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REVIEW PAPER

Hormonal regulation of sexual reproduction in *Phytophthora*

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CONTENTS

INTRODUCTION

The genus of *Phytophthora* contains several devastating plant pathogens of worldwide importance. Among them are late blight of potato caused by *P. infestans*, black pod of cacao caused by *P. plamivora*, and blight and root rot of peppers caused by *P. capsici* (Erwin and Ribeiro, 1996). Some species of *Phytophthora*, such as *P. sojae*, *P. heveae*, and *P. katsurae*, are homothallic and capable of producing sexual propagules of oospores in single culture. Other species such as *P. infestans*, *P. parasitica* (= *P. nicotianae*) and *P. capsici* are heterothallic and require the presence of opposite mating types known as A1 and A2 for the formation of oospores (Gallegly and Galindo, 1958; Savage et al., 1968). Some species such as *P. megasperma* (Ho et al., 1986a) and *P. cinnamomi* (Ho et al., 1983) even contain both homothallic and heterothallic isolates. Among 59 species of *Phytophthora* listed by Erwin and Ribeiro (1996), 41 were homothallic and 16 were heterothallic. The other two species did not form sex organs.

Normal oospores are produced readily by heterothallic *Phytophthora* even when opposite mating types of morphologically and physiologically distinct species are paired, for example, between the primitive soil-borne *P. cinnamom*i and the advanced air-borne *P. infestans* (Ko, 1980b; Savage et al., 1968). Such an unusual phenomenon shows the possibility that members of *Phytophthora* are unique in the biological world in having no genetic barrier in crosses involving different species. Another possible explanation is that chemical stimulation may be involved in oospore formation during mating, a hypothesis originally proposed by Ashby (1929).

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DISCOVERY OF SEXUAL HORMONES IN *PHYTOPHTHORA*

Although several lines of indirect evidence suggest the involvement of substances stimulatory to sexual reproduction in heterothallic *Phytophthora* (Ko, 1980b), early efforts, including my own, to detect the stimulatory substances were of no avail (Ko, 1988). Eventually, unequivocal proof of the production of sexual hormones was achieved by using a polycarbonate membrane that was impervious to mycelial growth but allowed free passage of water-soluble substances, to separate A1 and A2 cultures and to induce formation of oospores by both cultures (Ko, 1978).

Both A1 and A2 isolates of *P. parasitica*, *P. palmivora*, and *P. cinnamomi* formed selfed oospores when they were

paired with the other mating type of the same or different species on the opposite sides of polycarbonate membranes (Ko, 1978). The successful formation of oospores with the membrane method demonstrates the stimulation of sexual reproduction by substances produced by the other mating type and diffused through the membrane. The sex hormone produced by A1 isolates of *Phytophthora*, designatd hormone α1, can induce sexual reproduction of A2 but not A1 isolates. Similarly, sexual reproduction of A1 but not of A2 isolates can also be induced by hormone α2 produce by A2 isolates. Hormonal regulation of sexual reproduction subsequently has been confirmed with the polycarbonate membrane method in a number of *Phytophthora* species (Table 1), including *P. infestans* (Shaw et al., 1985; Shattock et al., 1986; Chang and Ko, 1990a, b; Shaw et al., 1985), *P. capsici* (Uchida and Aragaki, 1980), *P. cryptogea* (Ho and Jong, 1986), *P. drechsleri* (Ho, 1986a, b; Shattock et al., 1986), *P. megasperma* (Ho, 1986a), *P. citrophthora* (Ann, 1984), and *P. melonis* (Chang et al., 1984).

Homothallic species of *Phytophthora* also were able to induce oospore formation of A1 and /or A2 isolates, when pairings were carried out on the opposite sides of polycarbonate membranes, indicating their ability to produce α hormones (Table 2). This suggests that, like heterothallic *Phytophthora*, sexual reproduction in homothallic *Phytophthora* is also controlled by α hormones.

Attempts to detect α hormones in liquid or solid cultures of A1 and A2 isolates of *P. parasitic*a were not successful during the early phase of our study on this subject. However, when a culture block of *P. parasitica* was incubated with a piece of Millipore filter (cellulose nitrate and cellulose acetate) separated by a polycarbonate membrane, the filter was able to absorb hormone produced by the culture and stimulate oospore formation of the opposite type (Ko, 1983). It was also found that α hormones could be extracted from the hormone-loaded Millipore filters with ethyl ether, and that the isolated hormones were able to stimulate oospore formation when they were re-adsorbed on a piece of Millipore filter but not when they were added directly to agar cultures. Millipore filter probably can prevent inactivation of the hormones by agar media. This would explain why oospores were produced only on the agar medium surface in direct contact with the membrane when opposite mating types were paired on the polycarbonate membrane (Ko, 1978). Molecular weight estimation with the ultrafiltration and reverse osmosis membranes suggests that both hormones α1 and α2 have molecular weights between 100 and 500 (Ko, 1983).

The best solvents for extracting hormone α1 adsorbed on Millipore filter were methylene chloride, hexane, and 95% ethanol, followed by petroleum ether and chloroform (Chern et al., 1996). For extraction of hormone α2, 95% ethanol was the best, followed by methylene chloride, chloroform, and petroleum ether. Hexane was least effective in extracting hormone α2 from the filter. The observation that hormones α1 and α2 were soluble in organic solvents commonly used for lipid extraction (e.g. petroleum ether and chloroform), and the stability of both hormones in acid and base (pH 4-10), suggest that both hormones α1 and α2 are relatively stable, lipid-like natural products. Hormone α2 is more polar than hormone α1 because the former was less soluble in hexane than the latter, and after partition with hexane or petroleum ether, hormone α1 was present only in organic phase while hormone α2 was present in both organic and aqueous phases (Chern et al., 1996).

A method for the large scale extraction of hormones α1 and α2 from agar cultures of A1 and A2 isolates of *P. parasitica*, respectively, was subsequently developed (Chern et al., 1999). For hormone α1, approximately 120 L of the mixture of chloroform-methanol was used to extract 1,354 plates (20 L agar medium) of A1 culture, and for hormone α2, about 120 L of 95% ethanol was used to extract 1,296 plates (20 L agar medium) of A2 culture. The amounts of α hormones obtained were sufficient to permit characterization of their physical and chemical properties, but inadequate for structural elucidation. Results from this study suggest that both hormones α1 and α2 are neutral lipids with hydroxyl functional group(s) (Chern et al., 1999).

All the revealed properties of hormones α1 mentioned above were confirmed by the recent report of Qi et al. (2005). The authors used 1830 L of ethyl acetate to extract the same volume of liquid culture of A1 isolate of *P. parasitica*, and obtained 1.2 mg of pure hormone α1. Their analysis showed a diterpene structure with three hydroxyl functional groups for hormone α1, which supports the revelation of α hormones as neutral lipids with hydroxyl functional groups by Chern et al. (1999). According to the chemical structure, the molecular weight for hormone α1 is 344.3, which also confirms the estimated molecular weights for α hormones as between 100 and 500 by Ko (1983). The chemical structure of hormone α2 remains to be elucidated.

GROUPING BASED ON HORMONE PRODUCTION AND RECEPTION

Based on hormone production and reception, 16 sexuality types, divided into three groups, were proposed for members of the genus *Phytophthora* (Ko, 1980a). Members in Group I do not produce oospores in single culture. They can release hormones that stimulate others to produce oospores and /or produce oospores themselves when stimulated by hormones released by others. Their sexual reproduction requires cross induction by hormones and is therefore comparable to those commonly called "heterothallic." The A1 induction type may belong to sexuality type S1, S2 or S3, while A2 may belong to S4, S5 or S6. S7 is not responsive to either hormone α1 or α2 but can stimulate both A1 and A2 to from oospores while S8 can produce neither hormone α1 nor α2 but can

Table 1. Species of heterothallic *Phytophthora* with hormone-regulated sexual reproduction demonstrated with the polycarbonate membrane method.

Hormone producer		Oospore producer		
Species	Induction Type	Species	Induction Type	Reference
P. parasitica	A1	*P. parasitica*	A2	Ko, 1978[2]
SA[1]	A1	*P. palmivora*	A2	SA
SA	A1	*P. cinnamomi*	A2	SA
SA	A1	*P. colocasiae*	A2	Yu and Chang, 1980
SA	A2	*P. parasitica*	A1	Ko, 1978[2]
SA	A2	*P. palmivora*	A1	SA
SA	A2	*P. cinnamomi*	A1	SA
SA	A2	*P. melonis*	A1	Chang et al., 1984
P. palmivora	A1	*P. palmivora*	A2	Ko, 1978
SA	A1	*P. parasitica*	A2	SA[3]
SA	A1	*P. colocasiae*	A2	Yu and Chang, 1980
SA	A2	*P. cryptogea*	A1	Ho and Jong, 1986
SA	A2	*P. drechsleri*	A1	SA
SA	A2	*P. palmivora*	A1	Ko, 1978
SA	A2	*P. parasitica*	A1	SA[3]
P. cinnamomi	A1	*P. cinnamomi*	A2	Ko, 1978[4]
SA	A1	*P. parasitica*	A2	SA
SA	A1	*P. palmivora*	A2	SA
SA	A1	*P. colocasiae*	A2	Yu and Chang, 1980
SA	A1	*P. cryptogea*	A2	Ho and Jong, 1986
SA	A1	*P. drechsleri*	A2	SA
SA	A2	*P. cinnamomi*	A1	Ko, 1978[4]
SA	A2	*P. parasitica*	A1	SA
SA	A2	*P. palmivora*	A1	SA
SA	A2	*P. drechsleri*	A1	Ho and Jong, 1986
P. colocasiae	A1	*P. colocasiae*	A2	Yu and Chang, 1980
SA	A1	*P. parasitica*	A2	SA
SA	A1	*P. palmivora*	A2	SA
SA	A1	*P. cinnamomi*	A2	SA
SA	A2	*P. parasitica*	A1	SA
SA	A2	*P. palmivora*	A1	SA
SA	A2	*P. cinnamomi*	A1	SA
P. infestans	A1	*P. infestans*	A2	Shen et al., 1983[6]
SA	A1	*P. capsici*	A2	SA
SA	A1	*P. palmivora*	A2	SA
SA	A1	*P. parasitica*	A2	SA
SA	A1	*P. drechsleri*	A2	Shattock et al., 1986[8]
SA	A2	*P. infestans*	A1	Chang and Ko, 1990b[7]
P. capsici	A1	*P. capsici*	A2	Uchida and Aragaki, 1980
SA	A2	SA	A1	SA
P. citrophthora	A1	*P. parasitica*	A2	Ann, 1984
P. megasperma	A1	*P. megasperma*	A2	Ho, 1986a

Table 1. (Continued.)

Hormone producer		Oospore producer		
Species	Induction Type	Species	Induction Type	Reference
SA	A2	SA	A1	SA
P. melonis	A1	*P. parasitica*	A2	Chang et al., 1984
P. drechsleri	A1	*P. drechsleri*	A2	Ho, 1986b
SA	A2	SA	A1	SA
SA	A2	*P. infestans*	A1	Shattock et al., 1986[8]
P. cryptogea	A1	*P. cryptogea*	A2	Ho and Jong, 1986
SA	A2	SA	A1	SA
P. drechsleri	A1	*P. drechsleri*	A2	SA
SA	A2	SA	A1	SA

[1]SA= the same as above.
[2]Also reported by Ko et al., 1986; Chang and Ko, 1990a; Ko and Kunimoto, 1981; Ann, 1984.
[3]Also reported by Ko and Kunimoto, 1981; Ann, 1984.
[4]Also reported by Ann and Ko, 1989; Ho et al., 1983.
[5]Also reported by Yu et al., 1981.
[6]Also reported by Chang and Ko, 1990b; Shaw et al., 1985.
[7]Also reported by Shattock et al., 1986; Shaw et al., 1985.
[8]Also reported by Skidmore et al., 1984.

Table 2. Species of homothallic *Phytophthora* with hormone-regulated sexual reproduction demonstrated with the polycarbonate membrane method.

Hormone producer		Oospore producer		
Species	Induction Type	Species	Induction Type	Reference
P. heveae	A1A2	*P. parasitica*	A1	Ko, 1980a
P. cactorum	A1A2	SA[1]	A1	SA
P. katsurae	A1A2	SA	A1	SA
P. humicola	A1A2	SA	A1	Ko and Ann, 1985
P. citricola	A1A2	SA	A1	Ko et al., 1982
P. melonis	A1A2	SA	A1	Chang et al., 1984
P. heveae	A1A2	SA	A2	Ko, 1980a
P. sojae	A1A2	SA	A2	SA
P. insolita	A1A2	SA	A2	Ann and Ko, 1980
P. cinnamomi	A1A2	*P. cinnamomi*	A2	Ho et al., 1983

[1]SA= the same as above.

be stimulated by both A1 and A2 to form oospores. S7 and S8 are A1, A2 induction type. Members in Group II produce oospores in single culture and are comparable to those commonly called "homothallic" or A1A2 type. They form oospores by self-induction induced by self-produced hormones and are designated A1A2 induction type. This group includes S9 to S15. Those isolates belonging to Group III are neuter and are designated AO. They cannot stimulate others nor be stimulated to produce oospores. Only S16 belongs to this group. Prior to 1988, only S1, S2, S4, S5 and S16 have been found in species of *Phytophthora* (Ko, 1988). An unidentified species of *Phytophthora* belonging to S16 also was isolated from a diseased strawberry stem tissue (Chang, 1988).

Rubin and Cohen (2006) recently reported the isolation of "unusual mating type" of *P. infestans*. These isolates were sterile when inoculated alone but produced oospores in tomato tissue when co-inoculated with and A1 or A2 isolate. According to the grouping based on hormonal regulation mentioned above, the sexual behavior of these isolates are not unusual. They fit the description of sexuality type S8 of the A1, A2 induction type.

HORMONAL HETEROTHALLISM AND HOMOTHALLISM

Genetic exchange in the pairings between A1 and A2 types of different species of cross-inducing *Phytophthora* has been shown to be essentially nonexistent (Boccas and Zentmyer, 1976; Boccas, 1981; Erselius and Shaw, 1982; Chang and Ko, 1993). Consequently, all sexual progenies are produced by either A1 or A2 type by selfing induced by α hormones (Figure 1A). However, both hybrid and self progenies of A1 and A2 types are produced following induction by α hormones when isolates of the same species are paired (Chang and Ko, 1990a; 1992; Judelson, 1997) (Figure 1B). During the sexual reproduction of self-inducing *Phytophthora*, self progeny of A1A2 type is produced following induction by α hormones (Figure 1C).

Requirement of the presence of an opposite mating type in sexual reproduction of "heterothallic" *Phytophthora* is for production and reception of mating-type-specific hormones to initiate the process of selfing or hybridization. "Homothallic" species of *Phytophthora* do not require the presence of another mating type in sexual reproduction because they possess receptor(s) for self-produced α hormone(s) to initiate selfing. This is a novel mode of sexual reproduction in the biological world. It is a chemical heterothallism and homothallism, rather than the conventional biological heterothallism and homothallism.

FACTORS AFFECTING HORMONAL REGULATION OF SEXUAL REPRODUCTION

The influence of light on the sexual reproduction of *Phytophthora* is well documented (Harnish, 1965; Klisiewicz, 1970). Few or no oospores were found under continuous light while many were produced in darkness (Chang and Shu, 1988; Wang et al., 2005; Ko et al., 2006). A1 and A2 isolates of *P. parasitica* were exposed to light at different stages of sexual development to study the mode of action of light on hormonal regulation of sexual reproduction (Chern and Ko, 1993). Light was found to be inhibitory to the production of α hormones but not to the formation of the receptors of these hormones. After being produced, α hormones were stable under light. Light was also inhibitory to hormone induction of sexual reproduction but not oospore formation after hormone induction. Using A2 isolate of *P. colocasiae* as a hormone producer and A1 isolate of *P. parasitica* as a hormone receptor, Yu et al. (1981) also showed that hormone production, but not oospore formation, after hormone induction was greatly inhibited by light.

For most species of *Phytophthora* tested, the maximum temperature permiting vegetative growth is always inhibitory to sexual reproduction (Chern and Ko, 1994). Although both A1 and A2 isolates of *P. parasitica* were capable of growth at 34°C, no oospores were produced when they were paired at this temperature. These two isolates were, therefore, exposed to 34°C at different stages of sexual development to study the mode of action of high temperature on hormonal regulation of sexual reproduction (Chern and Ko, 1994). The production of α hormones and their receptors was inhibited at high temperature. However, after being produced, α hormones and their receptors were stable at high temperature. No oospores were produced when cultures were exposed to high temperature during hormone reception, or oospore formation after hormone induction.

CONVERSION OF INDUCTION TYPE

In 1981, the frequently observed phenomenon of oospore sectors in colonies originating from aged cultures of A1 or A2 isolates of *Phytophthora* was found to be due to the appearance of the opposite induction type during long term storage (Ko, 1981). During the same period, it was found that the fungicide chloroneb also could cause A2 isolate of *P. capsici* to change induction type and form oospore sectors on agar medium (Ko, 1981). Subsequently, other fungicides and the antibiotic streptomycin also were reported to have the ability to cause conversion of induction types (Table 3). Progeny derived from selfed oospores of the A1 or A2 induction type of *P. parasitica* (Ann and Ko, 1988) and *P. infestans* (Ko, 1994) induced by α hormones consisted of the parental induction type and the new opposite type, indicating the ability of selfing to cause conversion of induction type.

Some propagules of the A1 and A2 isolates of *P. parasitica* were converted to A1A2 type after long-term

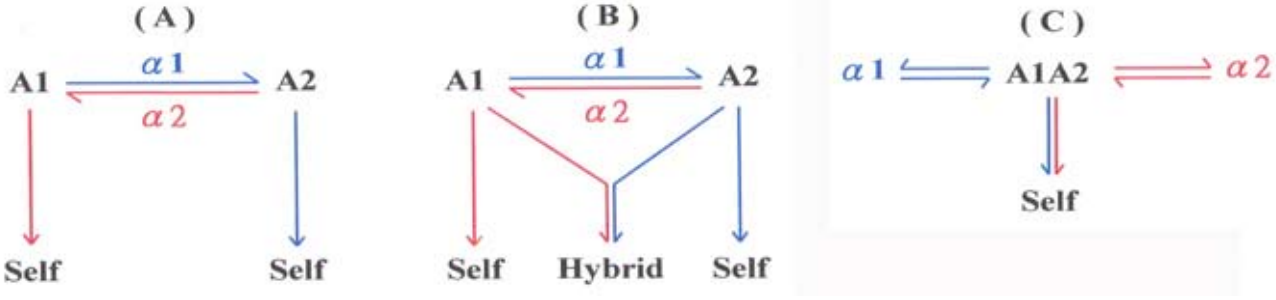

Figure 1. Schematic illustration of hormonal regulation of sexual reproduction in *Phytophthora*. (A), cross induction of different species; (B), cross induction of the same species; and (C), self induction.

storage and exposure to chlorneb, respectively (Ko, 1981). Selfing, segregation and certain fungicides also can cause conversion of A1 and A2 isolates of *Phytophthora* to A1A2 type (Table 4). This kind of A1A2 type is usually unstable, and some of its zoospore propagules may be converted to A1 or A2 again (Mortimer et al., 1977; Ko, 1981; Chang and Ko, 1990b).

When self-inducing (A1A2) *P. boehmeriae* was grown on medium containing the fungicide ethazol, the organism produced oospore-free sectors from which self-sterile cultures were obtained (Zhou et al., 1997). Single zoospore cultures derived from these self-sterile cultures were of the A1A2, A1, A2 and A0 types. So far this is the only example of a self-inducing species of *Phytophthora* being converted to cross-induction.

Conversion of induction type in cross-induing *Phytophthora* is a reversible process (Ko, 1981; Ko et al., 1986). When an induction type is converted to the opposite type, both hormone production and reception are changed (Ko, 1981; Ko et al., 1986). This indicates that genes for production of hormone α1 (*P1*) and reception of hormone α2 (*R2*) in A1 are linked and genes for production of hormone α2 (*P2*) and reception of hormone α1 (*R1*) in A2 also are linked, and that A1 (*P1R2*) and A2 (*P2R1*) genes are on the same chromosome. To account for the reversible nature of induction type conversion, it is further postulated that the transcription of such linked genes is regulated by a repressor that represses the expression of A2 (*P2R1*) gene in A1 induction type with one molecular configuration and A1 (*P1R2*) genes in A2 induction type with another configuration. Based on this hypothesis, the A1A2 induction type has either an inactive repressor or two chromosomes, one with A1 (*P1R2*) genes repressed and another with A2 (*P2R1*) genes repressed. The A0 induction type contains either a super repressor capable of repressing both A1 (*P1R2*) and A2 (*P2R1*) genes concomitantly, or a chromosome lacking the A1 (*P1R2*) and A2 (*P2R1*) genes, according to the same hypothesis.

MITOCHONDRIAL GENE CONTROL OF INDUCTION ("MATING") TYPES

Induction types in *Phytophthora* have been considered to be determined by either nuclear genes or cytoplasmic factors (Timmer et al., 1970). Based on cytological observations and segregation pattern, it was proposed that the A1 induction type is homozygous recessive aa and A2 is heterozygous Aa, while the self inducing A1A2 type is a trisomic Aaa (Mortimer et al., 1977; Sansome, 1980). This hypothesis was refuted by the observation of the induction of segregation of A2 and A1A2 from A1 by aging and chemical treatments (Ko, 1981; Ann and Ko, 1989; Chang and Ko, 1990b) because the homozygous recessive character should not segregate. Most of the data indicating nuclear inheritance of mating type were based on genetic mapping studies involving either the use of nuclear DNA

Table 3. Factors causing conversion of induction type A1 to A2 and vice versa in *Phytophthora*.

Type of conversion	Species	Causal factor	Reference
A1 → A2	*P. parasitica*	Aging	Ko, 1981
		Selfing	Ann and Ko, 1988
		Ethazol	Ko et al., 1986
		Streptomycin	Ann and Ko, 1992
		Metalaxyl	Chang and Ko, 1990
		Chloroneb	Ann and Ko, 1989
	*P. cinnamom*i	Ethazol, chloroneb	Ann and Ko, 1989
	P. infestans	Selfing	Ko, 1994
		Metalaxyl	Chang and Ko, 1990
		Mefenoxam	Groves and Ristaino, 2000
A2 → A1	*P. parasitica*	Aging	Ko, 1981
		Selfing	Ann and Ko, 1988
		Chloroneb	Ko et al., 1986
		Streptomycin	Ann and Ko, 1992
		Ethazol	Ann and Ko, 1989
	P. cinnamomi	Ethazol	Ann and Ko, 1989
	P. infestans	Selfing	Ko, 1994
		Metalaxyl	Chang and Ko, 1990
		Mefenoxam, benomyl	Groves and Ristaino, 2000

Table 4. Factors causing conversion from cross induction to self induction and vice versa in *Phytophthora*.

Type of conversion	Species	Causal factor	Reference
A1 → A1A2	*P. parasitica*	Aging	Ko, 1981
		Selfing	Ann and Ko, 1988
	P. infestans	Selfing	Ko, 1994
		Metalaxyl	Chang and Ko, 1996b
	P. cinnamomi	Ethazol, chloroneb	Ann and Ko, 1989
A2 → A1A2	*P. parasitica*	Chloroneb	Ko, 1981
		Selfing	Ann and Ko, 1988
	P. cinnamomi	Ethazol	Ann and Ko, 1989
	P. drechsleri	Segregation	Mortimer et al., 1977
A1A2 → A1	*P. parasitica*	Segregation	Ko, 1981
	P. infestans	Segregation	Chang and Ko, 1990b[1]
	P. drechsleri	Segregation	Mortimer et al., 1977
	P. boehmeriae	Ethazol	Zhou et al., 1997
A1A2 → A2	*P. parasitica*	Segregation	Ko, 1981
	P. infestans	Segregation	Fyfe and Shaw, 1992
	P. drechsleri	Segregation	Mortimer et al., 1977
	P. boehmeriae	Ethazol	Zhou et al., 1997
A1A2 → A0	*P. boehmeriae*	Ethazol	Zhou et al., 1997

[1]Also reported by Fyfe and Shaw, 1992.

markers for the construction of genetic linkage maps (Van der Lee et al., 2004) or the search for induction type linked nuclear DNA markers (Judelson, 1996). Their data, which were based only on association, do not prove that induction type is a nuclear trait. The putative induction type locus still has not been cloned.

Segregation in colony morphology during conidial propagation of a culture originating from a single conidium, referred to as a "dual phenomenon" in imperfect fungi, was found to be due to the dissociation of multinucleate organisms in a heterokaryotic condition (Hansen, 1938). Zoospores produced by an A2 culture of *P. drechsleri*, which originated from a zoospore, gave rise to A1, A2 and A1A2 cultures (Mortimer et al., 1977). The single-zoospore A1A2 cultures of *P. parasitica* (Ko, 1981) and *P. infestans* (Chang and Ko, 1990b) also produced A1 and A1A2 zoospore cultures. Such phenomena cannot be explained as the result of dissociation of heterokaryons because most zoospores are uninucleate (Mortimer et al., 1977; Zheng and Ko, 1997). It is conceivable that induction types are controlled by different mitochondrial DNA. Both A1 and A2 mitochondria probably exist in each isolate of *Phytophthora*, and the expression of induction type is the end result of the interaction between these two types of mitochondria, similar to the behavior of antibiotic resistance in *Phytophthora* (Gu and Ko, 2000c). Recently, we developed methods for protoplast fusion (Gu and Ko, 1998; 2000a), nuclear transplantation (Gu and Ko, 1998; 2000b), mitochondrial transplantation (Gu and Ko, 2000c), and isolation of single transplantation products (Ho and Ko, 1997). These techniques were used to test if induction type genes are located in nuclei or mitochondria.

Chang and Ko (1990b) showed that metalaxyl resistance (M^r) and chloroneb resistance (Cn^r) in *P. parasitica* were each conferred by a nuclear gene while streptomycin resistance (S^r) and chloramphenicol resistance (Cp^r) were each conferred by a cytoplasmic gene. When protoplasts carrying M^r nuclei from A1 isolate of *P. parasitica* were fused with protoplasts carrying Cn^r nuclei from A2 isolate of the same species, fusion products carrying M^r nuclei were either the A2 or A1A2 type while those carrying Cn^r nuclei were either the A1, A2, or A1A2 type (Gu and Ko, 2005). Fusion products carrying M^r and Cn^r nuclei also behaved as either an A1, A2 or A1A2 type. The result refutes the hypothesis that induction types in *Phytophthora* are controlled by nuclear genes. When nuclei from A1 isolate of *P. parasitica* were fused with protoplasts from the A2 isolate of the same species and vice versa, all the

nuclear hybrids expressed the induction type characterstics of protoplast parent. The same was true when the nuclei from the A1 isolate of *P. parasitica* were fused with protoplasts from the A0 isolate of *P. capsici* and vice versa. These results confirm the observation that induction type genes are not located in the nuclei and suggest the presence of induction type genes in the cytoplasms of the recipient protoplasts. When mitochondria from the A1 isolate of *P. parasitica* were fused with protoplasts from the A2 isolate of the same species, the induction type of three out of five regenerated protoplasts was changed from A2 to A1 type. The result demonstrated the decisive effect of the sexuality of mitochondrial donor on induction type characterstics of mitochondrial hybrids and suggested the presence of induction type genes in mitochondria. All of the mitochondrial hybrids resulting from the transfer of mitochondria from the A0 isolate of *P. capsici* into protoplasts from A1 isolate of *P. parasitica* were all of the A0 type. The result further supports the hypothesis of the presence of induction type genes in mitochondria in *Phytophthora*.

NUTRITIONAL SUBSTANCES NEEDED FOR SEXUAL REPRODUCTION

Prior to 1983 when Ko and Ho (1983) reported the discovery that lecithin was stimulatory to oospore formation of *P. parasitica* and *P. cactorum*, sterols were the only nutritional substances known to be beneficial to sexual reproduction in *Phytophthora* (Ko, 1998). Subsequently, cephalin was reported stimulatory to oospore formation of *P. cactorum* (Ko, 1985). In 1997, Jee et al. (1997) found that the 99% pure commercial lecithin from one of the shipments was partially inhibitory to the growth of *P. cactorum* on basal medium and was not stimulatory to oospore formation of the fungus. When it was dissolved in ether and then washed with deionized water or NaCl solution, the inhibitory effect disappeared, and the compound became strongly stimulatory to oospore formation. After removal of similar inhibitory substance, a number of fatty acids and related compounds became stimulatory to oospore formation of *Phytophthora* (Jee and Ko, 1997; Jee et al., 2002; Wu et al., 2003).

Previously, failure of *Phytophthora* to produce oospores in basal medium was believed to be due to their inability to synthesize the substances required for sexual reproduction (Hendrix, 1970; Elliott, 1983). However, when mycelium of *P. cactorum* produced in liquid basal medium was transferred to nutrient-free water agarose, oospores were produced (Jee and Ko, 1998). The organism also produced oospores on solid basal medium near the edge of the plates after a prolonged incubation period. These results showed that *P. cactorum* does not need special exogenous nutrients for sexual reproduction but does require a stress factor like nutrient deprivation or aging to trigger the sexual process. Furthermore, extracts from the mycelia of a *Phytophthora* species grown in liquid basal medium were found to be stimulatory to their own sexual reproduction, indicating their ability to synthesize substances needed for sexual reproduction (Jee and Ko, 1998; Wu et al., 2003).

FUTURE OUTLOOK

Following the detailed characterization of chemical and physical properties of α hormones (Ko, 1983; Chern et al., 1996; 1999), the chemical structure of hormone α1 was revealed (Qi et al., 2005) although hormone α2 remains to be determined. Hormone α2 appears to be more difficult to work with (M. Ojika, Nagoya University, *Personal communication*). The following information may be useful for future elucidation of the chemical structure of hormone α2. Solvent different from that used for extraction of hormone α1 probably should be used. According to tests with α hormones adsorbed on Millipore filter, 95% ethanol was best for extraction of hormone α2, followed by methylene chloride (Chern et al., 1996). For detecting the activity of hormone α2 in an extract, the hormone should be adsorbed on a piece of Millipore filter composed of cellulose nitrate and cellulose acetate (Ko, 1983; Chern et al., 1996). Ability of hormone α2 adsorbed on this type of Millipore filter to induce oospore formation increased about 25 fold in comparison with the same amount of hormone added directly to the receptor culture (Chern et al., 1996). It is also important to know that although hormones α1 and α2 have several similar characteristics, hormone α2 has a polarity greater than that of hormone α1, and is too polar to be analyzed by gas chromatography (Chern et al., 1999).

The discovery of control of induction types by mitochondrial genes in *Phytophthora* points to a new direction for research on genetics of sexual reproduction in this group of organisms. Reversible conversion of induction types led to the hypothesis that both A1 and A2 induction types contain A1 and A2 genes, and that the expression of A1 induction type is the result of repression of A2 gene by a repressor referred to as A2 repressor and the expression of A1 gene by another repressor referred to as A1 repressor (Ko, 1981; 1988). The other possibility is that both A1 and A2 induction types contain two kinds of mitochondrial DNA, one with A1 gene (A1 mtDNA) and another with A2 gene (A2 mtDNA). Based on this hypothesis, A1 induction type has A1 mtDNA as the predominant type and A2 mtDNA as the minor type, and A2 induction type is just opposite, having A2 mtDNA as the predominant type and A1 mtDNA as the minor type, a system similar to that proposed for mitochondrial gene control of antibiotics (Gu and Ko, 2000c). The validity of each hypothesis remains to be tested.

Relatively little is known about hormonal regulation of sexual reproduction in the closely related genus of *Pythium*. Using the polycarbonate membrane technique, hormonal regulation of sexual reproduction has been demonstrated in heterothallic *Pythium splendens* (Guo and Ko, 1991). Aging (Guo and Ko, 1991) and selfing (Guo and Ko, 1996) also caused mating type change in *Py. splendens*, and lecithins were found to be stimulatory

to sexual reproduction in *Pythium aphanidermatum* (Ko, 1986). Whether similar events also occur in other species of *Pythium* remains to be investigated.

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Characterization of MRT, a new non-LTR retrotransposon in *Monascus* spp.

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ABSTRACT. A new non-LTR retrotransposon, named MRT, was discovered in the filamentous fungus *Monascus pilosus* BCRC38072. The entire nucleotide sequence of the MRT element was 5.5-kb long, including two open reading frames. These two ORFs showed homologies to *gag*-like and *pol*-like gene products, and an A-rich sequence at the 3′ end of *pol*-like gene. ORF1 encoded a protein of 517 amino acids and contained a cysteine-rich zinc finger motif. ORF2 encoded a protein of 1181 amino acids and contained apurinic/apyrimidinic endonuclease (APE), reverse transcriptase (RT), RNaseH domains, and a CCHC motif. The phylogenetic analyses demonstrated that the MRT element should be classified into the Tad1 clade. The results of Southern hybridizations showed that MRT elements were distributed within *M. pilosus*, *M. ruber*, *M. sanguineus*, and *M. barkeri*. In addition, the species of *Monascus* can be grouped by the presence or absence of MRT elements in the hybridization pattern according to phylogenetic subgroups established with the partial β-tubulin gene.

Keywords: Bacterial artificial chromosome; *Monascus pilosus*; Non-LTR retrotransposon; Phylogenetic analysis.

INTRODUCTION

Retrotransposons are found in most eukaryotes and in some cases constitute a major part of the genome (e.g. 40-50% of the human genome). They have been divided into two subclasses based on their differences in overall structure. LTR retrotransposons closely relate to retroviruses and non-LTR retrotransposons, also called LINE-like elements. All these elements use reverse transcription to propagate. Non-LTR retrotransposons have been found in many groups of eukaryotic organisms, including mammals, insects, amphibians, plants, and also fungi.

Five non-LTR retrotransposons have been characterized in filamentous fungi, including *Tad1-1* in *Neurospora crassa* (Cambareri et al., 1994), MGR583 in *Magnaporthe grisea* (Hamer et al., 1989), CgT1 in *Colletotrichum gloeosporioides* (He et al., 1996), *marY2N* in *Tricholoma matsutake* (Murata et al., 2001), and Mars1 in *Ascobolus immerses* (Goyon et al., 1996) though the entire element is not described for this last species. These non-LTR retrotransposons usually contain two ORFs encoding *gag*-like and *pol*-like proteins. Moreover, they generally have poly (A) or A-rich regions at their 3′ terminus and generate truncation in 5′ UTRs. Phylogenetic analysis of non-LTR retrotransposons based on the RT (reverse transcriptase) domain, the only sequence found in all elements, defined eleven clades by Malik et al. (1999). More recently, Burke et al. (2002) proposed an additional classification in which the various clades fall into five groups on the basis of both the phylogenetic relationship of their RT sequence and the nature and arrangement of their protein domains. Based on sequence, structure, and phylogenetic analyses, the non-LTRs elements from filamentous fungi are grouped in the Tad1 clade. Recently, two non-LTR retrotransposons of yeast, Zorro in *Candida albicans* (Goodwin et al., 2001) and Ylli in *Yarrowia lipolytica* (Casaregola et al., 2002), have been placed into the L1 clade of mammalian elements.

According to the distribution of CgT1 in *C. gloeosporioides*, the presence or absence of CgT1 can be used to distinguish biotypes A and B that cause different anthracnose diseases on *Stylosanthes* in Australia (He et al., 1996). Moreover, DNA fingerprint analysis of CgT1 reveals that Australian isolates of biotype B are monomorphic. In addition, the analysis of the genetic relations and evolutionary history of many species has been facilitated by repetitive DNA fingerprinting probe (Cizeron et al., 1998; Blesa et al., 2001; Daboussi and Capy, 2003).

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Monascus spp. belongs to the ascomycetes, and has been used in Chinese fermented foods such as anka, anka pork, and rice wine for thousands of years. Thirteen *Monascus* species have been reported. They are known as producers of various secondary metabolites with polyketide structures, such as monacolins, and have medical importance (Endo, 1979). In this study, we report the discovery of a new non-LTR retrotransposon named MRT (*Monascus* Retrotransposon) in *Monascus* spp. The structural, genomic and phylogenetic analysis of the MRT elements was presented. Moreover, the distribution of MRT in *Monascus* species was also analyzed.

MATERIALS AND METHODS

Strains, media, and growth conditions

The nineteen strains of *Monascus* listed in Table 1 were used in this study. All strains were maintained on YM (DIFCO, Detroit, Michigan) agar for one week, and spore suspensions were obtained by washing cultured YM agar plates with distilled water. Mycelia for DNA isolation were harvested from YM broth after incubating for 8 days at 28°C with constant agitation and then frozen at -80°C.

BAC library construction and shotgun sequencing

The methods of Peterson et al. (2000) were used to make a BAC library of *Monascus pilosus* BCRC 38072. DNAs of eleven BAC clones were extracted for shotgun sequencing by a Qiagen Large-Construct kit (Qiagen, Valencia, CA). DNA sequencing was performed with an ABI Prism 3700 Sequencer (Applied Biosystems, Foster City, CA). The Phred-Phrap-Consed system developed by the Phil Green Laboratory was used to assemble DNA fragments (Gordon et al., 2001). Nucleotide and deduced amino acid sequences were used to interrogate the non-redundant database at GenBank using BlastN and BlastX. Sequence analysis was done using VectorNTI 9.0 (InforMax, Frederick, MD) software. Prediction of nucleic acid secondary structure was performed with Mfold server (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). The nucleotide sequences of MRT non-LTR retrotransposons found in this study have been submitted to GenBank under the accession numbers AY900582 and DQ299897 to DQ299900.

Genomic DNA preparation and Southern hybridization

Monascus genomic DNA was extracted according to the method developed by Bingle et al. (1999). Approximately 0.5 g (squeezed wet weight) of frozen mycelia was ground to a fine power under liquid nitrogen using a mortar and pestle. Protein was removed by successive rounds of extraction with phenol and chloroform. Genomic DNA

Table 1. Strains used and GenBank accession numbers for the β-tubulin gene.

Strain	Species[a]	MRT non-LTR retrotransposon[b]	Accession number of β-tubulin gene
BCRC 38072 (Taiwan isolate)	*Monascus pilosus*	+	DQ299886
BCRC 31502 (ATCC 16363)	*Monascus pilosus*, Type	+	AY498596
BCRC 31503 (ATCC 16364)	*Monascus pilosus*	–	DQ299887
BCRC 31533 (ATCC 16246)	*Monascus ruber*, Type	+	AY498589
BCRC 31523 (ATCC 16378)	*Monascus ruber*	+	DQ299888
BCRC 31534 (ATCC 16366)	*Monascus ruber*	+	AY498587
BCRC 31535 (ATCC 18199)	*Monascus ruber*	+	DQ299889
BCRC 33314 (ATCC 16371)	*Monascus ruber*	+	AY498588
BCRC 33323 (ATCC 18199)	*Monascus ruber*	+	DQ299890
BCRC 31542 (ATCC 16365)	*Monascus purpureus*, Type	–	DQ299891
BCRC 31541 (ATCC 16379)	*Monascus purpureus*	–	AY498598
BCRC 31615 (DSM 1379)	*Monascus purpureus*	–	DQ299892
BCRC 33325 (IFO 30873)	*Monascus purpureus*	–	DQ299893
BCRC 31506 (CBS 302.78)	*Monascus kaoliang*, Type	–	DQ299894
BCRC 33446 (ATCC 200613)	*Monascus sanguineus*, Type	+	AY498602
BCRC 33309 (ATCC 16966)	*Monascus barkeri*	+	DQ299895
BCRC 33310 (IMI 282587)	*Monascus floridanus*, Type	–	DQ299896
BCRC 33640 (ATCC 204397)	*Monascus lunisporas*, Type	–	AY498604
BCRC 33641 (ATCC 200612)	*Monascus pallens*, Type	–	AY498601

[a]"Type" indicates type strain.
[b]+, presence of MRT non-LTR retrotransposon; –, absence of MRT non-LTR retrotransposon.

was recovered by precipitation with ethanol and dissolved in TE buffer. For Southern hybridizations, genomic DNA (7.5 µg per lane) was digested with *Eco*RI and *Bam*HI restriction enzymes and separated through 1.0% agarose gels by electrophoresis. Southern hybridization analysis was performed using the DIG system (Roche Diagnostics, Mannheim, Germany). The probe of the MRT element was labeled by PCR amplification from genomic DNA of *M. pilosus* 38072 using a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The primer set of the MRT probe was MRT1-1F: CAGGGGGAGGCTAGGATGTA, and MRT1-1R: CACAGGTGGGTAGAGCCACAG. All other DNA manipulations were performed as described in Sambrook et al. (1989).

Phylogenetic analysis

In addition to MRT element, β-tubulin gene was chosen for phylogenetic analysis of *Monascus* spp. The partial β-tubulin genes were amplified with the primer set, btubulinF: 5′-CAACTGGGCTAAGGGTCATT and btubulinR: 5′-GTGAACTCCATCTCGTCCATA (Wu et al., 1996; Park et al., 2004). Sequences of partial β-tubulin genes obtained from *Monascus* strains used in this study have been submitted to GenBank under the accession numbers DQ299886 to DQ299896. The other accession numbers of partial β-tubulin genes, AY498587 to AY498589, AY498596, AY498598, AY498601, AY498602 and AY498604, were obtained from the GenBank database. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA 3.1 software with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

During the whole genome sequencing of *M. pilosus* BCRC38072, two repetitive sequences (mps01-1 and mps01-2) were observed in a ca. 160 kb BAC, mps01. They were found to have a homology similar to CgT1 (He et al., 1996), a non-LTR retrotransposon from *Colletotrichum gloeosporioides* (mps01-1, 31% identity and mps01-2, 32% identity by BlastX). The sequence homology indicated that the repetitive sequences were non-LTR retrotransposons. The new non-LTR retrotransposon was designated MRT. Eleven BAC clones of *M. pilosus* BCRC38072 were sequenced covering 1.55 Mb; and six positive BACs were identified, and 15 copies of the MRT element were found in determining the relative abundance and diversity of the non-LTR retrotransposon. The entire nucleotide sequence of the MRT element was 5.5-kb long. Given that the size of the *M. pilosus* BCRC38072 genome was ~30 Mb, the number of copies of the genome was estimated to be ~290, which occupied about 5% of the *M. pilosus* BCRC38072 genome.

The translation frames of the entire set of MRT elements revealed that four of them in BACs—mps02-1, mps07-1, mps11-1, and mps13-1—contained two

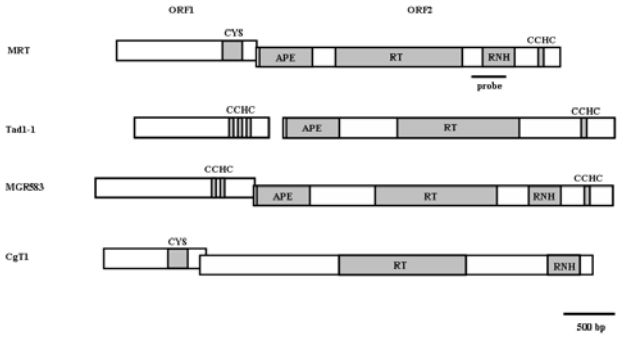

Figure 1. Structure of the MRT element. Schematic representation of the structural organization of the filamentous fungi non-LTR retrotransposons. Tad1-1, MGR583 and CgT1 in *Neurospora crassa*, *Magnaporthe grisea* and *Colletotrichum gloeosporioides* were obtained from the GenBank database using the following accession numbers, L25662, AF018033 and L76205, respectively. Southern hybridization analysis of MRT in the genomes of *Monascus* species hybridized with the probe indicated by small black bar. The abbreviation of CYS indicated the cysteine-rich region, RNH indicated the RNaseH, APE indicated the apurinic/apyrimidinic endonuclease domain, RT indicated the reverse transcriptase domain, and CCHC indicated the Cys-His region.

open reading frames (Figure 1), like other non-LTR retrotransposons that have been found in fungi, CgT1, Tad-1, and MGR583 (Hamer et al., 1989; Cambareri et al., 1994; He et al., 1996). Numerous stop codons were found in other copies of the MRT elements. A conceptual translation demonstrated that the first ORF in the MRT element may encode a protein of 517 amino acids. The deduced amino acid sequence of the MRT ORF1 contained one zinc finger motif that was cysteine-rich (Figures 1 and 2). The arrangement of cysteines indicated that the consensus sequence $CX_6HXCX_6CHX_2HX_6C$ represented an NF-X1-type zinc finger, based on Pfam analysis (Song et al., 1994). However, no 5′ UTR was clearly identified, suggesting that all of the active element may be rare in *M. pilosus* BCRC38072. A conceptual translation demonstrated that the second ORF in the MRT element may encode a protein of 1181 amino acids. The ORF1 and ORF2 overlapped by 1 bp. The deduced amino acid sequence of the MRT ORF2 contained an apurinic/apyrimidinic endonuclease (APE) domain, a reverse transcriptase (RT) domain, an RNaseH domain, and a CCHC motif (Figures 1 and 3). The APE domain at the N-terminal of ORF2 in the MRT element was proven not to be well conserved in other organisms. The RT domain located downstream of the APE domain contained conservation of the deduced amino acid sequences, YXDD, which were a part of the active site in the RT domain (He et al., 1996). The 3′ UTRs of the MRT elements were around 300 bp, and the 3′ ends were well conserved. Two parts, the A-rich sequence and the stem-loop region, were present in the conserved tail (Figure 4A). Interestingly, the A-rich stretch represented two sequences,

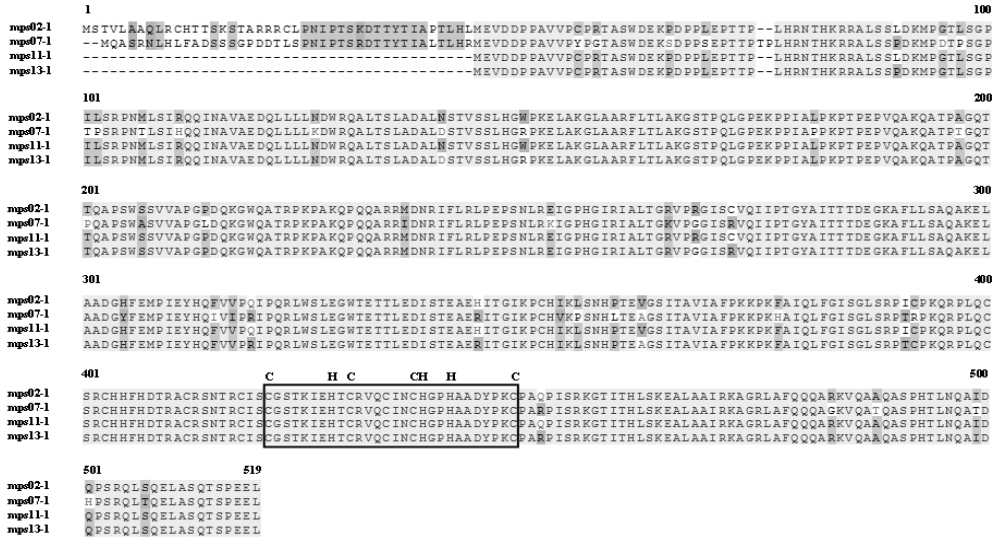

Figure 2. Deduced Amino acid sequences alignment of the MRT elements ORF1. The cysteine-rich nucleotide-binding domain represented a zinc finger with the consensus sequence $CX_6HXCX_6CHX_2HX_6C$ shown boxed.

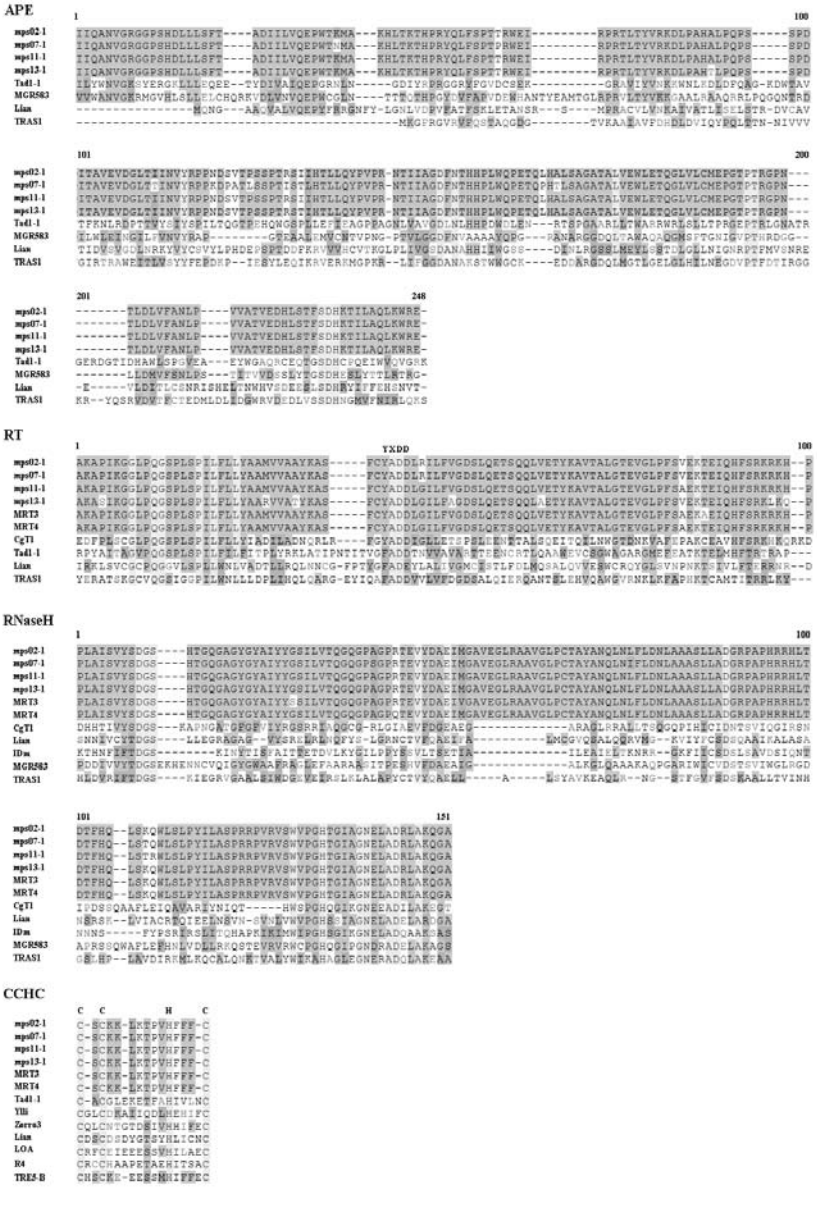

Figure 3. Multiple alignment of deduced amino acid sequences of the MRT elements with related organisms. Comparison of the N-termial apurinic/ apyrimidinic endonuclease (APE) domains based on an alignment of approximately 210 amino acid residues. Comparison of the reverse transcriptase (RT) domain. Conserved YXDD residues, active site of reverse transcriptase domain, were indicated above the alignment. Comparison of the RNaseH domains based on an alignment of approximately 130 amino acid residues. Conserved residues of RNaseH were described by Malik et al. (1999). Comparison of the C-terminal Cys-His region (CCHC). Conserved $CX_1CX_7HX_3C$ residues, putative zinc finger of the MRT element, were indicated above the alignment.

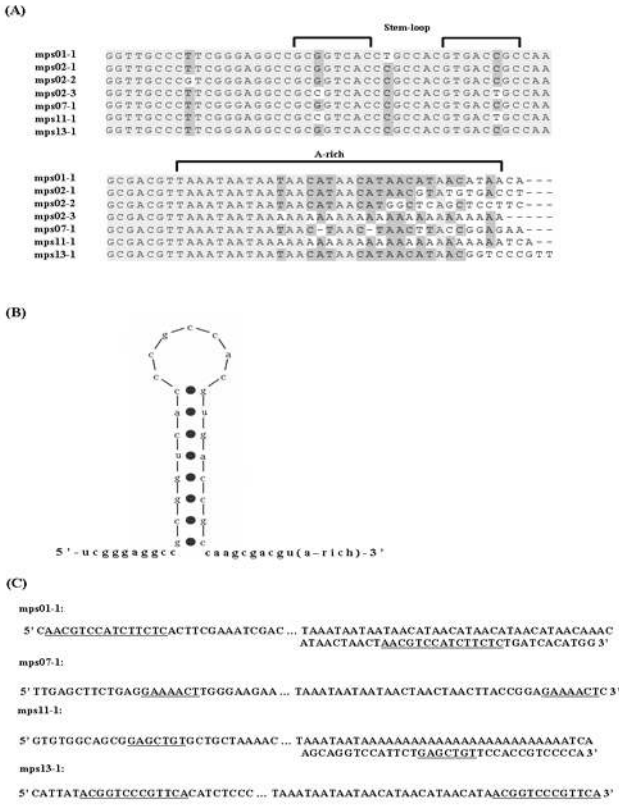

Figure 4. (A) Comparison of 3′ conserved region of the MRT elements from BACs. The A-rich sequences and stem-loop were marked. (B) Putative secondary structure of 3′ conserved region of the MRT element RNA as indicated in (A) was depicted. (C) Sequences at the ends of the MRT elements. Four elements from BACs were shown. Putative target site duplications were indicated by underlining.

TAAATAATAA(CATAA)n and TAAATAATAA(A)n. Additionally, the RNA transcribed from the conserved tail of the MRT element was proposed to form a stem-loop (Figure 4B). This stem-loop region can be recognized by the reverse transcriptase of the non-LTR retrotransposon (Baba et al., 2004). Furthermore, most non-LTR retrotransposons have target site duplications (TSDs) of variable lengths from 4 to 49 bp (Eickbush, 1992). The sequence results also revealed that four MRT elements presented 7 to 15 bp target site duplications (Figure 4C).

Since reverse transcriptase (RT) is a fundamental component of the machinery required to synthesize DNA, it is strongly conserved and used in analyses of retrotransposon phylogeny (Flavell, 1995). In particular, the eleven conserved block sequences of the reverse transcriptase domain defined by Malik et al. (1999) are extensively used for the construction of retrotransposon phylogeny. This study analyzed the phylogenetic relationships between members of the non-LTR retrotransposons, including the four MRT elements described above, using shared reverse transcriptase domains. The phylogenetic tree was rooted using RT sequences of bacterial and fungal group II introns (Xiong and Eickbush, 1990). Eleven clades were clearly distinguished (Figure 5A). This result was consistent with the phylogeny constructed by Malik et al. (1999). The phylogenetic tree further suggested that the MRT element belonged to the Tad1 clade known from filamentous fungi. Moreover, the apurinic/apyrimidinic endonuclease (APE) domain is believed to cleave DNA in the reverse transcription reaction at the chromosome target site (Maita et al., 2004) and is commonly employed to construct the phylogenetic analysis (Malik et al., 1999). Since CRE,

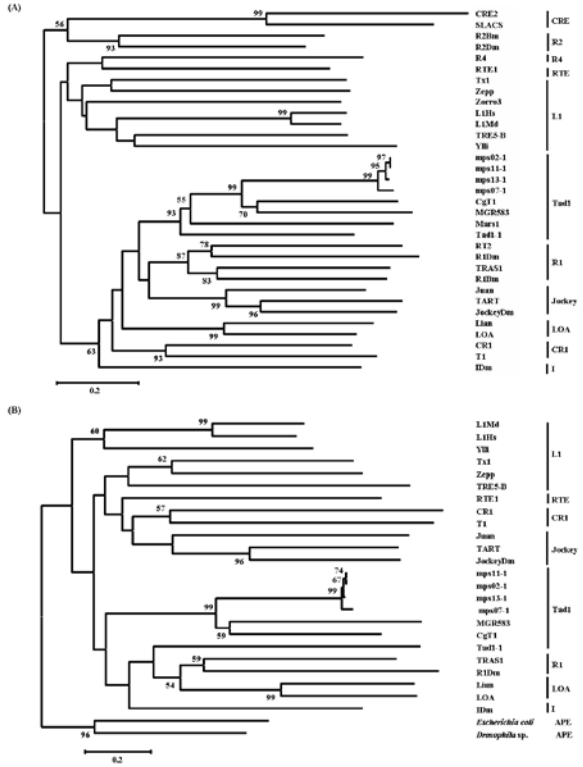

Figure 5. Phylogenetic tree of non-LTR retrotransposons from *M. pilosus* and various organisms. (A) The phylogeny of non-LTR retrotransposons based on the eleven conserved blocks of the reverse transcriptase domains defined by Malik et al. (1999) was constructed. The nucleotide sequences of MRT non-LTR retrotransposons were used in this study under the accession numbers DQ299897 to DQ299900. The tree was rooted using RT sequences of bacterial and fungal group II introns with *Lactococcus lactis* (P0A3U0), *Escherichia coli* (NP053121), *Sinorhizobium meliloti* (CAC49872) *Neurospora crassa* (NP041729), *Saccharomyces cerevisiae* (NP009310), and *Schizosaccharomyces pombe* (S78199). (B) The phylogeny of non-LTR retrotransposons based on an alignment of approximately 210 amino acid residues of apurinic/apyrimidinic endonuclease (APE) domain. Accession numbers for the apurinic/apyrimidinic endonuclease used as the outgroup following: *Drosophila* sp. (AAB19427), and *Escherichia coli* (P09030). Bootstrap values were shown in the nodes according to the 1000 replications. Only bootstrap values >50 were shown. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

R2 and R4 clades lacked an APE domain, other clades were applied to construct a phylogenetic tree that was rooted using the apurinic/apyrimidinic endonuclease of *Drosophila* sp. (AAB19427), and *Escherichia coli* (P09030). Figure 5B depicts the phylogenetic tree of the non-LTR retrotransposons according to the APE domain. It demonstrates that the close relationship between members of the Tad1 clade is consistent with the phylogeny obtained from the reverse transcriptase domain. The APE phylogeny had lower resolution than the RT phylogeny, perhaps because that APE domain was smaller and less conserved than the RT domain (Malik et al., 1999). Additionally, even though the APE and RT domains of CgT1 contained termination codons in the ORF1 and ORF2 (He et al., 1996), the phylogeny analysis of the deduced amino acid sequence of the CgT1 element also revealed that belonged to the Tad1 clade.

In order to study the distribution of MRT elements in *Monascus* species (Table 1), their genomic DNA was extracted and digested by *Eco*RI and *Bam*HI. The results of Southern hybridization showed that the MRT elements were widely distributed over *M. pilosus*, *M. ruber* and *M. barkeri* (Figure 6), and a large number of high-intensity bands were detected in these species. In contrast, the MRT element was absent in the species of *M. purpureus*, *M. kaoliang*, *M. floridanus*, *M. lunisporas*, and *M. pallens*. The intensity and diversity of hybridization patterns also shown in *M. sanguineus*, a newly found species of *Monascus*, were weaker than those in the species of *M. pilosus*, *M. ruber*, and *M. barkeri*. The weaker intensity of bands implied that the copy number of MRT in *M. sanguineus* was lower than in *M. ruber*, *M. pilosus*, and *M. barkeri*. The fingerprints of the DNA hybridizations demonstrated that the band patterns were distributed between 500 bp to 10 kb. However, the species *M. pilosus* BCRC31503 was an exception that could not detect the presence of any MRT elements (Figure 6). A phylogenetic characterization using the partial β-tubulin gene as a molecular differentiation marker was conducted to elucidate the evolutionary history of MRT elements among different species. According to the study of Park et al. (2004), the partial β-tubulin gene can be adopted to examine the phylogenetic relationship among the *Monascus* species without gaps in the alignment of partial β-tubulin genes. *Aspergillus flavus* (M38265), *Aspergillus parasiticus* (L49386), *Aspergillus fumigatus* (AY048754) and *Penicillium digitatum* (D78154) were used as outgroups of phylogenetic analysis. Interestingly, the result of the phylogenetic analysis of the partial β-tubulin gene showed that *M. pilosus* BCRC31503, *M. purpureus*, and *M. kaoliang* were placed into the same clade (Figure 7). This finding was in agreement with the results obtained by grouping species into a Southern hybridization pattern by the presence or absence of MRT elements. Therefore, *M. pilosus* BCRC31503 may have been misidentified and should be reconsidered as *M. purpureus*. Since *M. pilosus*, *M. ruber*, and *M. barkeri* could not be differentiated using the partial β-tubulin genes, three species have been suggested to be synonymous, meaning that they should be classified as a single species. DNA hybridization among

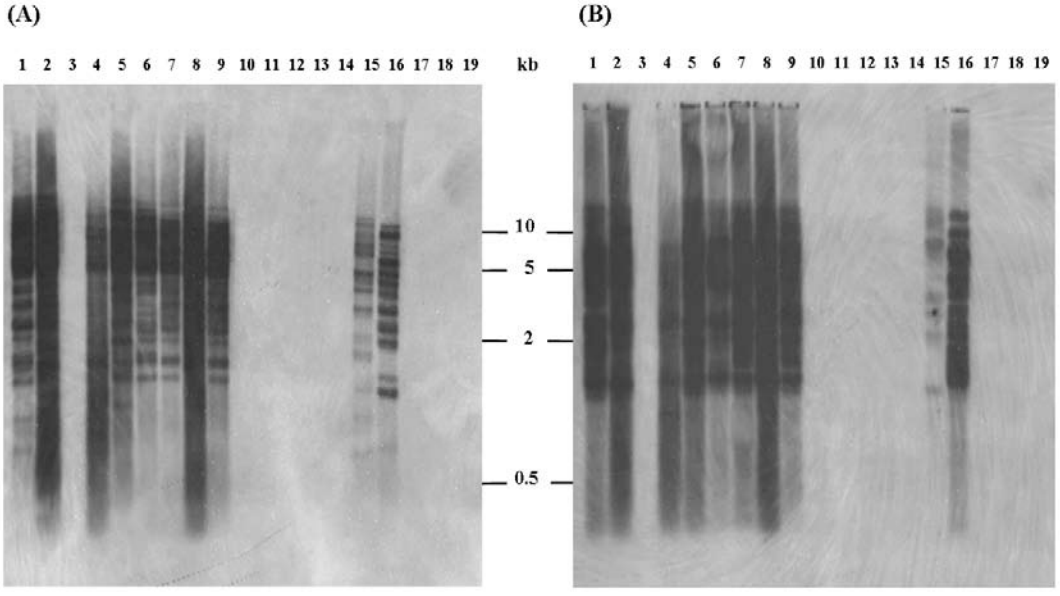

Figure 6. Southern hybridizations analyses of the MRT elements. Chromosome DNAs extracted from *Monascus* species were digested by *Eco*RI (A) and *Bam*HI (B) separated on electrophoresis gel and hybridized respectively with 422-bp probe. *Monascus* species—lane 1: *M. pilosus* BCRC38072; lane 2: *M. pilosus* BCRC31502; lane 3: *M. pilosus* BCRC31503; lane 4: *M. ruber* BCRC31533; lane 5: *M. ruber* BCRC31523; lane 6: *M. ruber* BCRC31534; lane7: *M. ruber* BCRC31535; lane 8: *M. ruber* BCRC33314; lane 9: *M. ruber* BCRC33323; lane 10: *M. purpureus* BCRC31542; lane 11: *M. purpureus* BCRC31541; lane 12: *M. purpureus* BCRC31615; lane 13: *M. purpureus* BCRC33325; lane 14: *M. kaoliang* BCRC31506; lane 15: *M. sanguineus* BCRC33446; lane 16: *M. barkeri* BCRC33309; lane 17: *M. floridanus* BCRC33310; lane 18: *M. lunisporas* BCRC33640; lane 19: *M. pallens* BCRC33641.

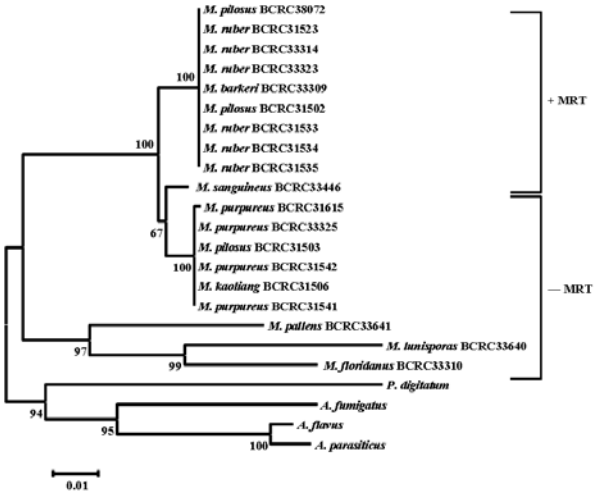

Figure 7. Phylogeny of *Monascus* species based on the partial β-tubulin gene amplified by PCR. The partial β-tubulin genes were used by the following accession numbers, DQ299886 to DQ299896, AY498587 to AY498589, AY498596, AY498598, AY498601, AY498602 and AY498604. Accession numbers for the β-tubulin genes were used as the outgroup following: *Aspergillus flavus* (M38265), *Aspergillus parasiticus* (L49386), *Aspergillus fumigatus* (AY048754), and *Penicillium digitatum* (D78154). Bootstrap values were shown in the nodes according to the 1000 replications. Only bootstrap values >50 were shown. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). + MRT: presence of MRT non-LTR retrotransposon; – MRT: absence of MRT non-LTR retrotransposon.

Monascus species (our unpublished data) also supported the identity of *M. pilosus* and *M. ruber*. The other genetic markers have been found to distinguish *Monascus* species using the D1/D2 region of the large subunit (LSU) rRNA genes (Park and Jong, 2003) and the ITS region (Park et al., 2004). These results also demonstrate that *M. ruber* and *M. pilosus* could not be differentiated. Moreover, the MRT non-LTR retrotransposons were widely distributed over *M. pilosus* and *M. ruber*. Hence, the two species were determined to be synonymous. In the taxonomy of *Monascus* (Hawksworth and Pitt, 1983), *M. barkeri* goes by the name of *M. ruber.* The results herein were consistent with their theory. Furthermore, *M. sanguineus* was placed on a branch that was separate from *M. pilosus*, *M. ruber*, and *M. barkeri,* while the difference of phylogenetic distance corresponded to the results of Southern hybridization with varying intensities of the bands of *M. pilosus*, *M. ruber*, and *M. barkeri*.

According to the phylogenetic subgroups established with the partial β-tubulin gene, the species were grouped by the presence or absence of MRT elements in the hybridization pattern (Figure 7). Since the MRT element was only detected in *M. pilosus*, *M. ruber*, *M. barkeri* and *M. sanguineus*, the MRT element was suggested to have been present in the ancestors of the *Monascus* species, and absent from most other species or to have diverged from *M. pilosus*. This phenomenon may have been caused by the genetic drift that is itself associated with small effective populations, which are responsible for an increase in the numbers of copies of elements in some species and a decline in others (Cizeron et al., 1998; Le Rouzic and Capy, 2005). However, horizontal transfer (HT) has been observed with the variable distribution of retrotransposons between different classes, phyla, or kingdoms. The horizontal transfer is believed to be present in members of the RTE clade (Župunski et al., 2001). Bov-B LINEs of the RTE clade exhibit a very low divergence between Ruminantia and Squamata, strongly indicating horizontal transfer. In this study, the result with the various species of *Monascus* revealed that MRT non-LTR retrotransposon was restricted to *M. sanguineus*, *M. pilosus*, *M. ruber*, and *M. barkeri*, the last three species of which were synonymous. These observations suggested that the MRT element was introduced into the ancestor of the *Monascus* species, and then diverged into *M. sanguineus*, *M. pilosus*, *M. ruber*, and *M. barkeri*. Although the evidence for horizontal transfer events is present in non-LTR retrotransposons of RTE clade, different taxonomic groups must be sampled to determine whether the horizontal transfer events also occurred in the Tad1 clade.

Transposable elements may be regarded as genetic components that evolve in an ecological community of the host genome (Brookfield, 1995). During evolution, the spread and distribution of the elements depend on not only their ability to amplify, but also the complex interactions between various families of elements, and between the elements and the hosts (Tu et al., 1998). Analyses of endogenous retrotransposable elements in *Monascus* species can be potentially used as markers in genetic mapping and in population studies. MRT was consistent with CgT1, which can distinguish between two groups based on presence or absence of non-LTR retrotransposon (He et al., 1996). Practically, our findings indicate that MRT has great potential in strain characterization and in studies of population structure and evolution in *Monascus* spp.

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MRT，紅麴菌中新穎的 non-LTR 逆轉位子之特徵分析

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本研究於紅麴菌中發現新的 non-LTR 逆轉位子，並命名為 MRT。其序列長度約為 5.5 kb，包含有兩個開放讀架 (ORFs)。此兩個開放讀架與 gag 及 *pol* 基因相似，並且於 *pol* 相似基因的 3′ 端具有很多的腺嘌呤。第一個開放讀架為 517 個氨基酸的蛋白質，包含有多量半胱氨酸的鋅指區域。第二個開放讀架為 1181 個氨基酸的蛋白質，包含有脫嘌呤/脫嘧啶核酸內切酶、反轉錄酶、核糖核酸酶 H 及 CCHC 區域。根據 MRT non-LTR 逆轉位子的氨基酸序列所建立的親緣關係，可將其歸屬於 Tad1 群叢。南方雜交法則進一步發現只有四種紅麴菌 *M. pilosus*，*M. ruber*，*M. sanguineus* 與 *M. barkeri* 具有 MRT non-LTR 逆轉位子。除此之外，利用 β-tubulin 基因所建立的親緣關係則可歸群出 MRT non-LTR 逆轉位子之存在與否。

關鍵詞：紅麴菌；細菌人工染色體基因庫；Non-LTR 逆轉位子；親緣分析。

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Basidiomatal formation in *Antrodia cinnamomea* from the perspective of gene expression

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ABSTRACT. In order to understand the phenomenon of morphological differentiation in medicinal fungus *Antrodia cinnamomea*, two complementary DNA (cDNA) libraries were constructed from the liquid-cultured mycelia (AM) and natural basidiomes (AT) produced from the infested wood. Using single-passed sequencing of cDNA clones, 821 and 993 high-quality expressed sequence tags (ESTs) were generated from the liquid-cultured mycelial and wild basidiomatal cDNA libraries, respectively. The results from BLASTX search revealed that only 32.5% to 33.7% of ESTs showed significant similarity to protein sequences in public databases (*E values* $\leq 10^{-10}$). The cDNAs encoding genes related to metabolism were found to be most abundant, followed by genes involved in protein fate and protein synthesis in each category. Genes related to the "Cell fate, cell cycle, and DNA processing" category showed the greatest difference between the liquid-cultured mycelial and wild basidiomatal cDNA libraries, followed by genes involved in metabolism. The results from this study have provided valuable sequence information, which may lead to improved production of basidiomes of *A. cinnamomea* and regulation of metabolites in the future.

Keywords: *Antrodia cinnamomea*; Expressed sequence tags; Medicinal fungus; Morphological differentiation.

INTRODUCTION

Antrodia cinnamomea is a resupinate to effused-reflexed basidiomycete with porous hymenium (Chang and Chou, 2004). The basidiomes of *Antrodia cinnamomea* (Chinese name, chang-chih) are well-known in Taiwan as a highly-prized folk medicine. This medicine has been used to treat drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer (Tsai and Liaw, 1982). *Antrodia cinnamomea* grows in the inner cavity of a decayed tree trunk of *Cinnamomum kanehirai* Hay (Lauraceae), and its large-scale cultivation by artificial means has been unsuccessful so far. Also, due to overexploitation, *C. kanehirai,* an endemic species of evergreen tree, is becoming rare. Hence, it is being conserved by the Taiwan government. The basidiomes of *A. cinnamomea* are becoming a scarce and high priced folk medicine in Taiwan, due to the species' host specificity and rarity in nature and to the failure of artificial cultivation (Chang and Chou, 2004).

Investigation of the differential mechanism of *A. cinnamomea* would be beneficial for the development of a new technique for the regulation of porous-hymenium basidomatal formation. However, no molecular characterization of the fruiting bodies from *A. cinnamomea* has been reported so far. Expressed sequence tag (EST) analysis has been successfully applied to the study of gene expression in animals, plants, and fungi subjected to various stresses or in different stages of development, as is evident from the increased number of reports on ESTs (Adams et al., 1991; Cooke et al., 1996; Yamamoto and Sasaki, 1997; Lee et al., 2002). In addition to the identification of pathogenicity of fungi, fundamental aspects of fungal development have also been examined using the EST-guided approach (Lee et al., 2002; Li et al., 2004). In edible mushrooms, the process of fruiting body formation is very important from a scientific and commercial point of view, and many scientists have therefore studied specifically expressed genes in the fruiting bodies of *Lentinula edodes*, *Schizophyllum commune*, and other basidiomycetes (Wessels, 1992; Kondoh and Shishido, 1995; Fernandez Espinor and Labarere, 1997). The EST-based approach investigated in the present study may lead to a fundamental understanding of basidiomatal formation in *A. cinnamomea*.

In the present study, two complementary DNA (cDNA) libraries from liquid-cultured mycelia (AM) and natural basidiomes (AT) were established in order to characterize the gene expression during porous-hymenium basidiomatal formation in *A. cinnamomea.* The potential functions of the EST clones were determined by comparing them with the sequences of other fungi and by obtaining specific genes for *A. cinnamomea*. The results obtained in the study may lead to the identification of genes and analysis

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of gene expression during basidiomatal formation in *A. cinnamomea*.

MATERIALS AND METHODS

Strains and culture conditions

Antrodia cinnamomea strain TFRIB 479 obtained from the rotten wood of *C. kanehirai* at Dawu, Taitung was identified in the study. The cultures were maintained as reported earlier (Chang and Chou, 2004). The natural basidiomes were obtained from the infested wood. Liquid-cultured mycelia (AM) and natural basidiomes (AT) were frozen in liquid nitrogen and stored at -80°C until use.

cDNA library construction

Total RNA for cDNA library construction was prepared following the method described by Chang et al. (1993) and modified by Chen et al. (2004). Poly $(A)^+$ RNA was then purified with Oligotex mRNA Mini Kit (Qiagen). The single strand cDNA was synthesized by SuperScript™ II Reverse Transcrpitase (Invitrogen) while the double strand cDNA was amplified using PCR Plus Master Mix Kit (GeneMark) (Sized-fractionation > 100 bp). Purification of the cDNA was performed using a DNA Clean/ Extraction Kit (GeneMark). The resulting cDNA was cloned into the pGEM-T Easy vector system (Promega) following the protocol for an overnight ligation. The ligation mixture was transformed into High Efficiency DH5α Competent cells (Hopegen) and then plated onto LB-ampicillin plates containing IPTG and X-gal.

DNA sequencing

After a cDNA library was plated onto LB media plates, the transformed white colonies were transferred into test tubes containing 5 mL LB-amp medium. Plasmid DNA was isolated from overnight cultures by the Plasmid Miniprep Purification Kit (GeneMark). Sequencing was performed with an ABI 377 automatic sequencer (Perkin Elmer) using a T7 sequencing primer.

Sequence analysis

Nucleotide sequences of less than 100 bp were excluded, and the remaining ones were analyzed. The leading vector and poor quality sequences were removed manually or via ChromasPro software (Technelysium Pty Ltd). Individual EST was assembled into contigs using the ContigExpress program of Vector NTI Suite 8 (InforMax, Inc.) with parameters optimized for ESTs rather than for genomic clones. The cDNA sequences were compared to non-redundant (nr) protein sequence databases at the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997). *E*-value results obtained from the BLASTX were categorized into $\leq 10^{-10}$, which represented significant homology, and $> 10^{-10}$, which implied no hit. The former results were then grouped according to putative function. A unique set with significant matches was annotated on the basis of their most similar functions following the general rules from the Functional Catalogue by the Munich Information Center for Protein Sequences (MIPS) (www.mips.biochem.mpg.de/proj/yeast/catalogues/funcat/index.html) and with the aid of the Gene Ontology Consortium (www.geneontology.org).

RESULTS

Sequencing and assembling of ESTs sequences

Total RNAs were isolated from liquid-cultured mycelial (AM) and natural basidiomes (AT) of *A. cinnamomea* for the construction of cDNA libraries. Initially, a total number of 1823 clones were randomly picked and subjected to 5′ end single-pass sequencing from the libraries. The leading vector, tailing of the sequence, and poor-quality sequences were excluded. After excluding fragments shorter than 150 nucleotides, 1,809 high-quality ESTs were submitted to dbEST (GeneBank accession nos. DV629596-DV631404). Most of the ESTs were 300-700 bp in length with an average of 538 bp. Among 817 clones from the mycelia library, 537 clones assembled into 104 contigs while 180 ESTs were unigenes. In the basidiomatal library, with its 992 clones, 742 clones assembled into 162 contigs while 250 ESTs remained as unigenes. As a result of the contig analysis, 604 different genes (unigene) could be obtained out of 1,809 analyzed cDNA clones. Among the 604 unigenes, 512 (84.8%) expressed differently in the mycelial and basidiomatal libraries.

Characterization of the cDNA library and the ESTs sequences

In this study, the putative function of the cDNAs, which is based on BLASTX's result, was assigned to those with E-values less or equal to 10^{-10} (Sterky et al., 1998). In AM and AT libraries, only 267 (32.7%) and 325 (32.8%) ESTs, respectively, showed similarity when these were compared with the public sequence databases. A total of 1809 ESTs matched with 592 distinct sequences in the non-redundant protein database and were assigned to 259 functional genes from both the libraries. The developmental stage specificity and redundancy of these 259 unigenes were analyzed to characterize the developmental gene expression from the EST data (Table 1). The results indicated that lanosterol 14-alpha-demethylase, and nucleolar protein nhp2 occurred more frequently in the basidiomatal library than in the mycelial library. On the other hand, peroxiredoxins, ATP-binding cassette transporter ABC1, phytase, and H/ACA snoRNP component occurred more redundantly in the mycelial library. The potential roles of these in basidiomatal formation in *A. cinnamomea* will be explored in the future.

Cellular roles of ESTs in *A. cinnamomea*

To obtain an overview of how *A. cinnamomea* functions at the cellular level, the transcripts identified with putative functions were assigned nine putative cellular roles based on those assigned them by the MIPS FunCat scheme

Table 1. Annotation of ESTs from liquid-cultured mycelia (AM) and wild basidiomes (AT) of *A. cinnamomea.*

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
I. Cell fate, cell cycle and DNA processing				
Anaphase promoting complex subunit 10	*Oryza sativa*	AAU10698	1	
ATP-dependent DNA helicase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43036		1
Brefeldin a resistance protein	*Schizosaccharomyces pombe*	CAB44765	1	4
Bud site selection-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43110		1
Cell cycle regulatory protein	*Ustilago maydis*	AAN10186		1
DNA topoisomerase I	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45618		1
Fibrillarin (NOP1)	*Neurospora crassa*	CAC18188	1	
Flap endonuclease-1	*Coprinopsis cinerea*	BAD14303		1
Microtubule end-binding protein EB1	*Coturnix japonica*	AAU12573		1
Opa-interacting protein OIP2	*Cryptococcus neoformans* var. *neoformans* B-3501A	EAL23044	1	
Prohibitin PHB1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40684	1	
RAB18	*Mus musculus*	BAC32402	1	1
Ras1p	*Suillus bovinus*	AAF65465		1
Topoisomerase I	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45618		1
Tubby-like protein 2	*Arabidopsis thaliana*	AAK98801		1
II. Cell rescue, defense, and virulence				
Catalase	*Campylobacter jejuni*	CAA59444	1	
Cytochrome P450, alkane-inducible	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43969	1	1
Cytochrome P450 monooxygenase	*Coriolus versicolor*	BAB59027	1	2
Cytochrome P450 oxidoreductase	*Coriolus versicolor*	BAB83588		1
Glutathione reductase	*Onchocerca volvulus*	CAA72516		1
Heat shock protein HSS1	*Puccinia graminis* f. sp. *tritici*	AAB93665	1	
Hob1p	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40855		1
Lanosterol 14-alpha-demethylase	*Phanerochaete chrysosporium*	AAU01160	3	17
Methylenetetrahydrofolate reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41236		5
O-methylsterigmatocystin oxidoreductase	*Neurospora crassa*	CAE76231	1	1
Osmotic stress-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41050	1	3
Peroxiredoxin Q	*Triticum aestivum*	AAV66923	1	
Peroxiredoxins	*Phanerochaete chrysosporium*	AAV53576	19	9
Protoplast regeneration and killer toxin resistance gene	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44305		1
Snodprot-FS	*Gibberella pulicaris*	AAV83793	8	3
WD-repeat protein-like	*Arabidopsis thaliana*	BAB09052		1
III. Cellular transport, transport facilitation and transport routes				
Allantoate permease	*Neurospora crassa*	CAE81935		1
ATPase of the ABC class	*Thermoanaerobacter tengcongensis*	ZP_00178674		1
ATP-binding cassette transporter ABC1	*Venturia inaequalis*	AAK62810	11	1
ATPase of the CDC48/PAS1/SEC18 (AAA) family	*Saccharomyces cerevisiae*	NP_013501		1
ATP synthase gamma chain	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43492		2

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Carnitine/acyl carnitine carrier	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41054	15	12
ER to Golgi transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC211	AAW44532	3	3
GTPase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46779	1	
Intracellular transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41812		1
Late endosome to vacuole transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42196	2	
Membrane zinc transporter	*Aspergillus fumigatus*	AAT11930		1
Nucleoporin	*Ustilago maydis*	CAG26761	1	
Phosphate transporter	*Pholiota nameko*	BAB43910		2
Protein-vacuolar targeting-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40999	1	
P-type cation-transporting ATPase	*Blastocladiella emersonii*	CAA04499		1
Sugar transporter	*Neurospora crassa*	CAB88582		1
Synaptobrevin (v-SNARE) homolog Bos1	*Schizosaccharomyces pombe*	CAB77004	1	1
Transmembrane transporter Liz1p	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43513		1
Tricarboxylate transport protein	*Schizosaccharomyces pombe*	CAB10116	2	
Triose phosphate translocator	*Cryptococcus neoformans* var. *neoformans*	AAR82906		1
Vacuolar ATP synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45529	1	
V-ATPase subunit A	*Fundulus heteroclitus*	BAB62103		1
Vesicle-mediated transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW47173	1	
Urea transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43008		1
IV. Cellular communication/signal transduction				
Calcium ion binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44613	1	1
Guanine nucleotide-binding protein alpha-4 subunit	*Ustilago maydis* 521	EAK86634		1
Sensory histidine kinase	*Mesorhizobium loti*	NP_103743	2	3
Serine kinase (hPAK65)	*Homo sapiens*	AAA75468		1
Rho GTPase activator	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44732		1
V. Metabolism				
Acetamidase	*Schizosaccharomyces pombe*	AL023592		1
Acid sphingomyelinase	*Neurospora crassa*	CAD70921		1
Acyl-CoA transferases/carnitine dehydratase	*Ralstonia metallidurans* CH34	ZP_00273947	1	
Agmatinase precursor	*Schizosaccharomyces pombe*	NP_593990	1	
Aldo-keto reductase	*Cryptococcus neoformans* var. *neoformans* B-3501A	AAW46629	2	3
Allantoicase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43136		2
Amidophosphoribosyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42423		1
Aryl-alcohol dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46369	1	1
Beta-1,3-mannanase	*Paecilomyces lilacinus*	BAD06516	1	
Chorismate synthase	*Schizosaccharomyces pombe*	T41268		3
Cycle propane fatty acid synthase	*Coprinopsis cinerea*	AAL73238		1
Dihydrokaempferol 4-reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43891		4

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Dolichyl-phosphate beta-glucosyltransferase	*Schizosaccharomyces pombe*	NP_596707	2	1
Exo-1,3-beta-glucanase	*Agaricus bisporus*	CAA63536		1
Endo-1,4-β-xylanase A	*Phanerochaete chrysosporium*	AAG44993	1	
Flavin-containing monooxygenase	*Aspergillus fumigatus*	CAE47890		2
Glucosidase 1	*Caenorhabditis elegans*	NP_502053	1	
Glutamate synthase (NADH)	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46054		1
Glycine dehydrogenase-like protein	*Pleurotus djamor*	AAS46734		2
Homoserine O-acetyltransferase	*Saccharomyces pastorianus*	Q06736	1	
Hydrolases	*Pseudomonas syringae* pv. *syringae* B728a	ZP_00126253		1
3-hydroxyanthranilate 3,4-dioxygenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41998	1	1
3-hydroxyisobutyrate dehydrogenase	*Novosphingobium aromaticivorans* DSM 12444	ZP_00305232		1
Hydroxymethylglutaryl-CoA lyase	*Pseudomonas mevalonii*	AAA25896	1	
IMP dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40949	1	
Laminarinase	*Phanerochaete chrysosporium*	BAC67687	1	
Lysosomal alpha-N-acetyl glucosaminidase	*Gallus gallus*	XP_418147		1
Mannosyl-oligosaccharide glucosidase	*Schizosaccharomyces pombe*	NP_594106		1
Nicotinate-nucleotide diphosphorylase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42323		1
Oxidoreductase	*Agrobacterium tumefaciens* str. C58	AAL43649	9	9
Phenol 2-monooxygenase	*Neurospora crassa*	CAF06102	1	
Phospho-2-dehydro-3-deoxyheptonate aldolase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46670	1	1
Phosphatidylinositol phosphate phosphatase	*Schizosaccharomyces pombe*	NP_596431		1
Phosphoglycerate mutase GPM2	*Mycobacterium tuberculosis* H37Rv	CAE55568	1	3
Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase	*Saccharomyces cerevisiae*	NP_012244	1	2
Phosphotyrosyl phosphatase activator	*Oryza sativa*	BAD37240		2
Phytase	*Trametes pubescens*	CAC48234	46	4
Polygalacturonase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42245		1
Polyketide biosynthesis associated protein	*Agrobacterium tumefaciens* str. C58	NP_532458	2	
Prenyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42863		1
Riboflavin aldehyde-forming enzyme	*Lentinula edodes*	BAD11818		1
Short-chain alcohol dehydrogenases	*Ralstonia metallidurans* CH34	ZP_00271907	1	3
Urate oxidase	*Aspergillus flavus*	CAA43896	1	1
UTP-glucose-1-phosphate uridylyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42292		1
Zinc-binding dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42578	1	
VI. Protein fate and synthesis				
Alanine-tRNA ligase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44283	1	
Arginyl tRNA synthetase	*Candida albicans* SC5314	EAK99505		1
Aspartyl-tRNA synthetase	*Saccharomyces cerevisiae*	NP_015221		1
Aspartic proteinase precursor	*Botryotinia fuckeliana*	AAG43236	6	6
ATP-dependent protease proteolytic subunit ClpP	*Arabidopsis thaliana*	BAC43126	1	

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Calmodulin-dependent protein kinase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45617		1
Carboxypeptidase C	*Saccharomyces cerevisiae*	S46008		1
Carboxypeptidase Y	*Trichophyton rubrum*	AAS76668		1
Chaperone regulator	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42057		2
Dipeptidyl aminopeptidases/ acylaminoacyl-peptidases	*Microbulbifer degradans* 2-40	ZP_00315190	1	
Elongation factor 1-gamma	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40932	3	7
Endopeptidasee	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43542	1	4
Eukaryotic translation initiation factor SUI1 family protein	*Arabidopsis thaliana*	NP_177291	1	
FKBP-type peptidyl-prolyl cis-trans isomerases 1	*Rubrivivax gelatinosus* PM1	ZP_00245218		1
Glutaminyl-trna synthetase	*Schizosaccharomyces pombe*	NP_596745		1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	*Schizophyllum commune*	P32638		1
Insulin degrading enzyme	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46588	2	2
Leucine aminopeptidase	*Coprinopsis cinerea*	BAB87833	1	
Nucleolar peptidyl-prolyl cis-trans isomerase (PPIase)	*Saccharomyces cerevisiae*	NP_013637	2	3
Polyubiquitin 6	*Gracilaria verrucosa*	S53719	1	
20S proteasome alpha-type subunit	*Saccharomyces cerevisiae*	NP_014604		3
Ribosomal large subunit assembly and maintenance-related protein	*Cryptococcus neoformans* var. *neoformans* B-3501A	AAW41360		2
Ribosomal protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42014		3
40S ribosomal protein S8	*Schizophyllum commune*	AAC69196	5	6
Ribosomal protein l15 homologue	*Aspergillus fumigatus*	CAE47918		1
50S ribosomal protein L22	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43715	1	
60s ribosomal protein l5-b	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42426		1
60s ribosomal protein l27	*Cryptococcus neoformans* var. *neoformans* JEC21	EAK83063	1	
LSU ribosomal protein L1P	*Thermus thermophilus* HB27	YP_005708	1	
RING/C3HC4/PHD zinc finger-like protein	*Cucumis melo*	AAO45753	1	
t-complex protein 1, beta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40957	1	1
t-complex protein 1, delta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44504	6	6
t-complex protein 1, theta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42275		2
Translation initiation factor	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43820	2	1
Ubiquitin-like protein	*Plasmodium yoelii*	EAA18158		1
Ubiquitin-like protein 5	*Drosophila melanogaster*	Q9V998		1
Ubiquitin carboxyl-terminal hydrolase	*Schizophyllum commune*	AF077976	1	
VII. Transcription				
Cofilin	*Pichia angusta*	AAK85273	1	1
DNA-directed RNA polymerase I	*Schizosaccharomyces pombe*	NP_594382		1
Glia maturation factor beta	*Cyprinus carpio*	BAA95482	1	1
H/ACA snoRNP component	*Candida albicans* SC5314	EAK98515	16	
Hexamer-binding protein HEXBP	*Leishmania major*	A47156	1	1

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Leucine zipper protein	*Oryza sativa*	BAD38167	1	1
Mammalian swi/snf complex 60 kda subunit homolog	*Schizosaccharomyces pombe*	T50184		2
mRNA processing-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43157		2
nhp2	*Schizosaccharomyces pombe*	AL158056	3	7
Nucleus protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40929	1	3
Reptin	*Apis mellifera*	XP_395860	1	
Pre-mRNA splicing factor RNA helicase PRP28	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41184	1	6
TATA box binding protein (TBP)	*Homo sapiens*	AB010067	2	
tRNA dihydrouridine synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45443	1	
trp-asp repeats containing protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46166	1	
VIII. Energy				
Cytochrome-b5 reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45007	1	
Cytochrome c1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44407	1	2
Glyceraldehyde 3-phosphate dehydrogenase	*Arabidopsis thaliana*	NP_172801	2	
NADP-dependent oxidoreductases	*Pseudomonas syringae* pv. *syringae* B728a	ZP_00124416	3	8

[a] Redundancy number of the sequences in liquid-cultured mycelia ESTs.
[b] Redundancy number of the sequences in wild-type basidiomatal ESTs.

(Figure 1). A major portion (79 ESTs) in the AM library represented transcription, which is in the metabolism category, corresponding to 9.6% of all ESTs. The next one was involved in protein fate and protein synthesis. Except for the "unknown function" category, ESTs related to metabolism were found to be the most abundant, followed by the category "protein fate and protein synthesis" categories in the AT library. In these categories, 40S ribosomal protein, S8, and the delta subunit of t-complex protein 1 (tcp-1-delta) were found in both libraries. In the metabolism category, there were 23 and 33 unigenes in the AM and AT libraries, respectively (Table 1). However, in the differential expression during the mycelial and basidiomatal stages, the cell fate, cell cycle, and DNA processing category was found to be the most divergent, followed by those involved in cellular transport, transport facilitation, and transport routes (Table 2). There were 15 kinds of ESTs in the cell fate, cell cycle, and DNA processing category while only two unigene ESTs occurred in both stages. The difference between the mycelial and basidiomatal stages was 86.7%.

DISCUSSION

From a scientific and economic point of view, differentiation of sexual fruiting bodies from mycelia at vegetative is an interesting phenomenon in homobasidiomycetes, especially while *A. cinnamomea* possesses a porous-hymenium basidiome without stipe. Although several mycologists have made efforts to study gene expression during the formation of fruit bodies (Lee et al., 2002; Sunagawa and Magae, 2005; Yamada et al., 2006), porous-hymenium basidiomatal formation and biosynthesis of active components at the molecular level have not yet been explained.

High-throughout single-run partial sequencing, generation, and analysis of ESTs have proven to be a rapid and efficient approach to obtaining information on mRNA expression (Adams et al., 1991). To study the gene regulation during porous-hymenium basidiomatal formation in *A. cinnamomea*, cDNA clones were randomly sequenced at different stages of the culture of *A. cinnamomea*. In total, 1,809 ESTs matched with 592 distinct sequences in the non-redundant protein database and assigned to 259 functional genes from all libraries. Although the number of ESTs in this study is low, one may obtain some useful information from the genes encoded by the *A. cinnamomea* genome. The cDNA libraries and the accompanying database are valuable resources for researchers hoping to understand the genetic control of basidiomatal formation and secondary metabolism in *A. cinnamomea*.

It is customary in genome annotation to establish a cutoff for "statistically significant" database hits. By correlating an observed alignment score with the expected distribution, we can quantify statistical significance in

Table 2. Divergency analysis of unigenes in each category from mycelia and basidiomatal stages.

Category	I	II	III	IV	V	VI	VII	VIII
Total[a]	15	16	24	5	45	37	15	4
Both[b]	2	7	4	2	10	9	7	2
Divergency (%)[c]	86.7	56.3	83.3	60	77.8	75.7	53.3	50

(Mention all the categories in Table 1)
[a]Total number of independent unigenes derived from two libraries.
[b]Number of independent unigenes appearing in both stages.
[c]Percentage of unigenes appearing only in mycelia or basidiomatal stage.

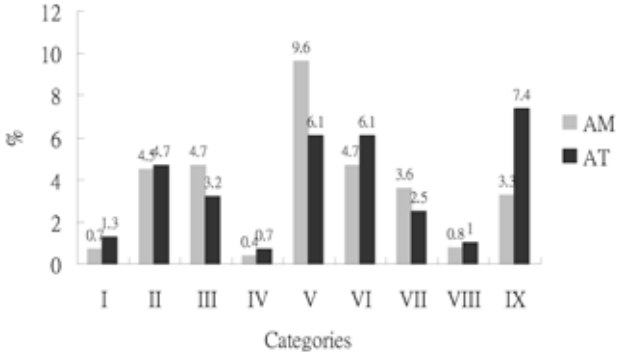

Figure 1. Functional categories of ESTs from different stages of *A. cinnamomea*. I: Cell fate, cell cycle and DNA processing; II: Cell rescue, defense and virulence; III: Cellular transport, transport facilitation and transport routes; IV: Cellular communication/signal transduction; V: Metabolism; VI: Protein fate and protein synthesis; VII: Transcription; VIII: Energy; IX: Unknown. AM: liquid-cultured mycelia; AT: wild basidiomes of *A. cinnamomea*. The number listed on top of the bar indicates the percentage of EST.

the form of an *E* value. It can be expressed in terms of the false-positive expectation value for the BLAST searches and is set routinely at values such as $E = 0.001$ or $E = 10^{-5}$. The problem with this approach is that the distribution of similarity scores for evolutionarily and functionally relevant sequence alignments is very broad, and a considerable fraction of them may fail the *E*-value cutoff, resulting in undetected relationships and missed opportunities for functional prediction (false negatives) (Galperin and Koonin, 2001). In the present study, to minimize the false-positive rate, it was critical to set the *E* values at less or equal to 10^{-10} while filtering low-complexity sequences. BLASTX analysis revealed that only 22.1% and 33.7% of ESTs showed significant similarity to published protein sequences in the AM and AT libraries, respectively. The absence of these sequences from public databases might indicate specific roles for these proteins in *A. cinnamomea*. Similarly, with E values less than or equal to 10^{-5} as the cutting point, only 39.5% to 40.8% ESTs showed no significant similarity to any known proteins in the existing databases.

In redundant ESTs analysis, most ESTs occurred one to four times while only few occurred several times in the cDNA libraries. In this study, 16 unigenes occurred more than five times in the libraries. There were 46 instances when cDNAs were identified as phytase among these unigenes. These unigenes showed high expression in the liquid-cultured mycelial library compared to the basidiomatal library. BLASTX analysis showed that these ESTs of phytases had 62~67% identity with *Trametes pubescens* phytase. Moreover, cDNA was identified as lanosterol 14-alpha-demethylase 17 times in the basidiomatal library, but only thrice in the liquid-cultured mycelia library. The lanosterol 14-alpha-demethylase belongs to the CYP51 family of the cytochrome P450 superfamily, and it is notable that lanosterol 14-alpha-demethylase is the only member of the cytochrome P450 family. Also, this is true in all biological kingdoms (Revankar et al., 2004). In addition, the ESTs identified as putative methylenetetrahydrofolate reductase were found five times in the basidiomatal libraries only. This suggests the possibility that this gene was expressed specifically at the basidiomatal stage and may be related to basidiomatal formation.

According to the number of unigenes in each category, cDNAs encoding for the genes related to "metabolism" were found to be the most abundant. Among the 45 unigenes isolated from these two libraries, 22 unigenes (48.9%) were expressed only in the basidiomatal library. In another study, riboflavin aldehyde-forming enzyme specifically and abundantly was expressed in the mature basidiomes of *Lentinula edodes* (Hirano et al., 2004). Thus, the promoter region of the riboflavin aldehyde-forming enzyme may be the emitter of the expressing signal of the vector for gene manipulation in a mature fruiting body. In our study, the putative riboflavin aldehyde-forming enzyme appeared only in the wild-type *A. cinnamomea.* This result indicates that the promoter of the aldehyde-forming enzyme may be the vector regulator when the mature basidiome is being manipulated.

Regardless of the cultural conditions or different expression patterns during the mycelial and basidiomatal stages, the group of ESTs tentatively assigned to the cell fate, cell cycle, and DNA processing-related category varied the most. Among the 27 unigenes isolated from these two libraries, only 18 unigenes (66.7%) were expressed in the basidiomatal library. On comparing our results with the sequence expression of *P. ostreatus* in the same category, the putative Ras 1p gene also appeared only at the fruiting body stage (Lee et al., 2002).

The EST database established in this study provides the sequence information of genes expressed during basidomatal formation at the whole-gene level. Combining the analysis with metabolomics, proteomics, microarray analysis, transformation system, and bioinformatics, we hope to explore the genes and to understand how basidiomatal formation can be regulated in the future.

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樟芝子實體形成過程基因表現之解析

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為了釐清樟芝不同型態之分化過程，我們分別自液態培養的樟芝菌絲體及野生之樟芝子實體建構二個互補股 DNA 的基因資料庫。經由單向之解序結果，分別穫得了 821 及 993 個高品質之表現序列標籤，續由 BLASTX 的分析結果顯示，與現有已知序列的資料庫之相似度僅有 32.5%-33.7% (以期望值 $\leqq 10^{-10}$ 為界)。不論是菌絲體或是子實體的資料庫，其中，屬於代謝相關的基因數目最多，接著是屬於與蛋白質的表現時期和蛋白質生合成有關之基因數目次多，而兩組互補股 DNA 的基因資料庫之間則以「細胞時期、細胞週期及 DNA 生成」一組所表現的基因差異最大，接著是與代謝有關之基因數目次多。藉由本研究所建構的序列資訊，將有助於克服樟芝所面臨難以促進子實體形成之難題，並可提供未來調控代謝物研究時之重要參考訊息。

關鍵詞：樟芝；基因表現標籤；藥用真菌；型態分化。