using primers Hox2-A3-R1 and 163mipF6. Lane 6, NGW19-6 (*A4*); lane 7, NGW12-163 (*A3*); lane 8, control (transformants with no clamps); lane 9, Hox2-1 (transformants with pseudoclamps); lane 10, Hox2-2 (transformants with pseudoclamps). Lane 11 to 15 was PCR results of wild-type strains and transformants with pMBsiphox1 using primers lp-d1R and A3-hox1-SacII. Lane 11, NGW19-6 (*A4*); lane 12, NGW12-163 (*A3*); lane 13, control (transformants with no clamps); lane 14, Shox1-1 (transformants with clamps); lane 15, Shox1-2 (transformants with clamps). Lane 16 to 20 was PCR results of the host strain and transformants introduced with pMBsiphox2 using primers lp-d1R and A3-hox2-SacII. Lane 16, NGW19-6 (*A4*); lane 17, NGW12-163 (*A3*); lane 18, control (transformants with no clamps); lane 19, Shox2-1 (transformants with clamps); lane 20, Shox2-2 (transformants with clamps). Lane 21 to 26 was amplification of DNA fragments containing *A3-hox1* and *A3-hox2* in the wild-type strains and the transformants using primers Hox1-A3-R1 and Hox2-A3-R1. Lane 21, NGW19-6 (*A4*); lane 22 NGW12-163 (*A3*); lane 23, control (transformants with no clamps); lane 24~26, Hox1,2-1, Hox1,2-2, Hox1,2-3 (transformants with increased fusion hook-cell). Panel B, The PCR results of four homeodomain protein genes (*A4-hox1*, *A4-hox2*, *A3-hox1* and *A3-hox2*) in the host strains and the transformants. The amplification order of each strain is *A4-hox1*

(using primers A4-hox1FNdeI and A4-hox1RBamHI), *A4-hox2* (using primers A4-hox1FNcoI and A4-hox1RBamHI), *A3-hox1* (using primers A3-hox1FNcoI and A3-hox1RBamHI) and *A3-hox2* (using primers A3-hox2FEcoRI and A3-hox2RBamHI). Lane a, NGW19-6 (*A4*); lane b NGW12-163 (*A3*); lane c Hox2-1 (the host of second transformation); lane d~f, Hox2-hox1-1, Hox2-hox1-2, Hox2-hox1-3 (transformants with increased hook-cell fusion). Panel C, The PCR results of *A3-hox1* and partial actin gene in Hox1-1 and *A3-hox1* transformants without clamps. Primers MipF and 163mipR6, actin2 up F2 and actin2 down R2, were used for amplification of *A3-hox1* and partial actin gene, respectively. The dashed arrow indicated the PCR amplification band of *A3-hox1* and the solid line arrow showed the PCR amplification band of partial actin gene. Lane 1, Hox1-1; lane 2~11, the *A3-hox1* transformants without clamps.





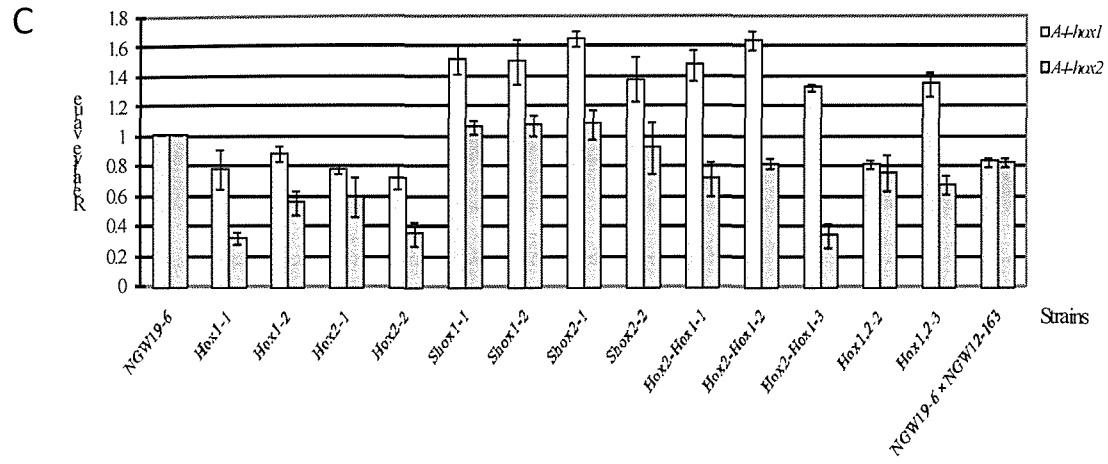


Fig. 4-5 The quantity of transcription of four homeodomain protein genes (*A3-hox1*, *A3-hox2*, *A4-hox1* and *A4-hox2*) in the host strain, dikaryon and transformants. Panel A, The quantity of transcription of *A3-hox1*. The transcription of *A3-hox1* in the *A3* strain NGW12-163 was used as reference values and set at 100%. Panel B, The quantity of transcription of *A3-hox2*. The transcription of *A3-hox2* in the *A3* strain NGW12-163 was used as reference values and set at 100%. Panel C, The quantity of transcription of *A4-hox1* and *A4-hox2*. The transcription of *A4-hox1* and *A4-hox2* in *A4* strain NGW19-6 were used as reference value for *A4-hox1* and *A4-hox2* in other transformants and dikaryon, respectively and set at 100%. The error bars indicate standard deviations (n = 3). The source of strains and co-transformants was as follows: NGW12-163 (*A3* strain); NGW19-6 (*A4* strain); Hox1-1 and Hox1-2 (introduced with *A3-hox1*); Hox2-1 and Hox2-2 (introduced with *A3-hox2*); Shox1-1 and Shox1-2 (introduced with pMBsiphox1); Shox2-1 and Shox2-2 (introduced with pMBsiphox2); Hox2-hox1-1, Hox2-hox1-2 and Hox2-hox1-3 (separately introduced with *A3-hox1* and *A3-hox2*); Hox1,2-2 and Hox1,2-3 (introduced with combined *A3-hox1* and *A3-hox2*); NGW19-6 × NGW12-163 (wild dikaryon).

4-5 Discussion

In bipolar mushroom *C. disseminatus*, the functions of mating type were studied in a tetrapolar mushroom, *C. cinerea*, instead of in a homologous bipolar species (James et al. 2006). In this research, we used a homologous transformation system to determine the functions of the *A* mating type in bipolar mushroom *P. nameko*. It provides an identity to individual and the functions of HD proteins we verified are truly the mating-type determinants.

In previous study, pheromone receptor protein genes in the *P. nameko* are not part of *MAT* locus and only homeodomain protein genes are involved in the mating incompatibility (Aimi et al. 2005). But how do the homeodomain proteins in these species determine the mating identity? Can it be confirmed that homeodomain proteins control dikaryosis and clamp cell formation through transformation studies in this bipolar species? With these questions, firstly, a single homeodomain protein gene (*A3-hox1* or *A3-hox2*) from *A3* strain was introduced into *A4* strain. Unfortunately, few fusion hooks were detected in the co-transformants expressing the introduced homeodomain protein gene. So we considered both homeodomain protein gene (*A3-hox1* and *A3-hox2*) are needed for hook-cell fusion and separately introduced both *hox* genes into *A4* strain. The co-transformants expressing both introduced *hox* genes were with significantly increased ratio of clamps among total clamps-like cells,

approximately 50%. The similar results were also detected in co-transformants introduced with *A3-hox1* and *A3-hox2* gene fragments that are linked together like the native genes. This also excluded the possibility that the promoter region of the homeodomain protein gene not only exists in the homologous spacer region between *A3-hox1* and *A3-hox2*, but also in the opposite homeodomain protein gene region, because *A3-hox1* or *A3-hox2* containing part of the promoter region can be expressed at a low level. When two combined *hox* genes were used for transformation and similar results were obtained, it excluded the possible problem caused by the promoter.

When we connected the *sip* promoter to the coding region of the *A3-hox1* and *A3-hox2* genes and introduced the fused fragment into the *A4* strain NGW19-6, more than 85% of the clamp-like cells in transformants were true clamps, and each cell contained two nuclei. The real-time RT-PCR results indicated that the promoter activity of *sip* is higher than the homeodomain protein gene in *P. nameko*. Based on these results, we concluded that complete clamp cell formation is controlled by the expression level of homeodomain protein genes and that altered expression of *A* mating-type genes is sufficient to drive true clamp cell formation.

In Shox1-1, Shox1-2, Shox2-1 and Shox2-2 strain, only the *A3-hox1* or *A3-hox2* gene was under control of the *sip* promoter. However, the amount of

A4-hox1 and *A4-hox2* gene expression was increased, exceeding the corresponding levels in the host strain NGW19-6 (*A4*) and the wild-type strain (NGW19-6 × NGW12-163). There are two possible reasons why *A4-hox1* and *A4-hox2* gene expression were increased; it could be caused by two nuclei in the same cell, or it could be caused by the self-regulation of homeodomain protein genes. These reasons may also explain the increased gene expression of *A4-hox1* gene in Hox2-hox1-1, Hox2-hox1-2, Hox1-hox1-3, and Hox1,2-3 strain.

Although a pair of homeodomain protein genes is needed for clamp-cell formation in *P. nameko*, only approximately 50% clamps were detected in the co-transformants. In the wild-type dikaryon, most clamp-like cells were clamps (Table 4-3). These findings raise the question of how clamp cell formation is completed in the wild-type dikaryon. Perhaps in wild-type dikaryon, it can also be done by changing expression levels, but the wild-type situation is still not completely determined and needed to further research.

When protein-protein interactions of homeodomain proteins in *Schizophyllum commune* were tested using filter and liquid assays for β-galactosidase, moderate β-galactosidase activity (around 0.44 U/hour × cell in yeast strain HF7c and more than 1.3 U/hour × cell) between two homeodomain proteins from different *Aα* were detected, confirming the ability of two different mating proteins to form a heterodimer (Magae et al. 1995). In our present

research with yeast two-hybrid systems, we used α-galactosidase assay, which is more sensitive than β-galactosidase assay, to detect the Hox proteins interaction. But a low-intensity interaction between A4-Hox2 and A3-Hox1 (about 0.033 mU/ml × cell), A3-Hox2 and A4-Hox1 (about 0.023 mU/ml × cell) was detected using α-galactosidase assay. So, we hypothesized that it is possible that the low interaction intensity between homeodomain proteins from two compatible monokaryon strains requires more heterodimer production. To achieve this requirement, gene dosage and the overexpression of homeodomain protein genes may induce the nearly 100% clamp cell formation in *P. nameko*. Also heterodimers between homeodomain proteins may regulate the expression of homeodomain protein genes and promote clamp cell formation.

In tetrapolar mushrooms, fusion is clearly a function of the pheromone receptor signaling pathway. But using the transformation studies, we confirmed that bipolar mushroom *P. nameko* do not use pheromone receptors to specify the mating type and fusion of hook cell is somehow accomplished via HD protein expression changes. The mating system of *P. nameko* is similar to semicompatible crosses with different *A* loci and common *B* loci (A≠ B=) in tetrapolar mushroom (Kothe 1999), because during mating crosses the nuclei migration which is controlled by *B* loci in tetrapolar mushroom is very slow in this species (data not shown) and monokaryotized mycelia can easily be isolated from the peripheral growing zone in a dikaryotic colony (Masuda et al. 1995).

If this species evolves from tetrapolar mushroom with semicompatible crosses, it is possible that in the tetrapolar mushroom *B* loci control the expression of *A* loci which affect the fusion of hook cell, while this species have common *B* loci and has to increase the expression amount of *A* loci by other ways.

In tetrapolar mushrooms, a heterodimer of compatible HD1 and HD2 proteins is assumed to be a transcription factor that binds unique target sites within the promoters of genes that commit cells to a new developmental pathway. Although we know that the overexpression of homeodomain protein genes may induce the nearly 100% clamp cell formation in *P. nameko*, we do not know if the genes regulated by the heterodimer of homeodomain proteins have corresponding changes in expression. Our future research will address this question so that we may understand the gene regulation of clamp-cell formation with homeodomain protein genes.

Table 4-1 Primers used in the present study.

Primer	Sequence (5'→3')	remark
A3-hox1FNcoI	CCATGGACGCACGAGTAACAGAAA	*A3-hox1* from NGW12-163 strain
A3-hox1RBamHI	GGATCCAAAATTTTCAATCAAGGTC	
A3hox2FEcoRI	GAATTCGCCATGGTATCCGATCTG	*A3-hox2* from NGW12-163 strain
A3hox2RBamHI	GGATCCAGCGACGAAAAGCATTAT	
A4hox1FNdeI	CATATGGCCTCCGCCGTGGACCTCAGA	*A4-hox1* from NGW19-6 strain
A4hox1RBamHI	GGATCCAGAAGATGGCAGATCAAT	
A4hox2FNcoI	ATTACAACCATGGTGTCGACCGCA	*A4-hox2* from NGW19-6 strain
A4hox2RSmaI	CCCGGGAATAGCAACAGAAAAGCAT	
MipF	GCAGAGCTAGCCAAATTACACGAA	Used for amplification of the fragment containing *A3-hox1*
163mipR6	TTGCTGGGACTGAACG	
Hox2-A3-R1	CGCAGGGGTAGGATGTTATGGATT	Used for amplification of the fragment containing *A3-hox2*
163mipF6	CATATGCTATTCCGGACA	
163mipF-d5	AAGGCTCAGGAAGAAGGGGAG	Used for amplification of the partial *A3-hox2*
163mipR-d7	TACCTCTGCACATCTTACCAATC	
Hox1-A3-3RACE1	CCGGGCTAACTGATTACTCCATG	Used for amplification of the partial *A3-hox1*
163mipF4	ATTTGATATGGGTAGCGG	
A3-hox1 forward	CGGAATGCTTGAACTTGAAGTAGAG	Used for real-time RT-PCR of *A3-hox1*
A3-hox1 reverse	ACTGGGATGGAATCTAGAACTTTGC	
A3-hox2 forward	GCTCAGGAAGAAGGGGAGAAATAG	Used for real-time RT-PCR of *A3-hox2*
A3-hox2 reverse	CAATCGGTCTAAGAAAGAGGGAATAC	
A4-hox1 forward	ATTCCAGAAGCCACCTCTAACG	Used for real-time RT-PCR of *A4-hox1*
A4-hox1 reverse	GCGGGTTGATGAATGTATGATTG	
A4-hox2 forward	CGCAAAAGCGTATCAGGCAG	Used for real-time RT-PCR of *A4-hox2*
A4-hox2 reverse	GCTGAAGGAGTGACTTTACCCAAT	
Actin forward	TCGGTCTTGAGGCTGCTGGT	Used for real-time RT-PCR of actin
Actin reverse	AGTCAACTCCTTCTGCATACGGTC	
ActindpF	CRGGTGTCMTGGTYGGWATGG	Used for partial actin gene amplification
ActindpR	CRRGVGGVGCRACGATCTTGAC	
Ip-d1R	TCGACGCAGATGGCACT	
Actin up F2	CTTCAATGTCAGGATACCACGCTTC	Used for partial actin gene amplification
Actin down R2	CACACCTTCCACAAAAAAAAACC	
Hox1-A3-R1	GGAACAGAGAGGCATAGTGATAGA	Used for amplification of the DNA fragment containing *A3-hox1* and *A3-hox2*.

Table 4-2 The ratio of clamps among total clamp-like cells.

Strains	Number of clamps	Total number of clamp-like cells	Ratio of clamps	Remark
NGW19-6 ×NGW12-163	141	165	85.4%	Wild-type dikaryon
Hox1-1	0	146	0	*A3-hox1* transformants
Hox1-2	1	127	0.8%	
Hox2-1	8	221	3.6%	*A3-hox2* transformants
Hox2-2	3	153	2.0%	
Hox2-hox1-1	59	112	52.7%	Transformants introduced with *A3-hox1* to Hox2-1
Hox2-hox1-2	64	150	42.7%	
Hox2-hox1-3	43	108	39.8%	
Hox12-1	69	133	51.9%	Transformants introduced wit pMBhox12
Hox12-2	52	99	52.5%	
Hox12-3	69	205	33.6%	
Shox1-1	120	138	89.1%	Transformants introduced wit pMBsiphox1
Shox1-2	107	123	89.1%	
Shox2-1	120	140	85.7%	Transformants introduced wit pMBsiphox2
Shox2-2	73	84	86.9%	

Table 4-3. Nuclei number per cell in the transformants with pMBsiphox1 and pMBsiphox2.

	No nucleus	One nucleus	Two nuclei	Three nuclei	Total cells counted	Percentage of two nuclei in total counted cells
Shox1-1	13	9	99	7	128	77.3%
Shox1-2	6	3	87	1	97	89.7%
Shox2-1	1	8	94	1	104	90.3%
Shox2-2	5	7	88	3	103	85.4%

Table 4-4 Two-hybrid analysis of A4-Hox1, A4-Hox2, A3-Hox1 and A3-Hox2 interaction.

GAL4 domain		α-Galactosidase activity
DNA-binding	Activation	(mU/ml cell)
GAD-T	GBK-lam	-
GAD-A4-hox2	GBK-A3-hox1	+
GAD-A4-hox2	GBK-A3-hox2	-
GAD-A4-hox2	GBK-A4-hox1	-
GAD-A4-hox2	GBK-A4-hox2	-
GAD-A3-hox2	GBK-A3-hox1	-
GAD-A3-hox2	GBK-A3-hox2	-
GAD-A3-hox2	GBK-A4-hox1	+
GAD-A3-hox2	GBK-A4-hox2	-

GAD and GBK denote the clontech cloning vector with the GAL4 DNA binding (GADT7) and activation domains (GBKT7), respectively. GAD-A4-hox2 and GAD-A3-hox2 denote vectors containing *A4-hox2* and *A3-hox2* cDNAs described in Materials and Methods.

Chapter 5

Conclusions and discussions

From an evolutionary perspective, mating-system switches from tetrapolar to bipolar in fungi are fascinating (Raper 1966). After switch, how the single mating type locus to act the functions before by two unlinked mating type locus (*A* and *B*), especially clamp cell formation, becomes interesting for research. James et al. (2005) failed to reveal the original functions of mating type genes in bipolar mushroom *Coprinellus disseminatus* due to test them in heterologous tetrapolar mushroom *Coprinopsis cinerea*. Based on the previous study by Aimi et al. (2005), in this study we focus on the composition of *A* mating type locus and their roles of clamp cell formation.

In the Chapter 2, using genomic walking, we got the flanking DNA sequence of *hox1* including upstream sequences and downstream sequences in *P. nameko*. A second homeodomain protein gene *hox2* was discovered upstream of *hox1*. Although a 39,882 bp nucleotide region containing *hox1* was amplified and sequenced, only a pair of homeodomain protein genes exist in the *A* mating type locus. These results negated the previous study about *P. nameko A* locus consisting of two functional subunits, *Aα* and *Aβ*, which appear to be located on the same chromosome within 0.3 cM (Masuda et al. 1995). Also, thirteen genes flanking around the *A* locus of *P. nameko* were discovered. Using GenBank

similarity searches to find homologues genes in other mushrooms, eight genes around *A* locus of *P. nameko* (including *β-fg*, *mip*, *up11*, *up10*, *up2*, *up8*, *sec61*, *glydh*) have homologues genes around *A* locus in the bipolar mushroom *C. disseminatus* and the tetrapolar mushrooms *C. cinerea* and *Laccaria bicolor* (Fig. 2-1) (Jame et al 2006; Niculita-Hirzel et al 2008; GenBank accession of *C. cinerea* is no. NW_001884709). Moreover, the order and transcription direction of these genes were very similar in these four species. Although four other genes (including *mmsd*, *lmwppp*, *hp1*, *amtp*) in *P. nameko* were not discovered around *A* locus in other three species, they were found upstream of *A* locus both in *C. cinerea* and *L. bicolor*. Conserved gene order around *A* locus discovered between the bipolar mushrooms and tetrapolar mushrooms agree with the idea that the ancestor of the homobasidiomycetes is accepted as having a tetrapolar mating system (Raper 1996; Raper and Flexer, 1971).

To clarify only homeodomain protein genes control clamp cell formation in *P. nameko*, we constructed the DNA-mediated transformation system in *P. nameko* described in Chapter 3. To further introduction of two homedomain protein genes, two selective markers, the homologous selective marker gene (carboxin resistant gene) and a heterologous drug selective marker gene [hygromycin B phosphotransferase gene (*hph*)], were used to construct transformation system, respectively. Both of these two constructed transformation systems have high transformation efficiency: efficiency of

carboxin resistant transformation was about 88.8 transformants per μg pMBsip2 DNA using 5×10^6 protoplasts in regeneration plates containing 1.0 μg/ ml carboxin; efficiency of hygromycin B resistant transformation was about 122.4 transformants per μg pMBhph1 DNA using 5×10^6 protoplasts in regeneration plates containing 150 μg/ ml hygromycin B.

In the chapter 4, using the constructed DNA-mediated transformation system in *P. nameko*, the roles of *P. nameko* homeodomain proteins genes during clamp cell formation were investigated *in vivo*. When a single homeodomain protein gene (*A3-hox1* or *A3-hox2*) from the *A3* monokaryon strain was introduced into the *A4* monokaryon strain, the co-transformants were observed many pseudo-clamps but very few clamps. When two homeodomain protein genes (*A3-hox1* and *A3-hox2*) were introduced either separately or together into the *A4* monokaryon, the ratio of clamps to the clamp-like cells in the co-transformants significantly increased to approximately 50%. We, therefore, concluded that the gene dosage of homeodomain protein genes is important for clamp cell formation in *P. nameko*. When the *sip* promoter was connected to the coding region of *A3-hox1* and *A3-hox2* and the fused fragments were introduced into NGW19-6 (*A4*), the transformants achieved more than 85% clamp cell formation and exhibited two nuclei per cell, similar to the dikaryon (NGW12-163 × NGW19-6). The results of real-time RT-PCR confirmed that *sip* promoter activity is greater than that of the native promoter

of homeodomain protein genes in *P. nameko.* So, we concluded that nearly 100% clamp cell formation requires high expression levels of homeodomain protein genes and that altered expression of the *A* mating-type genes alone is sufficient to drive true clamp cell formation.

In discussion of chapter 4, two hypotheses were proposed. One hypothesis is based on the weak interaction between Hox proteins in *P. nameko*, which perhaps cause requirement of more heterodimer production. This hypothesis is to explain why genes dosage and the over expression of homoedomain protein genes are needed for inducing the nearly 100% clamp cell formation in *P. nameko*. Another hypothesis is about one possible evolutionary of bipolar mushroom from tetrapolar mushroom. The mating system of *P. nameko* is similar to semicompatible crosses with different *A* loci and common *B* loci (A≠ B=) in tetrapolar mushroom (Kothe 1999), because during mating crosses the nuclei migration which is controlled by *B* loci in tetrapolar mushroom is very slow in this species (data not shown) and monokaryotized mycelia can easily be isolated from the peripheral growing zone in a dikaryotic colony (Masuda et al. 1995). If this species evolves from tetrapolar mushroom with semicompatible crosses, it is possible that in the tetrapolar mushroom *B* loci control the expression of *A* loci which affect the fusion of hook cell, while this species have common *B* loci and has to increase the expression amount of *A* loci by other ways.

In tetrapolar mushrooms, a heterodimer of compatible HD1 and HD2 proteins is assumed to be a transcription factor that binds unique target sites within the promoters of genes that commit cells to a new developmental pathway (Banham et al. 1995; Kamper et al. 1995; Magae et al. 1995). Although we know that the overexpression of homeodomain protein genes may induce the nearly 100% clamp cell formation in *P. nameko*, we do not know if the genes (including genes about hook cell formation, nucleus moving to hook cell, cell wall dissolving and so on) regulated by the heterodimer of homeodomain proteins have corresponding changes in expression. Our future research will address this question so that we may understand the gene regulation of clamp cell formation with homeodomain protein genes (see Fig. 5-1).

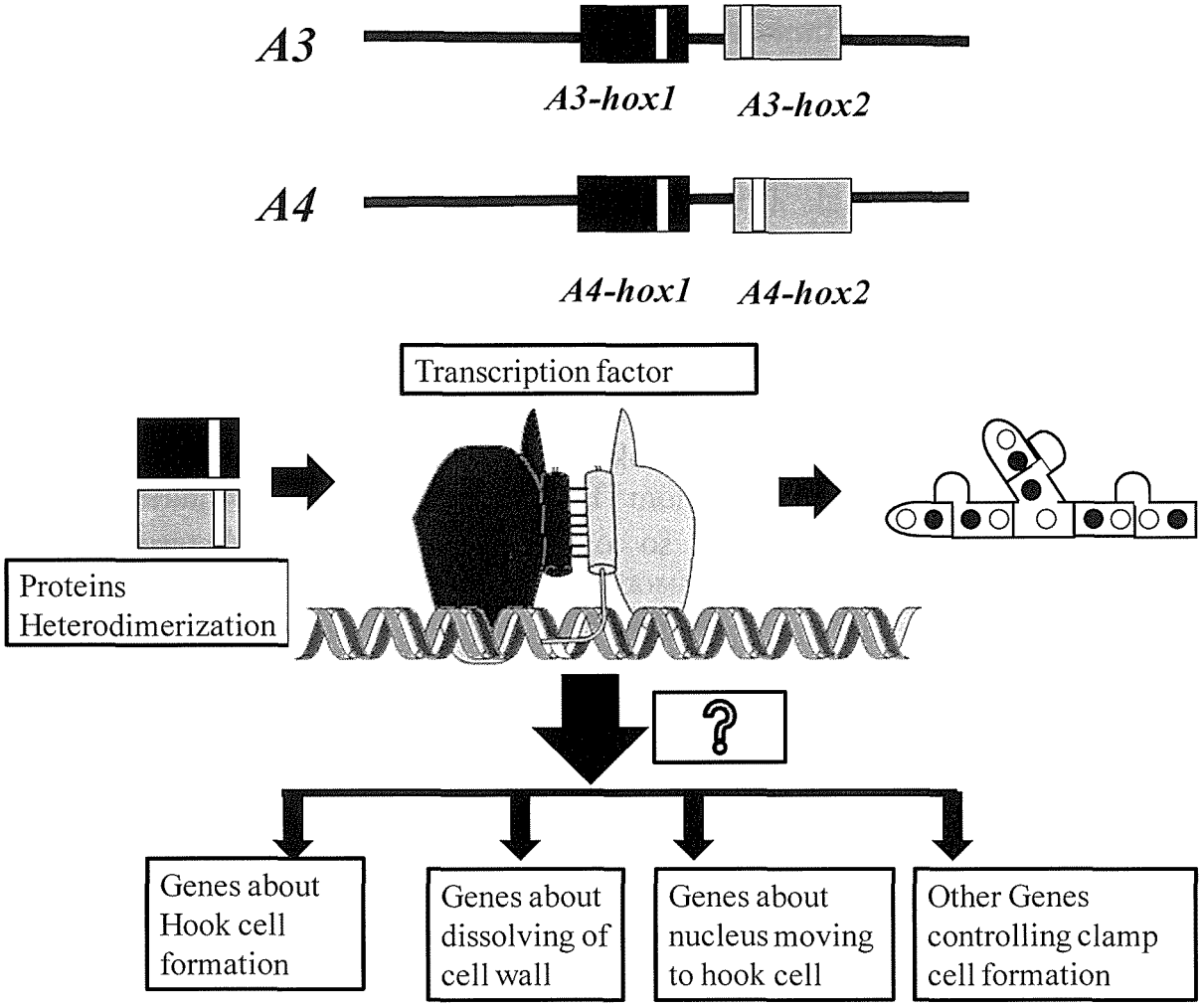


Fig. 5-1 The future study direction about specific genes regulated by the heterodimer of homeodomain proteins.

Abstract

The roles of homeodomain proteins during the clamp cell formation in a bipolar mushroom, *Pholiota nameko*

Mating is an essential step in the life cycle of sexually reproducing organisms. In the basidiomycete mushrooms, mating compatibility is controlled by one or two sets of multiple allelomorphic genes known as bipolar or tetrapolar mating systems, respectively. The mushroom *Pholiota nameko* (Strophariaceae) is known to carry a bipolar *A* incompatibility locus. In the previous study, the *P. nameko* genes encoding the homeodomain protein *hox1* were sequenced and characterized. Restriction fragment length polymorphism (RFLP) and linkage analyses indicated that only *hox1* is involved in regulating incompatibility in *P. nameko*. However, the composition of *A* mating type locus and how the homeodomain protein genes control clamp cell formation were still unknown before this study. In this study, chapter 2 described the genomic structure of *A* mating type in *P. nameko*; chapter 3 showed the exploit of the transformation system in *P. nameko*; in chapter 4 the roles of homeodomain proteins during the clamp cell formation *in vivo* were investigated; chapter 5 showed the conclusions and discussions.

In chapter 2, using genomic walking, the flanking DNA sequence of *hox1* including upstream sequence and downstream sequence was investigated in *P.*

nameko. A second homeodomain protein *hox2* was discovered upstream of *hox1*. Although a 39,882 bp nucleotide region containing *hox1* was amplified and sequenced, only a pair of homeodomain protein genes exist in the *A* mating type locus. Thirteen genes flanking around the *A* locus of *P. nameko* were discovered. Using GenBank similarity searches to find homologues gene in other mushrooms, eight genes around *A* locus of *P. nameko* (including *β-fg*, *mip, up11, up10, up2, up8, sec61, glydh*) have homologues genes around *A* locus in the bipolar mushroom *Coprinellus disseminatus* and the tetrapolar mushrooms *Coprinopsis cinerea* and *Laccaria bicolor*. Moreover, the order and transcription of these genes were very similar in these four species. Although four other genes (including *mmsd*, *lmwppp*, *hp1*, *amtp)* in *P. nameko* were not discovered around *A* locus in other three species, they were found upstream of *A* locus both in *C. cinerea* and *L. bicolor*. Analysis of the deduced protein sequences of the homeodomain protein genes from two strains of *P. nameko* show that the putative functional domains differ from those of the homeodomain proteins of the tetrapolar mushrooms, *C. cinerea* and *L. bicolor*.

In chapter 3, to investigate whether only homeodomain proteins control mating and clamp cell formation in *P. nameko*, a high efficient DNA-mediated transformation system was needed to constructed in this species. To further transformation of two mating types genes, two selective markers, the homologous selective marker gene (carboxin resistant gene) and a heterologous

drug selective marker gene [hygromycin B phosphotransferase gene (*hph*)], were used to construct transformation system, respectively. Both of these two transformation systems we constructed have high transformation efficiency; efficiency of carboxin resistant transformation was about 88.8 transformants per μg pMBsip2 DNA using 5×10^6 protoplasts in regeneration plates containing 1.0 μg/ ml carboxin; efficiency of hygromycin B resistant transformation was about 122.4 transformants per μg pMBhph1 DNA using 5×10^6 protoplasts in regeneration plates containing 150 μg/ ml hygromycin B. The transformation efficiency is believed to do the further transformation of mating type genes.

In chapter 4, using the DNA-mediated transformation system, the functions of homeodomain proteins genes were investigated. When a single homeodomain protein gene (*A3-hox1* or *A3-hox2*) from the *A3* monokaryon strain was transformed into the *A4* monokaryon strain, the transformants produced many pseudo-clamps but very few clamps. When two homeodomain protein genes (*A3-hox1* and *A3-hox2*) were transformed either separately or together into the *A4* monokaryon, the ratio of clamps to the clamp-like cells in the transformants was significantly increased to approximately 50%. We, therefore, concluded that the gene dosage of homeodomain protein genes is important for clamp cell formation. When the *sip* promoter was connected to the coding region of *A3-hox1* and *A3-hox2* and the fused fragments were introduced into NGW19-6 (*A4*), the transformants achieved more than 85% clamp cell

formation and exhibited two nuclei per cell, similar to the dikaryon (NGW12-163 × NGW19-6). The results of real-time RT-PCR confirmed that *sip* promoter activity is greater than that of the native promoter of homeodomain protein genes in *P. nameko.* So, we concluded that nearly 100% clamp cell formation requires high expression levels of homeodomain protein genes and that altered expression of the *A* mating-type genes alone is sufficient to drive true clamp cell formation.

In chapter 5, we concluded that in *P. nameko* the *A* mating-type locus comprises only a pair of homeodomain protein genes, and to drive nearly 100% true clamp cell formation in this species, both homeodomain proteins participated in the clamp cell formation and high expression levels of homeodomain protein genes is required. In a word, in bipolar mushroom, the regulation mechanism of the homeodomain proteins existing in *A* mating-type locus became clear on genetics and molecular biology.

和文摘要

二極性担子菌 *Pholiota nameko* のクランプ形成におけるホメオドメイン蛋白質の役割に関する研究

生物における交配は，有性生活環の基本的なステップであり，担子菌における交配型は，1組の複対立遺伝子により制御される 2 極性と 2 組の複対立遺伝子により制御されている四極性が知られる．これまでの RFLP（Restriction fragment length polymorphism）解析を用いた遺伝学的な研究で，ニ極性担子菌ナメコ（*Pholiota nameko*）の交配型（*A* 因子）は，ホメオドメイン蛋白質のみで制御されていることが示唆された．しかし，*P. nameko* の（*A* 交配型因子）のゲノム構造およびホメオドメイン蛋白質の *in vivo* での機能については，まだ明らかになっていない．そこで，本研究では，第 2 章で *P. nameko* の *A* 交配型因子のゲノム構造，第 3 章で *in vivo* で解析するための形質転換の開発，第 4 章では交配型をつかさどると思われるホメオドメイン蛋白質の役割の *in vivo* での解析を行い，第 5 章でそれを総括した．

第 2 章では，染色体歩行により，*A* 交配型因子の周辺領域 39,882 bp の塩基配列を決定した．これまでの研究で発見されていた *hox1* 遺伝子の上流域に 2 つ目のホメオドメインタンパク質遺伝子 *hox2* を見出した．その他に，13 個の遺伝子が発見され，その内，8 個の遺伝子（*β-fg*, *mip*, *up11*, *up10*, *up2*, *up8*, *sec61*, *glydh*）は，ニ極性担子菌 *Coprinopsis disseminatus* や四極性担子菌 *C. cinerea* and *Laccaria bicolor A* 交配型因子の周辺に見出された遺伝子のホモログ遺伝子であった.他の種の *A* 交配型因子の周辺領域に見いだされなかった 4 個の遺伝子（*mmsd*, *lmwppp*, *hp1*, *amtp*）は，ナメコ以外の他の種では *A* 交配型因子の 200 kb 以上上流に存在した．*A* 交配型因子の周辺領域の遺伝子構造は，進化の過程で組換えや転座等により入れ替わったものは存在するが，二極性，四極性，腐生菌，菌根菌を問わずかなり保存されていることが解った．

第 3 章ではホメオドメインタンパク質の役割を解明するために，ナメコにおける形質転換系を構築した．ホモロガスなマーカーとしてカルボキシン耐性，ヘテロロガスなマーカーとしてハイグロマイシン B 耐性を用い，プロトプラスト—ポリエチレングリコール(PEG)法で形質転換を行った．その結果，5×10^6 個のプロトプラストを使って，1.0 μg/ ml のカルボキシン濃度で 88.8 個／ μg pMBsip2 DNA の形質転換効率を得た．また，150 μg/ ml のハイグロマイシン B 濃度で，5×10^6 個のプロトプラストを用いて 122.4 個／ μg pMBhph1 DNA の形質転換効率を得た．これにより，ホメオドメイン蛋白質の役割が *in vivo* で解析できるようになった．

第 4 章では，構築した形質転換系を用いてホメオドメイン蛋白質の役割を調べた．*A3-hox1* および *A3-hox2* 遺伝子を，*A4* の交配型にをもつモノカリオン株に導入した．その結果，1 つのホメオドメイン蛋白質を導入した場合は，形成されたクランプのほとんどが偽クランプであった．*A3-hox1* および *A3-hox2* の 2 つのホメオドメイン蛋白質を導入した場合，約 50%の形質転換体が真正クランプを形成した．*A3-hox1* および *A3-hox2* 遺伝子の構造遺伝子をコハク酸デヒドロゲナーゼ IP サブユニットプロモーターの制御化で高度に発現させた場合，85%以上のクランプが，真正クランプとなった．以上のことから，クランプの形成には，ホメオドメイン蛋白質の発現量が，非常に重要であることが解った．

第 5 章で以上を総括した．ナメコにおける *A* 交配型因子は，1 対のホメオドメイン蛋白質からなっており，これらを和合性のモノカリオンに導入すると真正クランプの形成が確認できた．また，ホメオドメインタンパク質遺伝子の発現量は，真正クランプ形成の頻度に影響した．以上の結果から，ニ極性担子菌の交配型は，*A* 交配型因子内にコードされるホメオドメインタンパク質のみにより制御されていることが遺伝学的にも，分生生物学的にも明らかにすることができた．

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