Transformation of *Volvariella volvacea* with a Thermal Hysteresis Protein Gene by Particle Bombardment

LIQIONG GUO[1,2] JUNFANG LIN[1,2] SHENG XIONG[2] & SHOUCAI CHEN[2]
[1]College of Food Sciences, South China Agricultural University, Wushan, Tianhe, Guangzhou, Guangdong 510640, China; and [2] State Key Laboratory of Tropical Crop Biotechnology, The Chinese Academy of Tropical Agricultural Sciences, Chengxi, Haikou, Hainan 571101, China. Email: linjf@scau.edu.cn

Abstract: A cDNA encoding a thermal hysteresis protein gene was isolated from the Arctic insect spruce budworm by RT-PCR amplification. It was used to transform *Volvariella volvacea* through particle bombardment. PCR detection and Southern blotting analysis showed that the thermal hysteresis protein gene was integrated into *V. volvacea* genomes. A cold stress assay revealed that transgenic *V. volvacea* exhibited stronger cold tolerance than the host strain. The properties of transgenic *V. volvacea* were also changed in terms of growth rates and hyphal morphology. Growth rates of most *V. volvacea* transformants were significantly slower than those of a negative control strain. Furthermore, hyphae of most *V. volvacea* transformants were thinner than hyphae of the host strain. Experimental results also showed that *V. volvacea* transformants resistant to hygromycin were mitotically stable. Three-rounds of selection with the first selection on PDSA solid selective medium followed by second and third selections in PDSB liquid selective medium was the best selection procedure for *V. volvacea* transformant screening.

Key words: Thermal hysteresis protein gene, transformation, cold stress, *Volvariella volvacea*, hygromycin, Arctic insect spruce budworm

1 Introduction

V. volvacea, the straw mushroom, is one of the most important cultivated edible mushrooms in China, especially in Guangdong China. Total yield in Guangdong in 2001 was 30,000 tons, ranking second just behind *Lentinus edodes*. *V. volvacea* is a typical thermophilic mushroom. It can grow well at 43°C but it cannot tolerate cold stress under 10°C. Under conditions of 4°C, its mycelium will lyse and die, its fruiting body will soften, liquefy and decay.[1] This characteristic susceptibility to cold stress seriously limits the production seasons, production areas and use of refrigeration for maintaining the freshness of *V. volvacea*. Thus, it is very important to develop novel straw mushroom strains resistant to cold stress. However, no *V. volvacea* germplasm that is tolerant to cold stress is available. Moreover, the life circle of the straw mushroom is homothallic. Hyphae have no clamp connections and there is a scarcity of selectable markers for hybrid screening.[1] Therefore, it is very difficult to improve straw mushroom strains using conventional breeding methods. Fortunately, the development of genetic engineering provides a solution to this problem.

Thermal hysteresis proteins (THPs) possess the unique property of lowering the freezing point of water without significantly altering the melting point of water, and they play an important role in low temperature survival.[2] They can depress the internal freezing point in freeze avoiding organisms and/or prevent damage due to ice recrystallization in freeze-tolerant organisms.[2] They may protect membranes from low temperature damage by inhibiting thermotropic phase transitions and prevent leakage by blocking ion channels.[3, 4] Many types of thermal hysteresis proteins have been found in vertebrates, invertebrates, plants, fungi, bacteria and they have been fully studied.[2] Thermal hysteresis proteins from insects exhibit the most active antifreeze properties in cryoprotection.[5, 6]

Here we report our successful experimental results in the transformation of *V. volvacea* with a thermal hysteresis protein gene isolated from the Arctic insect spruce budworm from Sweden by RT-PCR amplification.

2 Materials and Methods

2.1 Strain and maintenance

V. volvacea strain V34 was used for transformation experiments and it is a commercial cultivation strain in China. It is routinely maintained on PDSA medium (g/L: potato, 200; dextrose, 20; KH_2PO_4, 3; $MgSO_4$, 1.5; vitamin B1, 0.005; agar, 20) in our laboratory.

2.2 Thermal hysteresis protein gene

Poly A+ mRNA from the spruce budworm from Sweden was directly isolated and purified by using Oligotex Direct mRNA Kits (QIAGEN) and its protocol. This mRNA was the template subjected to RT-PCR amplification. A cDNA fragment of ~410 bp was obtained by RT-PCR amplification using primers AFP1 (5'-TGCAGGAATCGGCACGAGGAA-3') and AFP2 (5'-GACTTTCATGGCTTAATTAGC-3'). This cDNA fragment was ligated with pGEM T-Vector (Promega) to be recombinant plasmid pGTHP4 and was sequenced using an ABI 377A DNA Automatic Sequencer (PE Applied Biosystems). pGTHP4 was transformed into competent cells of *E. coli* strain DH5α which were used for replication of the recombinant plasmids.[7] The DH5α was routinely grown on LB medium containing appropriate antibiotics. Stock solutions were stored at -80°C in small aliquots.

2.3 Plasmids

The binary vector pCAMBIA1301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia), containing the *E. coli* hygromycin B phosphotransferase gene (*hpt*) under the control of CaMV 35S promoter, and the β-glucuronidase gene (*gus*) with the CaMV 35S promoter, was used for the construction of the *V. volvacea* gene expression vector.
The expression vector (10254 bp), designated pCTH823 (Figure 1), consists of a pCAMBIA1301 backbone from which the β-glucuronidase gene fragment was removed with restriction enzyme *Bst*EII and *NcoI* and the thermal hysteresis protein gene excised from pGTHP4 with restriction enzyme *Bst*ZI and *NcoI*. These plasmids pCTH823 and pCAMBIA1301 were also transferred into competent cells of *E. coli* strain DH5α and they were grown on LB medium containing appropriate antibiotics and stored at -80°C in small aliquots.

2.4 Transformation of *V. volvacea* strain V34 by particle bombardment

The preparation of microprojectiles (gold particle,Φ=1.0μm) for bombardment in the Biolistic PDS-1000/He Particle Delivery System (BIO-RAD) was based on the protocol provided by the supplier with a slight modification. The bombardment explants were *V. volvacea* mycelia grown on PDSA plates and PDSB broth for 5 days at 28°C. These mycelia with medium were cut into pieces and placed in mycelial disks of 6cm diameter at the center of other PDA plates. The mycelial disks were then bombarded once or twice with plasmid pCTH823 under vacuum (26 inches of Hg) at 1,100 psi and at distances of 6cm, 9cm or 12cm from the stopping plate. At the same time, a negative control was bombarded under same conditions but without plasmid pCTH823.

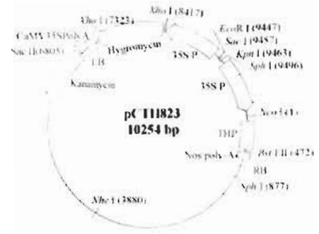

Figure 1. Physical map of the expression vector pCTH823

The vector (10254 bp) consists of a pCAMBIA1301 backbone containing the kanamycin resistance gene, the right border and left border sequences. The hygromycin resistance gene and thermal hysteresis gene (THP) are located between the border sequences. Both are controlled by the cauliflower mosaic virus 35S promoter (35S P). The THP gene is joined to the *nos* polyadenylation signal terminator and the hygromycin resistance gene is joined to the cauliflower mosaic virus 35S polyadenylation signal terminator. Restriction endonuclease sites are also shown with the map distances in base pairs.

2.5 Selection of transformants

The minimum inhibitory concentration (MIC) of hygromycin for *V. volvacea* was determined on PDSA and PDSB respectively. Mycelial disks of *V. volvacea* bombarded with plasmid pCTH823 were incubated on PDSA without hygromycin at 28°C for two days for recovery growth followed by one week of the first-stage selection incubation on the selective PDSA plates containing 60 μg/ml hygromycin B at 28°C. Then, disks of mycelia from the resulting growth were cut into thin slices (about 0.5cm x 0.1cm) and transferred to the selective medium PDSB (otato 200g/L, dextrose 20g/L, KH2PO4 3g/L, MgSO4 1.5g/L, vitamin B1 5mg/L, hygromycin 40 μg/ml) for three weeks of the second-stage selection incubation at 28°C with shaking (150 rpm). This was followed by the third-stage selection incubation using the same procedures but using DTB selective medium (dextrose 20g/L, tryptone 2g/L, KH_2PO_4 0.6g/L, $MgSO_4$ 0.5g/L, vitamin B1 0.5mg/L, hygromycin 40 μg/ml). After this three-round selection procedure, survival mycelia were considered as putative transformants.

2.6 Characterization of putative transformants by PCR

Genomic DNA was isolated from mycelia of the putative transformants and non-transformed control of *V. volvacea* as described by Raeder and Broda.[8] PCR amplification of the THP target gene for detection of integration was performed with primers AFP1 and AFP2 and the DNA templates from putative transformants and negative control using the PCR kit and its protocol recommended by the manufacturer (QIAGEN) with addition of an appropriate concentration of dimethylsulfoxide.[9] A positive control was included in the PCR detection with plasmid pGTHP4 as template.

2.7 Southern blotting analysis of transformants

About 15 μg of genomic DNA from putative transformants and non-transformed control was digested with *Hin*dIII (Bio-labs) overnight at 37°C and electrophoresed for separation on a 0.8% agarose gel. About 50 pg of plasmid pGTHP4 was digested with *Sal* I (Bio-labs) and included in the electrophoresis separation as a positive control. DNA transfer from agarose gel to Hybond N+ nylon membrane (Amersham) was performed using standard procedures[7] followed by filter baking for one hour at 80°C and UV cross linking at 1.2×10^5 μCi. A

410 bp fragment containing the THP gene was excised from plasmid pGTHP4 with restriction enzymes *Nco* I and *Bst*Z I (Bio-labs). The fragment was eluted and purified from the agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) for use as the hybridization probe. It was labelled with 50 μCi of [α-^{32}P]d-CTP at 3000 Ci/mmol by using Prime-It Random Primer Labeling Kit (Stratagene) and the method recommended by the supplier's instructions. Pre-hybridization and hybridization of the filter were carried out at 65°C for 6 hours and overnight respectively.

2.8 Bioassay for cold tolerance of transformants

Nine transformants, designated V34I-1, V34I-2, V34I-3, V34I-4, V34II-1, V34II-2, V34II-3, V34II-4, V34II-5 and the non-transformed negative control V34-CK were cultivated on PDSA medium at 28°C for 5 days. They were then cut into pieces with the medium (3mm x 4mm in size) and transferred to new PDSA plates for cold treatment at 4°C. One plate of each transformant and control was taken from 4°C conditions every 12 hours in the first three days and every 24 hours after three days of cold treatment and incubated at 28°C for the detection of survivors.

2.9 Morphological observation of transformants

Transformants and controls were incubated on the PDSA plates and slant tubes at 28°C with three replicates. Their morphologies and growth rates were investigated daily.

3 Results

3.1 Thermal hysteresis protein gene

A ~410 bp cDNA fragment was obtained by RT-PCR amplification with the mRNA template from the Arctic insect spruce budworm and using the primers AFP1 and AFP2 (Figure 2). DNA sequence analysis indicated that this cDNA fragment was 411 base pairs in length and contained a full-length open reading frame of 327 bp. A BLAST homology search showed that this cDNA fragment was highly homologous with several thermal hysteresis protein genes. The nucleotide sequence homologies were up to 99%, 98% and 97% with *Choristoneura fumiferana* thermal hysteresis protein precursor mRNA (accession number: AF263009), *C. fumiferana* antifreeze protein isoform 333 precursor mRNA (accession number: AF286208) and *C. fumiferana* antifreeze protein isoform 4 precursor mRNA (accession number: AF285838), respectively. The deduced protein sequence of the cDNA was 108 amino acid residues in length. The amino acid sequence homologies were 100%, 99% and 99% with *C. fumiferana* thermal hysteresis protein precursor (accession number: AAG32660), *C. fumiferana* antifreeze protein (accession number: AAK19296) and *C. fumiferana* antifreeze protein isoform 333 precursor (accession number: AAG32669), respectively.

3.2 Transformation by particle bombardment and selection of transformants

Particle bombardment experiments with *V. volvacea* showed that all stable transformants came from mycelium explants grown on PDSA but not PDSB. This result seemed to imply that mycelium explants from PDSA solid medium could produce genuine transformants but mycelium explants from PDSB liquid medium could not. The transformation efficiency of the particle bombardment with 6cm of distance from the stopping plate to the target shelf was significantly higher than those for 9cm or 12cm distances (data not shown). Single bombardments had similar transformation efficiencies to double bombardments.
V. volvacea is sensitive to hygromycin. Minimum inhibitory concentrations were 60 µg/ml in PDSA solid

selective medium and 40 µg/ml in PDSB liquid selective medium. Selection experiments indicated that three-round of selection with first selection on PDSA solid selective medium followed by second and third selection in PDSB liquid selective medium was the best selection procedure (Figures 3A, 3B, 3C). Selection with all stages on PDSA solid selective medium produced more false transformants due to more aerial hypha while selection with all stages in PDSB liquid selective medium gave the lowest transformation efficiency (data not shown). All negative control mycelia (host strain) are unable to re-grow on non-selective PDSA medium without hygromycin after the three-round of selection.

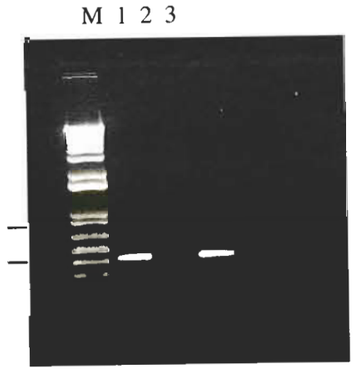

Figure 2. RT-PCR products from Swedish budworm mRNA
Lane M: DNA ladder. Lanes 1 and 3: PCR products derived from budworm mRNA template; Lane 2: negative control (PCR with H_2O as template).

3.3 Stability of transformants resistant to hygromycin

The stability of hygromycin resistant phenotypes of transformants was assessed by incubating transformants resistant to hygromycin in non-selective PDSA medium for three consecutive subcultures and then challenging with the selective medium again. All transformants retained the hygromycin resistant phenotype. This result suggested that all transformants were mitotically stable.

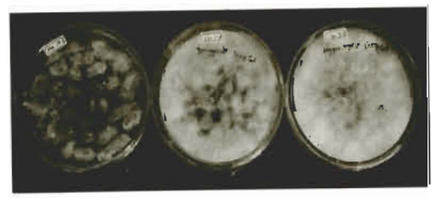

Figure 3A. First-stage selection on solid medium of three-round of selection procedure
CK refers to the negative control host strain bombarded without plasmid pCTH823 and incubated on selective medium containing 60 µg/ml of hygromycin for 7 days. I and II refer to mycelial disks bombarded with plasmid pCTH823 and incubated on selective medium containing 60 µg/ml of hygromycin for 7 days.

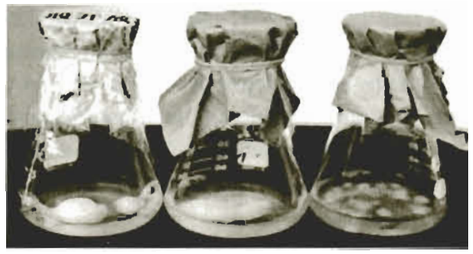

Figure 3B. Second-stage selection in liquid medium of three-round of selection procedure

CK refers to the control host strain incubated in selective medium containing 40 μg/ml of hygromycin for 3 weeks.I and II refer to mycelia resistant to hygromycin incubated in selective medium containing 40 μg/ml of hygromycin for 3 weeks.

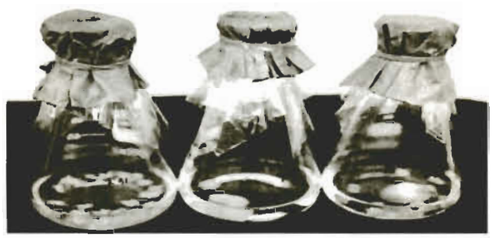

Figure 3C. Third-stage selection in liquid medium of three-round of selection procedure

CK refers to the control host strain incubated in selective medium containing 40 μg/ml of hygromycin for 3 weeks. I and II refer to putative transformants incubated in selective medium containing 40 μg/ml of hygromycin for 3 weeks.

3.4 Molecular analysis of transformants

PCR amplification results revealed that the THP gene was present in the genomes of all transformants examined (Figure 4). Southern blotting analysis confirmed that the THP gene was integrated into the genomes of all nine transformants detected (Figure 5). No false positive were detected by PCR amplification or Southern hybridization. Patterns of hybridization bands shown by the transformants demonstrated that multiple copies of the THP gene were integrated into the *V. volvacea* genome at different chromosomal sites. However, the intensity of hybridization signals was not very strong in most cases, suggesting that only a small number of copies of the THP gene had been integrated into the transformant genomes.[10] A number of fainter hybridization bands were obtained in the Southern blotting. These might represent border sequences that contained a fragment of the THP gene and genomic DNA sequences flanking the ends of the integrated THP gene fragments.[10] The multiple hybridization bands appeared in all transformants and could indicate the separate integration events of the THP gene or its re-arrangements.

3.5 Cold tolerance of transformants

Bioassay results showed that all transformants exhibited stronger cold tolerance than the host strain (Figure 6). Transformant V34I-1, the weakest cold tolerant transgenic line among all nine transformants, was able to endure 9 days of cold stress, or 7 days longer than the non-transformed negative control host strain. Furthermore, transformants V34I-3, V34I-4, V34II-4, V34II-5 were able to re-grow after 16 days of cold stress at 4°C, cold tolerance which was 13 days longer than the negative control.

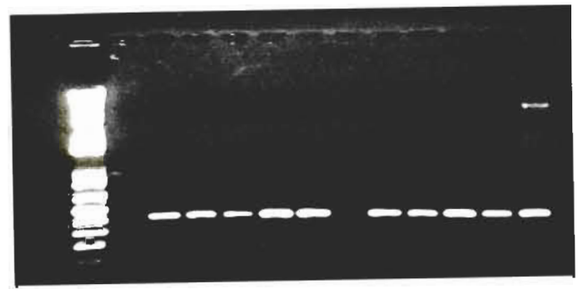

Figure 4. PCR detection of putative transformants

Genomic DNA were isolated from putative transformants and the non-transformed host strain of *V. volvacea*. PCR amplification was performed using primers AFP1 and AFP2 to specifically amplify a ~410 bp sequence of the target THP gene. Lane M: DNA ladder. Lane 1: PCR template DNA was isolated from non-transformed host strain. Lanes 2 to 6: PCR template DNA was isolated from putative transformants V34II-1, V34II-2, V34II-3, V34II-4 and V34II-5, respectively. Lane 7: negative control with water as PCR template. Lanes 8 to 11: PCR template DNA was isolated from putative transformants V34I-1, V34I-2, V34I-3 and V34I-4 ,respectively. Lane 12: PCR template DNA was isolated from plasmid pGTHP4.

Figure 5. Southern blotting analysis of transformants

Genomic DNA (15 μg) from putative transformants was digested with *Hin*dIII and probed with the 410bp ^{32}P-labeled THP gene fragment. Lane M: DNA ladder. Lanes 1 to 9: DNA isolated from putative transformants V34II-1, V34II-2, V34II-3, V34II-4, V34II-5, and V34I-1, V34I-2, V34I-3, V34I-4, respectively. Lane 10: plasmid pGTHP4. Lane CK: DNA isolated from the non-transformed host strain.

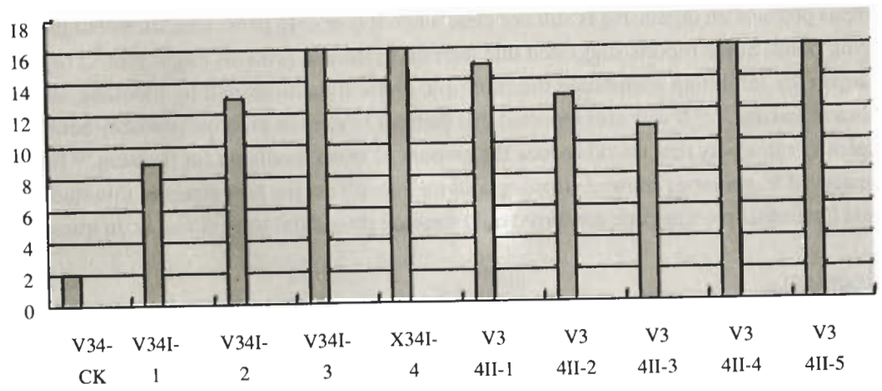

Figure 6. Cold tolerance of different transformants and non-transformed control

3.6 Morphology of transformants

Different transformants has different growth rates. Variance analysis showed that growth rates of transformants V34I-2, V34I-4, V34II-1, V34II-3, V34II-4, V34II-5 were significantly slower than that of the negative control host strain except that transformants V34I-1, V34I-3, V34II-2 had similar growth rates to the non-transformed control.

In comparison with the non-transformed host strain, hyphal morphologies of all nine transformants were altered. The hyphae of transformants V34I-1, V34I-3, V34II-2 were obviously thinner than the host in the first week when subcultured on fresh plates. The hyphae of transformants V34I-2, V34I-4, V34II-1, V34II-3 were much thinner than the control strain and almost no aerial hypha were observed in these four transformants. The transformant V34II-4 had dense mycelium and its hyphal color was white in the beginning and became light yellow later. The transformant V34II-5 possessed dense and thick mycelium and its colony exhibited appeared radiate in shape.

4 Discussion

Multiple hybridization bands appearing in the Southern Blotting suggested that the THP gene was integrated in the genomes of nine transformants at different copy numbers and at different genomic loci. This result was consistent with most transformation integration events in transgenic filamentous fungi.[10-15] This might be produced by microhomology-mediated illegitimate recombination and ectopic integration.[16-20]

The presence of both faint hybridization signals and a strong hybridization signal in the same transformant such as V34I-1, V34I-4, V34II-4 reflected the fact that complex integration events occurred during the transformations. As described above, the fainter hybridization bands probably represented border sequences that contained both part of the THP gene sequences and chromosomal sequences adjacent to the sites of THP gene integration, whereas the stronger hybridization bands might correspond to complete THP gene copies.[10, 20]

The appearance of a ~2.8 kb hybridization band in all nine transformants suggested that there might exist an integration hotspot in the *V. volvacea* genome. Similar observations have also been reported in *Oryza sativa*,[19, 21] *Arabidopsis thaliana*,[22] and *Schizosaccharomyces pombe*.[17]

It is well known that under freezing conditions, thermal hysteresis proteins protect organisms against freezing damage by lowering freezing points, inhibiting nascent ice crystal growth and re-crystallization, and reducing the percentage of freezing water in cells. However, the mechanisms of the cold damage protective effects of thermal hysteresis proteins on organisms is still not clear when it is in cold stress conditions but the temperature is above freezing point. Some reports suggested that thermal hysteresis proteins might protect organisms from cold stress damage by inhibiting membrane thermotropic phase transitions and by blocking ion channels to prevent membrane leakage.[3, 4] It was also reported that thermal hysteresis proteins probably bound and immobilized internal water in a way that would reduce the amount of water available for freezing.[2] In any case, all nine transformants of *V. volvacea* showed stronger cold tolerance than the host strain in this study, suggesting that the thermal hysteresis protein gene conferred cold damage protection to *V. volvacea* in transformants.

Acknowledgements

This work was supported by research grants from the National Natural Science Foundation of China, Grant Nos. 39960050 and 30060054.

References

[1] Chang ST. The biology and cultivation of edible fungi. New York:Academic Press, 1978, pp573-605.
[2] Barrett J. Thermal hysteresis proteins. Int. J. Biochem. Cell Biol. 2001, 33:105-117.
[3] Rubinsky B, Mattioli M, Arav A, *et al*. Inhibition of Ca2+ and K+ currents by antifreeze proteins. Amer. J. Physiol. 1992, 262: R542-545.
[4] Holmberg N, Lilius G, Bulow L. Artificial antifreeze proteins can improve Naes tolerance when expressed in *E. coli*. FEBS Letters, 1994, 349:354-358.
[5] Tyshenko MG, Doucet D, Davies PL, *et al*. The antifreeze potential of the spruce budworm thermal hysteresis protein. Nature Biotechnol. 1997, 15:887-890.
[6] Graham LA, Liou YC, Walker VK, *et al*. Hyperactive antifreeze protein from beetles. Nature, 1997, 388:727-728.
[7] Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning - A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory: Cold Spring Harbor, USA, 1989.
[8] Raeder U, Broda P. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1985, 1:17-20.
[9] Chakrabarti R, Schutt CE. The enhancement of PCR amplification by low molecular-weight sulfones. Gene, 2001, 274:293-298.
[10] Jia JH, Buswell JA, Peberdy JF. Transformation of the edible fungi, *Pleurotus ostreatus* and *Volvariella volvacea*. Mycol. Res. 1998,102:876-880.
[11] Bailey AM, Mena GL, Herrera-Estrella L. Transformation of four pathogenic *Phytophthora* spp by microprojectile bombardment on intact mycelia. Curr. Genetics, 1993, 23:42-46.
[12] van de Rhee MD, Graca PMA, Huizing HJ, *et al*. Transformation of the cultivated mushroom, *Agaricus bisporus*, to hygromycin B resistance. Mol. Gen. Genetics, 1996, 250:252-258.
[13] van de Rhee MD, Mendes O, Werten MWT, *et al*. Highly efficient homologous integration via tandem exo-β-1,3-glucanase genes in the common mushroom, *Agaricus bisporus*. Curr. Genetics, 1996, 30:166-173.
[14] Yanai K, Yonekura K, Usami H, *et al*. The integrative transformation of *Pleurotus ostreatus* using bialaphos resistance as a dominant selectable marker. Biosci. Biotechnol. Biochem. 1996, 60:472-475.
[15] Sato T, Yaegashi K, Ishii S, *et al*. Transformation of the edible basidiomycete *Lentinus edodes* by restriction enzyme-mediated integration of plasmid DNA. Biosci. Biotechnol. Biochem. 1998, 62:2346-2350.
[16] Fincham JRS. Transformation in fungi. Microbiol. Revs. 1989, 53:148-170.
[17] Virgin JB, Bailey JP. The M26 hotspot of *Schizosaccharomyces pombe* stimulates meiotic ectopic recombination and chromosomal rearrangements. Genetics, 1998, 149:1191-1204.
[18] Cormack BP, Falkow S. Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen *Candida glabrata*. Genetics, 1999, 151:979-987.
[19] Kohli A, Griffiths S, Palacios N, *et al*. Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals
a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology-mediated recombination. Plant J. 1999, 17:591-601.
[20] Prudhomme M, Libante V, Claverys JP. Homologous recombination at the border: Insertion-deletions and the trapping of foreign DNA in *Streptococcus pneumoniae*. Proc. Nat. Acad. Sci. USA, 2002, 99:2100-2105.
[21] Kohli A, Leech M, Vain P, *et al*. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Nat. Acad. Sci. USA, 1998, 95:7203-7208.
[22] Urawa H, Hidaka M, Ishiguro S, *et al*., Enhanced homologous recombination caused by the non-transcribed spacer of the rDNA in *Arabidopsis*. Mol. Gen. Genomics, 2001, 266:546-555.