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A simple and effective method for total RNA isolation of appressoria in *Magnaporthe oryzae**

Tong-bao LIU[1,2], Jian-ping LU[2], Xiao-hong LIU[1], Hang MIN[2], Fu-cheng LIN[†‡1]

([1]*State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University, Hangzhou 310029, China*)

([2]*College of Life Sciences, Zhejiang University, Hangzhou 310058, China*)

[†]E-mail: fuchenglin@zju.edu.cn

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Abstract: Appressorium formation is an important event in establishing a successful interaction between the rice blast fungus, *Magnaporthe oryzae*, and its host plant, rice. An understanding of molecular events occurring in appressorium differentiation will give new strategies to control rice blast. A quick and reliable method to extract total RNA from appressorium is essential for studying gene expression during appressorium formation and its mechanism. We found that duplicate film is an efficient substratum for appressorium formation, even when inoculated with high density conidia. When inoculated with conidia at 1×10^6 ml^{-1}, the percentages of conidium germination and appressorium formation were (97.98±0.67)% and (97.88±0.45)%, respectively. We applied Trizol before appressorium collection for total RNA isolation, and as much as 113.6 μg total RNA was isolated from the mature appressoria at 24 h after inoculation. Functional analysis of two genes, *MNH6* and *MgATG1*, isolated from the cDNA subtractive library, revealed that the quantity of RNA was good enough to construct a cDNA (complementary DNA) library or a cDNA subtractive library. This method may be also applicable for the appressorium RNA isolation of other pathogenic fungi in which conidia differentiate into appressoria in the early stages of host infection.

Key words: Appressorium, *Magnaporthe oryzae*, RNA isolation
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INTRODUCTION

Magnaporthe oryzae is the causal organism of rice blast disease, one of the most destructive diseases of rice (*Oryza sativa*) throughout the world (Ou, 1985). This fungus is the first plant pathogenic fungus to be fully sequenced (Dean *et al*., 2005), providing a detailed insight into its genome. The infection process begins when *M. oryzae* airborne conidia land on the rice leaf surface. After attached by spore tip mucilage (Hamer *et al*., 1988), a conidium germinates and forms a highly melanized, dome-shaped infection cell called an appressorium (Bourett and Howard, 1990). A penetration peg emerges from an appressorium and penetrates the plant cuticle with huge turgor pressure (8 MPa) inside the appressorium (Howard *et al*., 1991). Once entering the plant cells, the invaded hyphae swell and fill the host epidermal cells within 24 h. Under favorable conditions, *M. oryzae* conidium germinates within 1 h after incubation, followed by swellings evolving on the tips of germ tubes within 2 to 4 h. At approximately 6 to 8 h after incubation, melanin-pigmented, mature appressoria form.

The cues, which trigger appressorium formation in this fungus, have been intensively investigated during the last decade, but are still not well known. However, appressorium formation in *M. oryzae* in vitro was shown to be at least partially due to contact with a hard surface (Xiao *et al*., 1994) and the hydrophobicity of the substratum (Jelitto *et al*., 1994; Lee and Dean, 1994), as well as chemical components of the plant surface (Gilbert *et al*., 1996) and the ab-

‡ Corresponding author

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sence of exogenous nutrients (Dean, 1997).

A number of genes involved in the induction and function of appressoria have been identified by mutant analysis (Talbot *et al*., 1993; Sweigard *et al*., 1998; Balhadère *et al*., 1999; DeZwaan *et al*., 1999; Ahn *et al*., 2004; Gupta and Chattoo, 2007) and expression pattern analysis (Lee and Dean, 1993; Kamakura *et al*., 1999; 2002). However, knowledge of the molecular basis of conidial germination and appressorium formation remains superficial.

Since appressorium formation is a complex process from initiation to maturation, it would require the expression of many specific genes in each stage. Therefore, studying gene expression during appressorium formation will give some important insight into the mechanism of appressorium formation and morphological formation and/or function of appressoria. To discover the genes uniquely expressed during appressorium formation, mRNA isolation and suppression subtractive hybridization have been considered effective in identifying transcripts with differential expression profiles. However, extraction of good quality RNA from appressoria is troublesome and challenging, because it is extremely difficult to isolate cellular materials from the firmly attached germ tubes (Kamakura *et al*., 1999). To prepare total appressorium RNA, cellophane membranes (Kamakura *et al*., 1999) and dialysis membranes and cAMP (cyclic adenosine monophosphate) (Irie *et al*., 2003) were used to induce appressorium formation. The cellophane membrane was not an ideal substratum to induce appressorium formation, because the efficiency of appressorium formation was not reproducible between each experiment, and the membrane with the attached tube/appressorium must be ground altogether, which caused a poor yield of total RNA. Rice leaves were also used to induce appressorium differentiation and *M*. *oryzae* was ground with the infected leaves to isolate the appressorium RNA (Rauyaree *et al*., 2001). Since these methods were all limited, it is necessary to find a more efficient and reliable substratum for appressorium formation, and a simple and effective method for appressorium RNA extraction.

Suppression subtractive hybridization approach has been considered effective for generating differentially regulated or tissue-specific cDNA probes and libraries (Diatchenko *et al*., 1996). However, suppression subtractive hybridization strategy requires large amounts of pure mRNA and resulting methods for RNA isolation of the firmly attached germ tubes of earlier stages or mature appressoria are required. In this study, we report a simple and effective method for total RNA extraction from appressoria of *M*. *oryzae*.

MATERIALS AND METHODS

Fungus and culture conditions

M. *oryzae* isolate Guy 11, a rice pathogenic isolate, is a stock culture in our laboratory. For conidiation, the fungus was grown on complete media (CM) at 25 °C with a 12-h photophase. Conidia were collected in distilled water and washed twice by resuspension in distilled water and centrifugation.

Conidium germination and appressorium formation on duplicate film

For conidium germination and appressorium formation, droplets (20 μl) of conidium suspension with different concentration were placed on duplicate films and then kept in humid boxes at 25 °C for 24 h. Duplicate films were firstly disinfected by dipping in 70% (v/v) alcohol for 2~3 h followed by cleaning with liquid detergent and thoroughly washing with sterilized double distilled water. These films, designated cleaned membranes, were used in all experiments unless otherwise stated. In each experiment 20 μl conidial suspension was placed on a duplicate film and two layers of wet filter paper in a Petri dish, and covered with a lid containing two layers of wet filter paper. After incubating for 24 h at room temperature (25 °C), the conidia were examined under a microscopy for conidium germination and appressorium formation. Total numbers of germinated conidia with or without appressoria and ungerminated conidia were counted in a microscope field, and percentage was calculated as follows: conidium germination rate (%)=(total number of germinated conidia/total number of conidia)×100; appressorium formation rate (%)=(total number of conidia with appressorium/total number of germinated conidia)×100. Six microscopic fields were selected randomly for each duplicate film; for each data point two duplicate films were used and values were averaged. Experiments were repeated at least three times.

Preparation of appressoria

Conidia of *M. oryzae* were isolated and washed as described above and then suspended in sterile distilled water at 1×10^6 conidia/ml. Conidial aliquots (20 μl) were pipetted onto duplicate film sheets and then kept on wet sponges in closed plastic containers to provide and maintain a high-humidity environment at 25 °C. About 3×10^8 conidia were placed on duplicate film sheets.

Extraction of total RNA and preparation of cDNA

Total RNA was prepared from the appressoria formed on the duplicate film sheets at 24 h post-inoculation and the mixture of equal weights of conidia, aerial mycelia and substratum mycelia using the Trizol method following the manufacturer's procedure (Molecular Research Center, USA). To extract total RNA from appressoria, water of the droplets was removed with sterile tissue paper. Approximately 2 ml Trizol was added onto the duplicate films and the films were scraped with a cell scraper. Trizol containing appressoria and other cell components was collected and transferred to a 50-ml tube. After centrifuged at 12000×*g* for 15 min, the supernatant was transferred to a new centrifuge tube. Then 4 ml of chloroform was added, and the emulsion was mixed for 15 s and incubated at room temperature for 5 min. After centrifuged at 10000×*g* for 15 min at 4 °C, the upper aqueous phase was transferred to a new centrifuge tube, and the total RNA was precipitated by the addition of 10 ml isopropanol followed by incubation at 4 °C for 20 min. The pellet obtained was dissolved in 300 μl double distilled water and reprecipitated by the addition of 300 μl isopropanol. Centrifugation was repeated at 10000×*g* for 15 min, and the pellet was rinsed with 75% (v/v) ethanol and dried on ice for 10 min. To check the integrity of the total RNA, 5 μl of the isolated RNA was measured by ultraviolet (UV) detection using GeneQuant *pro* (Biochrom, UK). cDNA was synthesized using SMART cDNA library construction kit (Clontech, USA).

Construction of cDNA subtractive library

To construct the cDNA library of genes specially expressed during appressorium formation stage, cDNA subtractive hybridization was carried out following the protocol of the polymerase chain reaction (PCR)-select cDNA subtraction kit (Clontech, USA). The subtracted cDNA was cloned into the pBlueScript II SK(+) vector (Stratagene, USA).

RESULTS AND DISCUSSION

Conidium germination and appressorium formation by *M. oryzae*

Due to the variability in the frequency of appressorium formation when the conidial population density varied, we tested the effect of concentration on conidium germination and appressorium formation on duplicate films. At 1.0×10^5~1.0×10^6 conidia/ml, both conidium germination and appressorium formation were nearly complete (Table 1). As conidial concentration increased more than 1.0×10^6 ml^{-1}, a decreasing proportion of the conidial germ tubes underwent differentiation into appressoria, as shown in Table 1. Analysis of developmental gene expression required a large conidial population (>10^8 conidia per time point) to isolate an adequate quantity of RNA. A high population density (1.0×10^6 conidia/ml) was required to isolate sufficient amounts of RNA over a short time span and hence for accurate analysis of developmental gene expression. When inoculated on duplicate films with a concentration of 1.0×10^6 conidia/ml, the percentage of conidia germination and appressorium formation were (97.98±0.67)% and (97.88±0.45)%, respectively. The concentration, 1.0×10^6 conidia/ml, was suitable for appressorium preparation and total RNA extraction, which was consistent with the previous reports (Kamakura *et al*., 1999; Irie *et al*., 2003).

Table 1 Induction of *M. oryzae* conidia germination and appressorium formation by duplicate films

C (conidia/ml)	*n*	P_1 (%)*	P_2 (%)*
1.0×10^5	286	99.07±0.79^A	98.80±1.05^A
5.0×10^5	314	98.80±1.52^A	98.08±1.55^A
1.0×10^6	412	97.98±0.67^A	97.88±0.45^A
1.5×10^6	345	97.05±0.47^B	96.83±1.30^A
2.0×10^6	443	96.40±1.67^C	94.11±3.51^B
2.5×10^6	532	95.40±0.79^D	93.73±0.66^B
5.0×10^6	527	86.05±1.53^E	78.45±2.99^C
1.0×10^7	608	76.51±1.17^F	64.54±2.94^D

C: conidia concentration; *n*: number of conidia observed; P_1: percentage of conidium germination; P_2: percentage of appressorium formation. Assays were done on duplicate films as described in the text; *Values are the mean±*SD* for three independent experiments; The same superscript capital letters in the same column denote that the difference is not significant as estimated by Duncan's test ($P\leq0.01$)

Substratum attachment appears to be a prerequisite for appressoriation of most fungi (Jelitto *et al*., 1994; Lee and Dean, 1994; Xiao *et al*., 1994; Kim *et al*., 2000), although conidium germination and appressorium formation are triggered by different signals in different species of phytopathogenic fungi. We found that duplicate film is a suitable substratum for conidium germination and appressorium formation in our experiments, because when inoculated with a high conidium population density, e.g., 1.0×10^6 conidia/ml, conidium germination and appressorium formation were nearly complete. From Table 1, as conidial concentration increased from 1.0×10^5 ml^{-1}, conidium germination decreased and a decreasing proportion of the conidial germ tubes differentiated into appressoria. These results suggest that there are self-inhibitors of conidium germination and appressorium formation in the conidia. Fungal conidia often do not germinate in denser populations. This phenomenon, known as self-inhibition, is caused by chemicals inside the conidia (Hegde and Kolattukudy, 1997). These self-inhibitors are produced during sporulation and ensure that conidia germinate under conditions favorable to the fungus. Considering conidium germination, appressorium formation and total RNA isolation, we decided to use the inoculation of 1.0×10^6 conidia/ml for appressorium total RNA isolation.

Extraction of total RNA

Droplets (20 μl) of conidium suspension at 1.0×10^6 conidia/ml were placed on duplicate films and then kept in humid boxes at 25 °C for 24 h (Fig.1). Eighty μl total RNA was obtained from the mature appressoria 24 h after inoculation. To check the quality and quantity of the total RNA prepared by this method, 5 μl appressorium total RNA was measured by UV detection at 230, 260 and 280 nm and another 5 μl appressorium total RNA was electrophoresed on 1.2% (w/v) denaturing formaldehyde/agarose gel and stained with ethidium bromide. The concentration of the total RNA isolated from the mature appressoria was 1.42 μg/μl and the A_{260}/A_{280} (absorbance rate of 260 to 280 nm) and A_{260}/A_{230} (absorbance rate of 260 to 230 nm) were 1.83 and 2.25, respectively, indicating little contamination from proteins in the RNA isolated. From the gel, total RNA appeared as two bright bands (28S rRNA and 18S rRNA) and showed intact (Fig.2a). About 113.6 μg appressorium total RNA was obtained by this method and was good for the further molecular biological studies. Using this strategy, total RNA was isolated from the germinated conidia and/or apprressoria after the conidia were incubated for various periods of time (Fig.2b).

Fig.1 Droplets (20 μl) of conidium suspension (1.0×10^6 conidia/ml, approximate 15 ml) were placed on a 20 cm×30 cm duplicate film

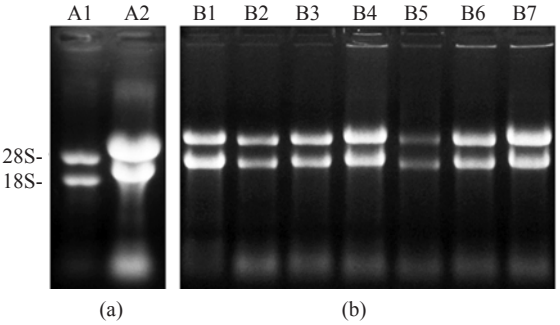

Fig.2 Total RNA isolated from the appressoria. Total RNA was analyzed with electrophoresis on a 1.2% (w/v) denaturing formaldehyde/agarose gel. (a) A1 indicates total RNA isolated from the mature appressoria 24 h after inoculation. A2 indicates total RNA isolated from the mixture of equal weights of conidia, aerial mycelia and substratum mycelia; (b) B1~B7 indicate the total RNA from the germinated conidia and/or appressoria 2, 4, 8, 12, 18, 24 and 30 h after inoculation, respectively

To find the genes that are expressed during the whole stage of appressorium formation, transcribed mRNA must be measured by reverse-transcript polymerase chain reaction (RT-PCR) or Northern hybridization analysis. However, it was extremely difficult to prepare enough total RNA from the germ tube and/or appressoria even for the RT-PCR analysis before because of the following two reasons: First, for efficient conidium germination and appressorium formation, the maximum concentration of conidium suspension was strictly limited to not more than

1.0×10^6 conidia/ml. Second, since the germ tube and the appressoria attached firmly onto the solid surface, e.g., cellophane membrane, it was almost impossible to remove those from the surface and thus the membrane with the attached germ tube and/or appressoria must be ground altogether (Kamakura *et al*., 1999; Irie *et al*., 2003). These two factors caused a poor yield of total RNA in average and the difficulty of achieving RT-PCR or Northern analysis for many candidate clones in the library. Because of this technical difficulty, few molecular biological studies on the whole developmental stage have been done to date.

To resolve this problem, we improved the method of total RNA preparation. By using duplicate films as a substratum for appressorium formation and adding Trizol before harvesting appressoria, we obtained the total RNA from the appressoria. This method was much easier than Kamakura *et al*. (1999)'s method and the yield was 4.0 μg per 100 cm^2 of duplicate films in average and was more reproducible. We know that during harvesting the germ tube and/or appressoria, time for removing might cause undesirable changes of the population of the mRNA in the cell. To avoid these problems, we added Trizol onto the substratum quickly after removing the water with sterile tissue paper so that every reaction in the cell could be halted with the presence of Trizol timely. Thus, germ tubes and/or appressoria were easily scratched away with an eraser from the surface of the substratum.

Enrichment of cDNA specifically expressed during the late stage of appressorium formation by cDNA subtraction

Total RNA of the mixture of conidia, aerial mycelia and substratum mycelia was isolated by a Trizol method following the manufacturer's procedure. Three μl total RNA (about 15 μg total RNA for the mixture of conidia/mycelia and about 5 μg for appressoria) was used for the synthesis of double-stranded cDNA with an SMART (switching mechanism at 5′-end of RNA transcript) cDNA library construction kit (Clontech, USA). One part of the appressorium cDNA was used to construct mature appressorium cDNA library (Lu *et al*., 2005a). Another part of cDNA prepared from the appressoria was subtracted with the excess of cDNA prepared from the mixture of conidia, aerial mycelia and substratum mycelia.

PCR products from the subtraction were ligated with the pBlueScript II SK(+) vector and introduced in the *Escherichia coli* DH5α, and then 338 colonies were stored as a grid subtractive library. After 250 clones from the subtracted appressorium cDNA library were sequenced, 155 non-redundant ESTs (expressed sequence tags) were obtained, of which 72 have not been previously isolated in *M. oryzae*. For details about the construction and the results of the subtractive library, please see Lu *et al*.(2005b). Among these ESTs, s121, an EST (GenBank accession No. CK828204) homologous to ESTs encoding 11-kDa nonhistone chromosomal protein, was isolated from this subtractive library. The corresponding gene of the EST was cloned and designated as *MNH6* (Lu *et al*., 2007). Functional analysis revealed that Mnh6 has an important role on fungal morphogenesis. When *MNH6* gene was knockout, the Δ*mnh6* mutants exhibited pleiotropic effects on fungal morphogenesis and pathogenicity, including reduction in mycelial growth, conidiation, appressorium development, infectious growth in host cells, and greatly reduced pathogenicity on barley and rice. s197, another EST (GenBank accession No. CK828251) for autophagy-related gene 1 *MgATG1*, was also found in the appressorium of the rice blast fungus (Liu *et al*., 2007). In the Δ*mgatg1* mutant, in which the *MgATG1* gene had been deleted, autophagy was blocked. Fewer lipid droplets in its conidia, lower turgor pressure of the appressorium, defects in morphogenesis as delayed initiation and slower germination of conidia were also found in the mutants. Furthermore, as a result of lower turgor pressure in appressorium, the Δ*mgatg1* mutant lost its ability to penetrate and infect both rice and barley.

Functional analysis of these two novel genes identified in this subtractive library in our laboratory strongly suggested that our total RNA isolation and subtraction strategy were successful in condensing the stage-specific cDNA from appressorium in the late stage of development. Total RNA from the germinated conidia and/or appressoria at other stage, e.g., 2, 4 and 6 h was obtained and the subtraction works are underway in our laboratory.

CONCLUSION

Duplicate film is an efficient and reliable substratum to induce appressorium formation in *M. oryzae*. When the *M. oryzae* conidia were inoculated on the duplicate film at concentration of 1.0×10^6 conidia/ml, percentages of conidium germination and appressorium formation 24 h after incubation at 25 °C were (97.98±0.67)% and (97.88±0.45)%, respectively. With the strategy of adding Trizol before harvesting, an adequate amount and good quality of appressorium total RNA were obtained. The results of cDNA subtractive library and function analysis of the genes isolated from the subtractive library also confirmed that this method was suitable for extracting appressorium total RNA in *M. oryzae* and may be applicable for other appressorium-formed pathogenic fungi, such as *Colletotrichum trifolii*, *Metarrhizium anisopliae*, *Erysiphe graminis*, and so on.

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