UDC 635.8:579.258
ISSN 1330-9862

preliminary communication

(FTB-1259)

Identification of Two Thermotolerance-Related Genes in *Agaricus bisporus*

*Rong Chen, Lanfen Chen and Siyang Song**

The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen, Fujian, 361005, P. R. China

Received: August 3, 2003
Accepted: November 10, 2003

Summary

To characterize thermotolerance-related genes in *Agaricus bisporus* strain 02, we employed differential display PCR (DD-PCR) to analyze total RNA samples extracted from the mycelia grown at different temperatures. Two partial DNA fragments (023-11A and 023-11B) were cloned thus far, the expression of which was correlated with the culturing temperature. The sequences of the two DNA fragments were determined and the results showed that the nucleotide sequence of 023-11A was unknown, and 023-11B was highly similar in nucleotide sequence (identities 24 %, positives 45 %) to a gene coding for the karyopherin docking complex of the nuclear pore complex of *Saccharomyces cerevisiae*. It is possible to use the two fragments for further characterization of full-length coding sequences, which can potentially be used for generating new thermotolerant mushroom strains by transgenic technique.

Key words: Agaricus bisporus, thermotolerance-related gene, fluoro differential display polymerase chain reaction

Introduction

Mushrooms are excellent food that can be used in well-balanced diets for their low content of fat and energy and high content of dietary fiber and functional compounds. As one of the most popular species on the international mushroom market, *Agaricus bisporus* has been undergoing a rapid increase in market share in recent years (*1*). However, successful cultivation of *Agaricus bisporus* is often limited by its own biological properties, in particular, its sensitivity to high temperature. The optimal temperature for cultivation of this commercially important mushroom is 22–25 °C. In general, temperature above 32 °C will impair the growth of mycelia and result in a fruit body of poor quality with long and thin stem and opened cap. For this reason, *A. bisporus* can only be cultivated seasonally in Southern China, where it accounts for 70 % of the production of the whole country. An introduction of the thermotolerant strain of *A. bisporus* will provide a significant boost to the Chinese mushroom industry.

In this paper, we described the employment of a new fluorescence-based differential display polymerase chain reaction (fluoro-DD-PCR) technique that we have developed for identifying thermotolerance-related genes in white button mushroom. This technique was adopted from the well-established DD-PCR technique (*2,3*) by fluorescein labeling. We employed the fluoro-DD-PCR on the thermotolerant strain 02, which produces a high quantity of fruity bodies at 32 °C. However, these fruit bodies are of low quality with caps that open readily. That is why we considered strain 02 to be a suitable

* Corresponding author; Phone: ++86 592 21 84 005; Fax: ++86 592 21 81 015; E-mail: sysong@xmu.edu.cn

model not only for quantity breeding, but also for the research of thermotolerance.

Materials and Methods

Fungus culture

The commercial strain of *Agaricus bisporus* 02 was supplied by the Mushroom Research and Development Station, Fujian Research Institute of Light Industry, China. Mycelia of the fungi were cultured in liquid potato dextrose agar (PDA) medium for 14 days at 24 °C and then divided into two halves. One half was continuously cultivated at 24 °C, and the other at 34 °C for 12 h to induce expression of the thermotolerance-related genes.

RNA extraction and reverse transcription

Total RNA was extracted from the fungi cultured at different temperatures by the $CsCl_2$ density centrifugation as described by Sambrook *et al.* (*4*). For reverse transcription, 0.2 μg of total RNA samples were mixed by pipetting gently with 4 μmol of reverse transcriptional primer AP3 (ACGACTCACTATAGGGCTTTTTTT TTTTTGG) in a nuclease-free microcentrifuge tube. The samples were then heated to 70 °C for 5 min and chilled rapidly with ice. The samples were then mixed with 7.8 μL of 5 × SuperScript II RT Buffer, 2 μL of DTT (100 mmol/L), 2 μL of dNTPs (10 mmol/L), 7.8 μL of nuclease-free water, and 0.2 μL of (200 U/μL) SuperScript II Reverse Transcriptase, as suggested by the manufacturer (Invitrogen). The reverse transcription was then performed by incubation first at 42 °C for 5 min, and then at 50 °C for 50 min. Finally, the reaction was terminated by heat inactivation at 70 °C for 15 min (*5*).

DD-PCR reaction

Each reaction contained 20 μL of reaction mixture, which included 2 μL of the first strand cDNAs directly from the reverse transcription, 2 μL of 10 × PCR buffer, 2 μL of $MgCl_2$ (25 mmol/L), 1.6 μL of dNTPs (10 mmol/L), 2 μL of TMR-3' oligo(dT) primer (2 μmol/L), 2 μL of random amplification primer (as indicated in Table 1), 10.2 μL of distilled water, and 0.2 μL (5 U/μL) of Taq DNA polymerase. Five separate reactions were carried out and each TMR of the 5 primers is indicated in Table 1. The reaction mixtures were incubated at 95 °C for 2 min. Then the PCR was carried out by incubation following a sequence of 92 °C for 15 s, 50 °C for 30 s, and 72 °C for 2 min for 4 cycles, followed by 30 cycles at 92 °C for 15 s, 60 °C for 30 s, and 72 °C for 2 min. A final incubation at 72 °C for 7 min (*5*) terminated the reaction.

Immediately after PCR amplification, 7 μL of sample were mixed with 4 μL of fluorescence DNA sequencing loading solution. After incubation at 95 °C for 2 min, samples were separated on a 4.5 % HR-1000 denaturing gel (GX 403). The gel was dried with a Gennomyx LR Sequencer. The DNA bands were visualized with a

Table 1. Sequences of random primers for DD-PCR

Primer	Nucleotide sequence
M13r-ARP9	ACAATTTCACACAGGATAAGACTAGC
M13r-ARP10	ACAATTTCACACAGGAGATCTCAGAC
M13r-ARP11	ACAATTTCACACAGGAACGCTAGTGT
M13r-ARP12	ACAATTTCACACAGGAGGTACTAAGG
M13r-ARP17	ACAATTTCACACAGGACTGCTAGGTA

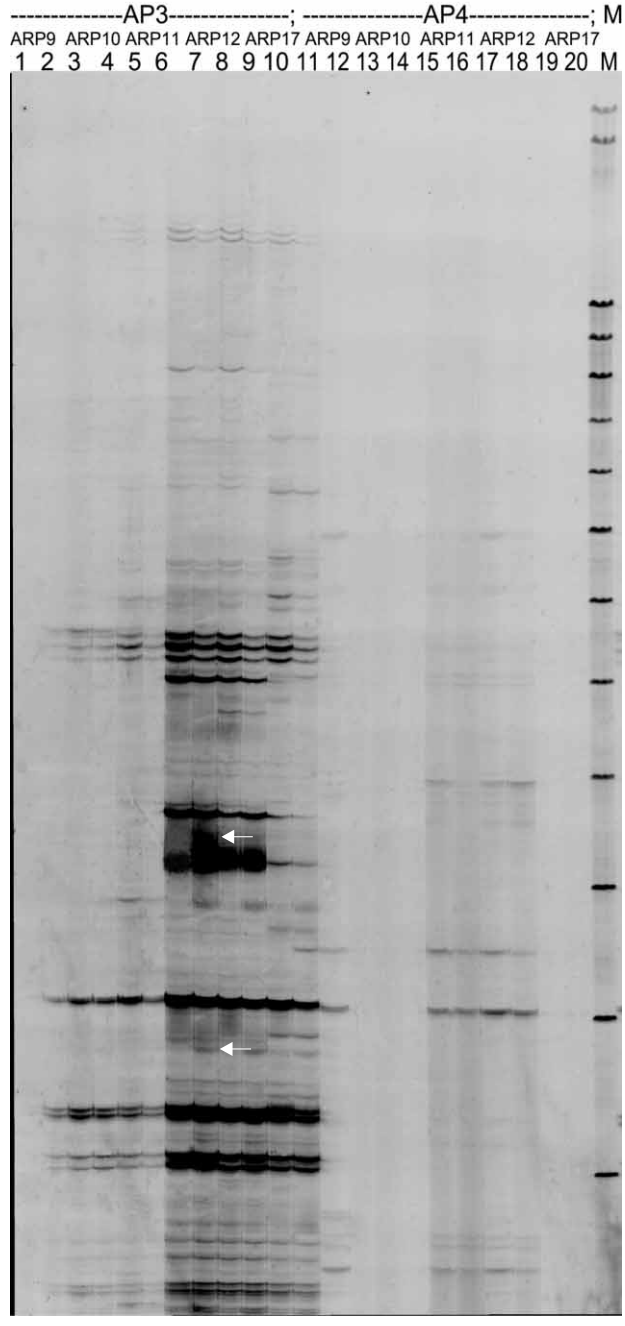

Fig. 1. The TMR-fluorescence-based differential display of *A. bisporus* strain 02 cultivated at different temperatures
Numbers 1, 3, 5, 7, 9 – RNA samples cultivated at 24 °C
Numbers 2, 4, 6, 8, 10 – RNA samples cultivated at 32 °C
M – Fluoro-marker in TMR-fluorescent anchored primer adaptor kit
Differential fragments are marked with arrow

GENOMYX SC fluorescent scanner, and then target bands were excised for further amplification.

Re-amplification, cloning and sequencing of differentially displayed DNA fragments

The differentially displayed bands were excised from the gel and the DNA fragments were extracted from the gel slices by soaking the gel with 100 μL of TE buffer in microcentrifuge tubes, and then precipitated with ethanol. After washing, 5 μL of each sample was used for re-amplification. The reaction was carried out as described above. The candidate cDNAs were cloned into plasmid pMD18-T vector (*4*), and customer sequenced by Bio-Asia (Shanghai) Co.

Results and Discussion

The expression of RNAs was higher at 34 than at 24 °C. To identify these RNAs, the RNA samples from mycelia cultured at 24 and 34 °C were extracted. The HIEROGLYPH™ mRNA Profile Kit was employed to identify the potential thermotolerant RNAs. TMR-fluorescent labeled primers were used to facilitate visualization of the PCR products. The differential display of fluorescence-labeled products was identified with a fluorescent scanner (Fig. 1). Each result was repeated twice under the same conditions.

Two fragments, identified from the product of Ap3--ARP11 amplification, were differently displayed when the mycelia were grown at 34 °C and were designated as 023-11A and 023-11B. The DNA fragments were recovered from the gel and cloned into pMD18-T vector. The sequences of both fragments were determined (Figs. 2 and 3). The sequences were compared with the NCBI database of GENEBANK and the NR gene bank to determine sequence similarity with known sequences. The results in Figs. 4 and 5 indicate that the sequence of fragment 023-11A is novel. Interestingly, the sequence showed similarity in amino acid sequence to the carboxyl-terminal proteinase gene of *Arabidopsis thaliana* (identities = 31 %; positives = 47 %) (*6*), suggesting that fragment 023-11A might encode a protease. Fragment

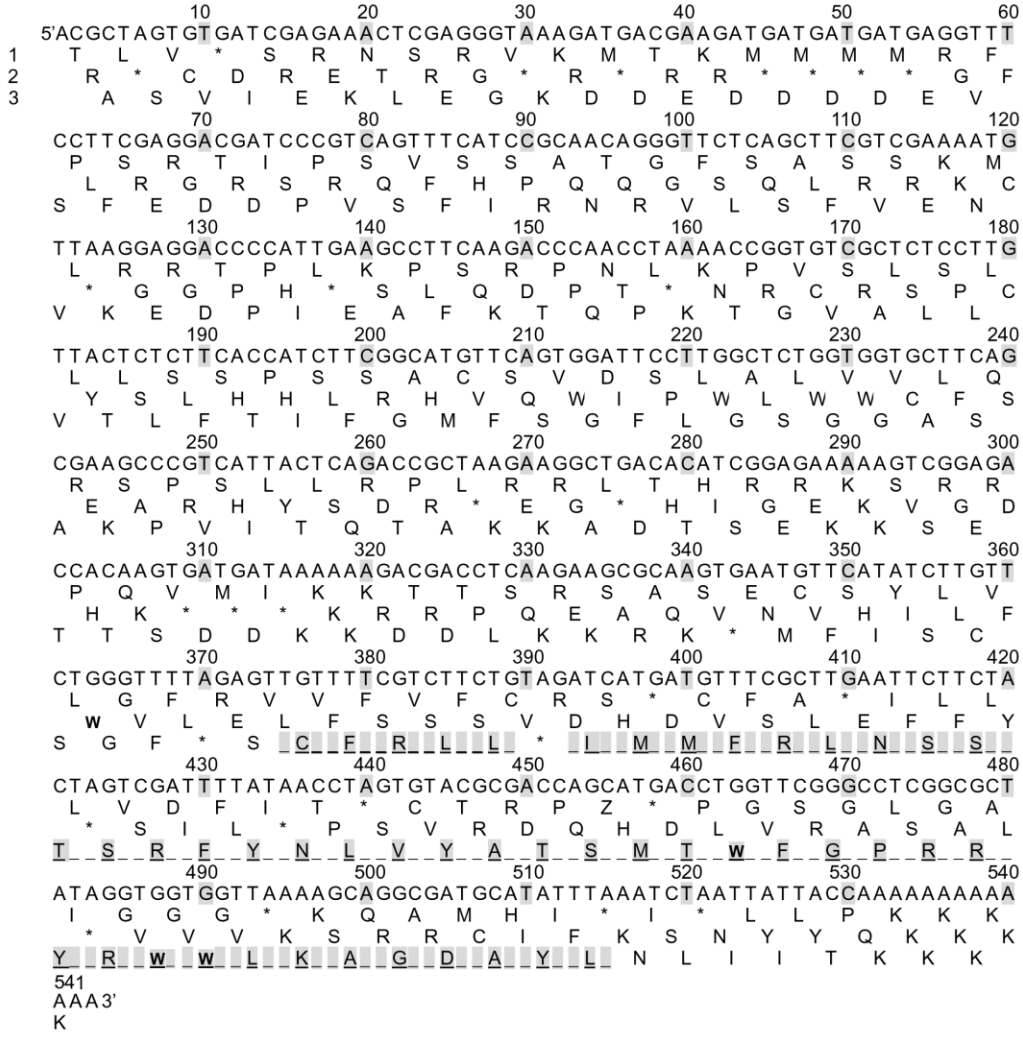

Fig. 2. The DNA sequence and the possible ORFs of differential fragment 023-11A
* – Terminal codon
__ – Homology sequence to partial carboxyl-terminal proteinase gene of *A. thaliana*

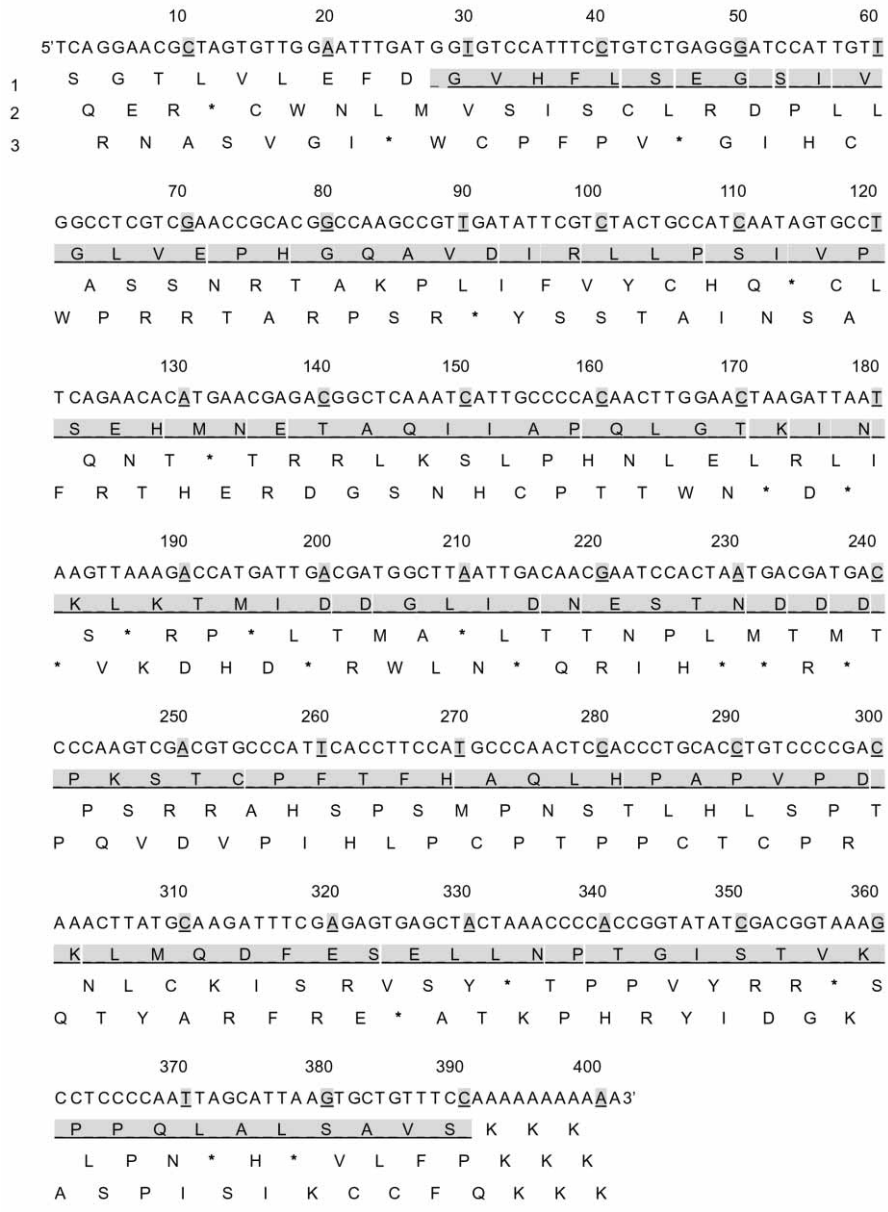

Fig. 3. The DNA sequence and the possible ORFs of differential fragment 023-11B
* – Terminal codon
__ – Homology sequence to partial karyopherin docking complex of the nuclear pore complex gene of *S. cerevisiae*

023-11B shares a high similarity in amino acid sequence with karyopherin docking complex of the pore complex gene of *Saccharomyces cerevisiae* (identities = 24 %; positives = 45 %) (7).

DD-PCR has been widely used to isolate differentially expressed genes between normal and abnormal mammal cells since it was first reported in 1992. Here we report a usage of fluoro-DD-PCR, in which the labeling radioactive isotope is substituted with the fluorescein so that the PCR products can be visualized with a fluorescent scanning. This allows efficient and effective isolation and the analysis of DD-PCR products. The primers for both reverse transcription and PCR were also modified. The sequence of T7 promoter with TMR on the 5′-terminal oligo-T was used in the anchor primers. The 5 primers in Table 1 could theoretically be used for amplification of almost all eukaryotic mRNAs. Random sequence of 10-bp oligonucleotides was also linked to the M13 general primer at 5′-terminal. A fluoro-DD--PCR technique was used for monitoring temperature for genes of spawns of a thermotolerant strain 02. The fluoro-DD-PCR is more sensitive than the extraction and purification of dsRNA from the treated material followed by gel electrophoresis, and is not limited to sporophores.

The fragment 023-11A contained an open reading frame of 47 codons, which shared a similarity of 31 % in nucleotide sequence with the 3′-end non-coding region of *A. thaliana* protease. In addition, the open reading frame of 023-11A was in the opposite direction of the *A.*

```
375                                                                  515
CFRLL*IMMFRLNSS----------TSRFYNLVYATSMT-WFGPRRYRWWLKAGDAYL
C+  LL      ++NS           S F+N   Y  S+T  W  P+    WW++  GD Y+
CYNLLCSGFIQINSQIAMGASISPVSGFHNPQYDISITIWKDPKEGHWWMQFGDGYV
255                                                                  311
```

Fig. 4. The comparison of homology between 023-11A partial sequence and partial carboxyl-terminal proteinase gene of *A. thaliana*
+ Positive site; -- Gap site
Score = 32.8 bits (73), Expected = 2.7, Frame = +3
Identities = 18/57 (31 %), Positives = 27/57 (46 %), Gaps = 10/57 (17 %)

```
          105
023-11B   GQAVDIRLLPSIVPSEHMNETAQIIAPQLGTKI----------NKL----KTMI----------DDG
          G   D R+L ++ P+++   +       +  LGT +        N+   KT +          +DG
Sbjct     GSKKDPRILKNVSPNDNNSANNNAHSSDLGTVVFDSNEAPPKTSLADWQKEDG
          125

023-11B:  LIDNESTNDDDPKSTCPFTFHAQLHPAPVPDKLMQDFESELLNPTGI STVKPPQLA
          +  +++  N  +DP  +    TF  +      P  P  +  ++    S +LN     +T    P   A
Sbjct:    I FSKTD N I EDPNLSSN ITFDGKPTATPSPFRPLEK---TSR ILNFFDKNTKTTPNTA

            416
023-11B:  LSAVS
          S  S
Sbjct:    SSEAS
            237
```

Fig. 5. The comparison of homology between 023-11B partial sequence and partial karyopherin docking complex of the nuclear pore complex gene of *S. cerevisiae*
+ Positive site; -- Gap site
Score = 31.3 bits (69), Expected = 5.6 Frame = +3
Identities = 28/114 (24 %), Positives = 52/114 (45 %), Gaps = 10/114 (8 %)

thaliana protease. Additional experiments are needed to characterize the gene and to identify the function of the protein.

The fragment 023-11B also had an open reading frame that shared 24 % similarity with yeast karyopherin, a component protein of the docking complex in the nuclear pore complex. The potential product of this open reading frame shared 45 % identity with the yeast karyopherin in amino acid sequence (*7*), suggesting that 023-11B is the counterpart of yeast karyopherin. The karyopherin docking complex of nuclear pore complex is important for transportation of proteins between the nucleus and cytoplasm of cells (*8*,*9*). It is possible that the cell needs to increase nuclear-cytosol trafficking at high temperature. In that case increased expression of karyopherin may be essential for cell to survive at high temperature. More experiments are needed to verify whether 023-11B encodes the karyopherin docking complex of the nuclear pore complex, and whether high expression of 023-11B contributes to thermotolerance of the organism.

In search of thermotolerance-related genes in *A. bisporus*, we focused on the differences of RNA transcription of the mushroom. Organisms always have differences in their gene expression under stress environment. The response of high temperature is known as heat shock response (HSR), which appears as a series of transcription and translation of Heat Shock Protein (HSP) genes. Generally, HSPs are molecular chaperones that participate in a variety of cellular functions, including protein folding and transport, and the repair of stress-induced damage. In our research, we discovered a karyopherin docking complex showing similarity to the counterpart of *Saccharomyces cerevisiae*. We also note that new reports show that karyopherin family can act as »chaperones« to sequester crucial factors for spindle formation (*10*). We suggest that to respond to the high temperature inducement, some proteins are over-expressed and linked to the increase of transport from plasmids into nucleus. As proteins of karypherin family play a central role in nucleocytoplasmic transport, karypherins may be related to stress response of *A. bisporus*.

As a preliminary research about thermotolerance-related genes of *A. bisporus*, we cannot affirm that these two genes have the direct relationship with thermotolerant feature of white mushrooms. More experiments are still needed to prove that these genes are expressed at a detectable level or are specifically related to the inducement of high temperature.

References

1. X. Wu, D. Xu, H. Liu, Anhui, *Agri. Sci. Bull. 8* (2002) 59–60
2. P. Liang, A. B. Pardee, *Science, 257* (1992) 967–970.
3. M. Strauss, D. Bauer, H. Muller, *Nucleic Acid Res. 21* (1993) 4272–4280.
4. J. Sambrook, E. F. Fritsch, T. Manistis: *Molecular Cloning, A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press (1989).
5. Beckman Corporation: *HIEROGLYH mRNA Profile System for Differential Display Analysis* (1998).
6. S. Sato, Y. Nakamura, T. Kaneko, T. Katoh, E. Asamizu, H. Kotani, S. Tabata, *DNA Res. 7* (2000) 31–63.

7. A. Goffeau, B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, S. G. Oliver, *Science, 274* (1996) 546, 563–567.

8. D. J. Sweet, L. Gerace, *Trens. Cell Biol. 5* (1995) 444–447.

9. N. Shulga, P. Roberts, Z. Gu, L. Spitz, M. M. Tabb, M. Nomura, D. S. Goldfarb, *J. Cell Biol. 135* (1996) 329–339.

10. M. V. Nachury, T. J. Maresca, W. C. Salmon, C. M. Waterman-Storer, R. Heald, K. Weis, *Cell, 104* (2001) 95–106.

Identifikacija dvaju srodnih termotolerantnih gena u *Agaricus bisporus*

Sažetak

Da bi se karakterizirali srodni termotolerantni geni u *A. bisporus*, soj 02, upotrijebljen je diferencijalni postupak PCR (DD-PCR) radi analize svih ekstrahiranih RNA uzoraka dobivenih iz micelija uzgojenih pri različitim temperaturama. Zasada su klonirana dva djelomična DNA fragmenta (023-11A i 023-11B), a njihova je ekspresija uspoređena s temperaturom uzgoja. Utvrđene su sekvencije tih dvaju DNA fragmenata. Pokazalo se da je nepoznata sekvencija nukleotida DNA fragmenta 023-11A, a fragmenta 023-11B vrlo slična sekvenciji nukleotida (identičnost 24 %, sigurnost 45 %), u genu koji kodira kompleks vezanja karioferina na kompleks pore nukleusa u *Saccharomyces cerevisiae*. Postoji mogućnost da se ta dva fragmenta koriste za daljnje utvrđivanje cjelokupnih sekvencija kodiranja, što bi se moglo upotrijebiti za proizvodnju novih termotolerantnih sojeva gljive transgenskom tehnikom.