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

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ABSTRACT: In the bipolar basidiomycete *Pholiota 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 formation. When a single homeodomain protein gene (*A3-hox1* or *A3-hox2*) from the *A3* monokaryon strain was introduced 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 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 altered expression of the A mating-type genes alone is sufficient to drive true clamp formation.

KEYWORDS : Bipolar mushroom, Homeodomain protein gene, *Pholiota nameko*, transformant

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).

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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.

Materials and methods

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.

Mycelium preparation, DNA and RNA extraction

To collect mycelium of auxotrophic mutant strain NGW19-6 and NGW 12-163, five mycelial agar blocks($5\times5\times5mm^3$) 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%, pH5.6) in a 100-ml Erlenmeyer flask. To collect mycelium of co-transformants, the MYG plates and liquid medium contained 2.0μg/ml carboxin or 150μg/ml hygromycin B(in the case of two-step transformations, both drug reagents were mixed). The mycelium were grown at 25℃ 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.*(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℃ for two weeks, after which the mycelium, along with 3 square agar blocks($5\times5\times5mm^3$), was transferred to a piece of sterilized cellophane(40×40 mm^2) on an MYG plate and grown at 25℃ 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.

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

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℃ for 5min, followed by 30cycles of 30s at 94℃, 30s at 58℃, and 5min at 72℃. The PCR product was subcloned into the pT7Blue(R) T-vector(Novagen, Darmstadt, Germany) to create pMBhox12.

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, 5×106 protoplasts, 5-10㎍ of plasmid DNA, and 10-15㎍ 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㎍/ml of carboxin and/or 200㎍/ml of hygromycin B, as appropriate. After a 7- to 10-day incubation at 25℃, 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.

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 20min with a solution of 50㎍/ml DAPI(4', 6-diamino-2-phenylindole)(Merck, Darmstadt, Germany), which stains nuclei, and 20㎍/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).

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℃ for 4min; followed by 30cycles of 94℃ for 30s, 57℃ for 30s, 72℃ for 2min; and a final extension at 72℃ for 10min. The amplified fragments were digested with EcoR52I and SacII. 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℃ for 4min, followed by 30cycles of 94℃ for 30s, 58℃ for 30s, 72℃ for 5min, and a final 10-min extension at 72℃. The PCR product was also digested with EcoR52I and SacII. 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.

Southern hybridization

Southern hybridization analysis of the transformants was performed to analyze the integration of the trans-

forming DNA. Genomic DNA(0.3-0.5μg) from NGW19-6, NGW12-163 and the co-transformants was digested for 5h at 37℃ in a 500-μ l 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.

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℃ for 30s to activate thermostable DNA polymerase, 61℃ for 20min to reverse transcrip-

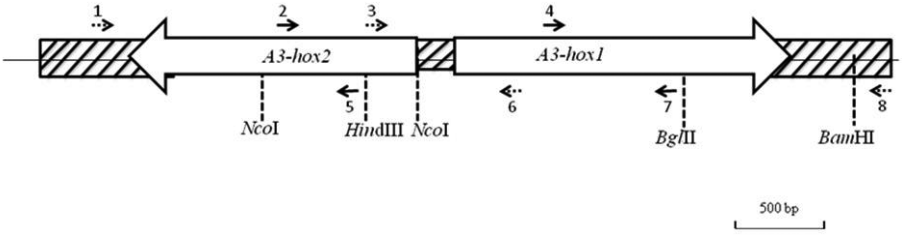

Fig. 4-1. Map of *A3*-hox1 and *A3-hox2*. The position of primers used for DNA amplification, the Southern hybridization probe, and the cutting sites of restriction enzymes. The dashed arrows show the primer position used for the amplification of DNA fragments, and the solid arrows indicate the primer 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.

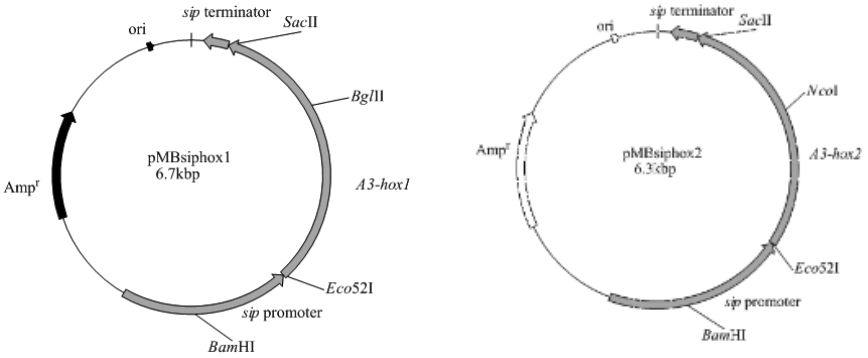

Fig. 4-2. The physical map of plasmid pMBsiphox1 and pMBsiphox2. The *Nco*I, *Bam*HI, *Eco*52I, *Sac*II, *Bgl*II recognition sites are shown. Arrows indicate direction of transcription.

Table 4-1. Primers used in the present study

Primer	Sequence (5'→3')	remark
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
MipF 163mipR6	GCAGAGCTAGCCAAATTACACGAA TTGCTGGGACTGAACG	Used for amplification of the fragment containing *A3*-hox1
Hox2-A3-R1 163mipF6	CGCAGGGGTAGGATGTTATGGATT CATATGCTATTCCGGACA	Used for amplification of the fragment containing *A3-hox2*
163mipF-d5 163mipR-d7	AAGGCTCAGGAAGAAGGGGAG TACCTCTGCACATCTTACCAATC	Used for amplification of the partial *A3-hox2*
Hox1-A3-3RACE1 163mipF4	CCGGGCTAACTGATTACTCCATG ATTTGATATGGGTAGCGG	Used for amplification of the partial *A3*-hox1
A3-hox1 forward A3-hox1 reverse	CGGAATGCTTGAACTTGAAGTAGAG ACTGGGATGGAATCTAGAACTTTGC	Used for real-time RT-PCR of *A3*-hox1
A3-hox2 forward A3-hox2 reverse	GCTCAGGAAGAAGGGGAGAAATAG CAATCGGTCTAAGAAAGAGGGAATAC	Used for real-time RT-PCR of *A3-hox2*
A4-hox1 forward A4-hox1 reverse	ATTCCAGAAGCCACCTCTAACG GCGGGTTGATGAATGTATGATTG	Used for real-time RT-PCR of *A4*-hox1
A4-hox2 forward A4-hox2 reverse	CGCAAAAGCGTATCAGGCAG GCTGAAGGAGTGACTTTACCCAAT	Used for real-time RT-PCR of *A4-hox2*
Actin forward Actin reverse	TCGGTCTTGAGGCTGCTGGT AGTCAACTCCTTCTGCATACGGTC	Used for real-time RT-PCR of actin
ActindpF ActindpR	CRGGTGTCMTGGTYGGWATGG CRRGVGGVGCRACGATCTTGAC	Used for partial actin gene amplification
Ip-d1R	TCGACGCAGATGGCACT	
Actin up F2 Actin down R2	CTTCAATGTCAGGATACCACGCTTC CACACCTTCCACAAAAAAAAACC	Used for partial actin gene amplification
Hox1-A3-R1	GGAACAGAGAGGCATAGTGATAGA	Used for amplification of the DNA fragment containing *A3*-hox1 and *A3-hox2*.

tion, 95℃ for 30s pre-denaturation, and then 35cycles of 95℃ for 15s, 60℃ for 15s, and 74℃ for 30s. 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. specificity. Data analysis was performed according to the manipulation's instructions.

Results and discussions

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 af-

ter 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 (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(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.

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).

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).

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 Hox1-2	0 1	146 127	0 0.8%	*A3*-hox1 transformants
Hox2-1 Hox2-2	8 3	221 153	3.6% 2.0%	*A3-hox2* transformants
Hox2-hox1-1 Hox2-hox1-2 Hox2-hox1-3	59 64 43	112 150 108	52.7% 42.7% 39.8%	Transformants introduced with *A3*-hox1 to Hox2-1
Hox12-1 Hox12-2 Hox12-3	69 52 69	133 99 205	51.9% 52.5% 33.6%	Transformants introduced with pMBhox12
Shox1-1 Shox1-2	120 107	138 123	89.1% 89.1%	Transformants introduced with pMBsiphox1
Shox2-1 Shox2-2	120 73	140 84	85.7% 86.9%	Transformants introduced with pMBsiphox2

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%

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).

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(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 ㎍/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%

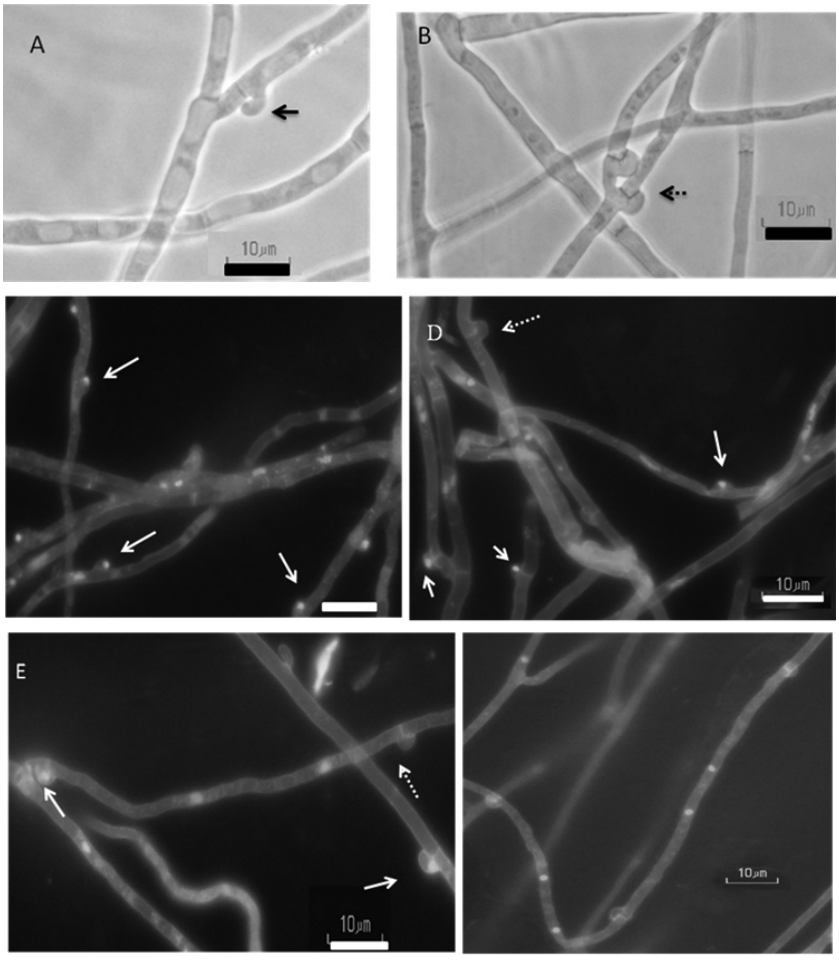

Fig. 4-3. The configuration of clamps and pseudo-clamps, and DAPI and Fluorescent Brightener 28 staining of nuclei and cell walls in the co-transformants. Panel A, Pseudo-clamps in Hox2-1. Panel B, Clamps in Hox2-1. Panel C, Pseudo-clamps with staining of nuclei and cell walls in Hox2-1. Panel B, Pseudo-clamps 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 μ m.

real clamps among the clamp-like cells(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 (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(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 (Fig. 4-4A, lane 11 and 12). In Southern hybridization, the partial *A3-hox1* gene sequence was used as the probe. BamHI and BglII, 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

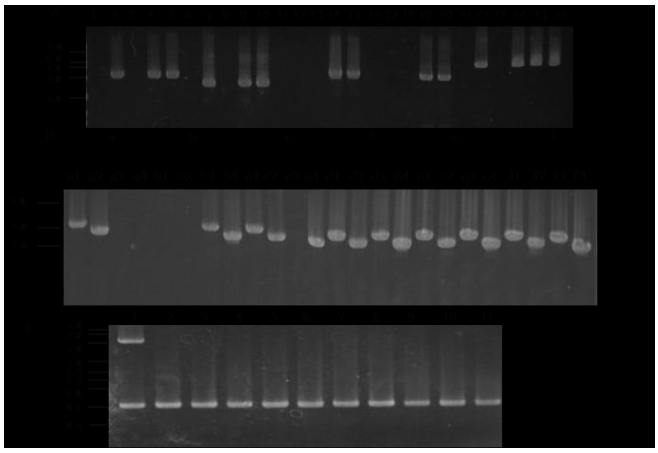

Fig. 4-4. Polymerase chain reaction(PCR) of the host strains, *A3*-hox1 and *A3-hox2* transformants. The position and size in kilobase pair(kbp) are indicated on the left. Panel A, PCR amplification of the host strains and transformants. Lane 1 to 5 was PCR results of host strains and *A3*-hox1 transformants 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 Ip-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 Ip-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*-hox1FNdel and *A4*-hox1RBamHI), *A4-hox2*(using primers *A4*-hox 1FNcoI and *A4*-hox1RBamHI), *A3*-hox1 (using primers *A3*-hox1FNcoI and *A3*-hox 1RBamHI) and *A3-hox2*(using primers *A3-hox2*FEcoRI and *A3-hox2*RBamHI). 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.

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(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.

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 0.13cm/day in the MYG plate without carboxin) than NGW19-6 (about 0.26cm/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 0.17cm/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).

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(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.

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

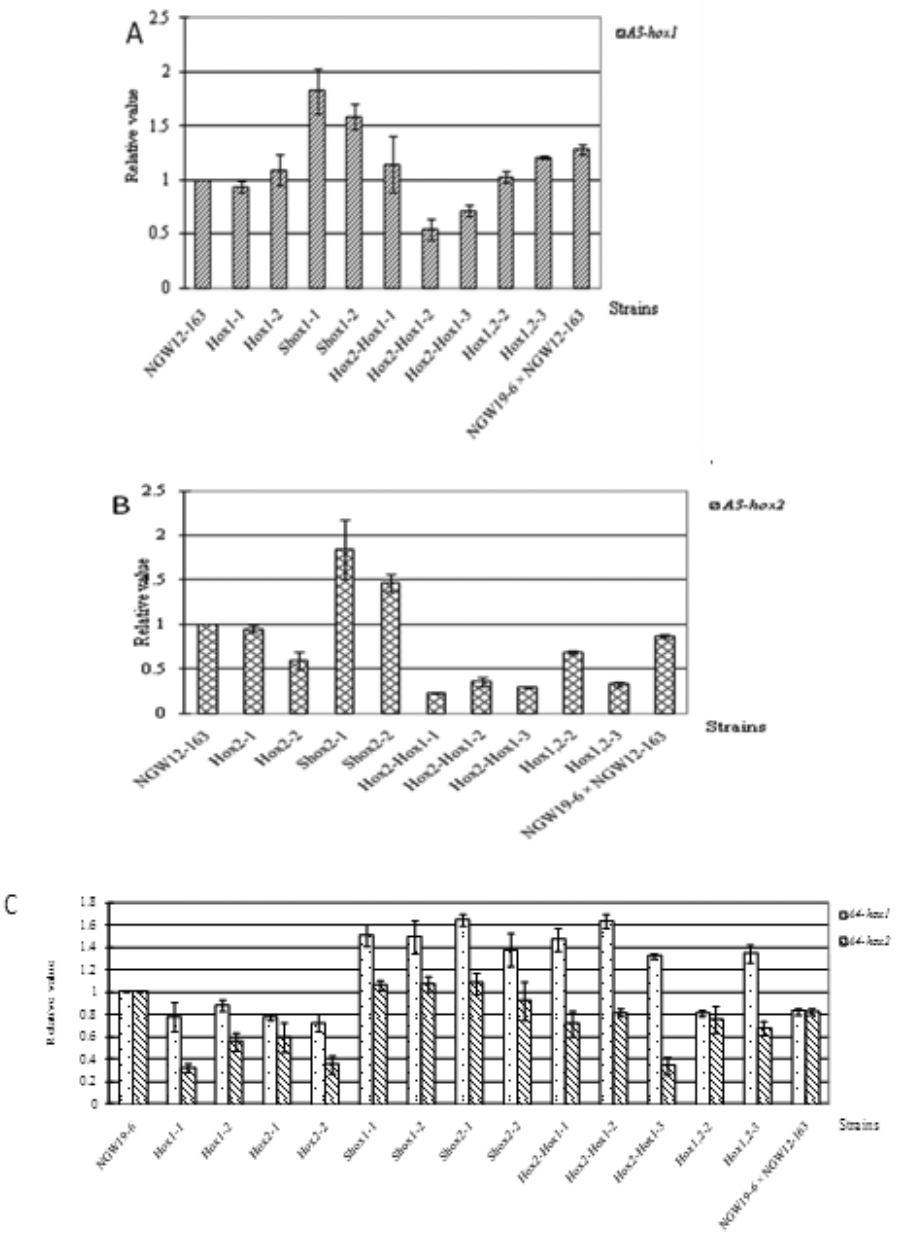

Fig. 4-5. The quantity of transcription of four homeodomain protein genes(*A3*-hox1, *A3-hox2*, *A4*-hox1 and *A4-hox2*) in the host strain, dikaryon and transformants. Panel A, The quantity of transcription of *A3*-hox1. The transcription of *A3*-hox1 in the *A3* strain NGW12-163 was used as reference 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).

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 de-

tected 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.

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.

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