A new approach for breeding low-temperature-resistant *Volvariella volvacea* strains: Genome shuffling in edible fungi

Ziping Zhu[1,2]
Xiao Wu[1,3]
Beibei Lv[1,3]
Guogan Wu[1,3]
Jinbin Wang[1,3]
Wei Jiang[1,3]
Peng Li[1,3]
Jianhua He[1,3]
Jianzhong Chen[1,3]
Mingjie Chen[4]
Dapeng Bao[4]
Jinsong Zhang[4]
Qi Tan[4]
Xueming Tang[1,3*]

[1] *Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, People's Republic of China*

[2] *School of Life Science, Taizhou University, Taizhou, People's Republic of China*

[3] *Key Laboratory of Agricultural Genetics and Breeding, Shanghai Academy of Agricultural Sciences, Shanghai, People's Republic of China*

[4] *National Engineering Research Center of Edible Fungi, Shanghai, People's Republic of China*

Abstract

Volvariella volvacea is difficult to store fresh because of the lack of low-temperature resistance. Many traditional mutagenic strategies have been applied in order to select out strains resistant to low temperature, but few commercially efficient strains have been produced. In order to break through the bottleneck of traditional breeding and significantly improve low-temperature resistance of the edible fungus *V. volvacea*, strains resistant to low temperature were constructed by genome shuffling. The optimum conditions of *V. volvacea* strain mutation, protoplast regeneration, and fusion were determined. After protoplasts were treated with 1% (v/v) ethylmethylsulfonate (EMS), 40 Sec of ultraviolet (UV) irradiation, 600 Gy electron beam implantation, and 750 Gy ^{60}Co-γ irradiation, separately, the lethality was within 70%–80%, which favored generating protoplasts being used in following forward mutation. Under these conditions, 16 strains of *V. volvacea* mutated by EMS, electron beam, UV irradiation, and ^{60}Co-γ irradiation were obtained. The 16 mutated protoplasts were selected to serve as the shuffling pool based on their excellent low-temperature resistance. After four rounds of genome shuffling and low-temperature resistance testing, three strains (VF_1, VF_2, and VF_3) with high genetic stability were screened. VF_1, VF_2, and VF_3 significantly enhanced fruit body shelf life to 20, 28, and 28 H at 10 °C, respectively, which exceeded 25%, 75%, and 75%, respectively, compared with the storage time of V23, the most low-temperature-resistant strain. Genome shuffling greatly improved the low-temperature resistance of *V. volvacea*, and shortened the course of screening required to generate desirable strains. To our knowledge, this is the first paper to apply genome shuffling to breeding new varieties of mushroom, and offers a new approach for breeding edible fungi with optimized phenotype. Volume 63, Number 5, Pages 605–615, 2016

Keywords: *genome shuffling, Volvariella volvacea, low-temperature-resistance, protoplast, edible fungi*

Address for correspondence: Xueming Tang, 2901 Beidi Road, Shanghai 201106, People's Republic of China. Tel.: +86 21 62202746; Fax: +86 21 62202746; e-mail: saas_xmtang@foxmail.com.

Received 26 August 2014; accepted 15 May 2015

DOI: 10.1002/bab.1420

Published online 18 October 2016 in Wiley Online Library (wileyonlinelibrary.com)

1. Introduction

Volvariella volvacea, the edible straw mushroom and a highly nutritious food source, is grown on an industrial scale in many tropical and subtropical regions of Southeast Asia because of its short production cycle and low output cost [1]. Earlier empirical observations on the health-promoting properties of *V. volvacea* have been reinforced by an increasing number of reports ascribing immunomodulating, antitumor, and hypocholesterolemic activity to various components isolated from mushroom fruit bodies and mycelium [2]. However, fruit body

is difficult to store fresh, as after harvest, the stipe grows rapidly and the cap opens and ages. This reduces nutritional value and shelf life greatly. Additionally, *V. volvacea* cannot remain fresh at low temperatures, the fruit body becomes soft and rots at 0–10 °C. Thus, maintaining freshness is a constraining factor in *V. volvacea* production [3].

V. volvacea is a homothallic fungus without clamp connections among mycelium, and the lack of genetic selection markers makes its crossbreeding difficult [4]. Many traditional mutagenic strategies have been applied in order to select out strains resistant to low temperature, but few commercially efficient strains have been produced [5–7]. Additionally, *V. volvacea* has been transformed with exogenous antifreeze proteins using a particle gun, but few desirable strains with low-temperature resistance were obtained [8]. Currently, screening for *V. volvacea* strains resistant to low temperature is a difficult task internationally [9]. Genome shuffling is a new efficient approach for breeding of microorganisms that involves multiple rounds of protoplast fusion to allow for recombination of genomes of the starting populations [10]. Genome shuffling is evolution at molecular level over the whole genome and can extend reorganization from a single gene to the entire genome by DNA recombination (more exchange of genome fragments between the parents) [10]. When there is a lack of genomic information concerning starting strains, genome shuffling offers a distinct advantage over recombinant DNA techniques [11]. Zhang et al. [10] successfully used genome shuffling for rapid enhancement of tylosin production in *Streptomyces fradiae*. Lactic acid was produced effectively from the shuffled strain, which was a recombinant of *Lactobacillus delbrueckii* and *Bacillus amyloliquefaciens* [12]. Improved ethanol production by a xylose-fermenting recombinant yeast strain was achieved using genome shuffling [13].

As the genetic characteristics of *V. volvacea* are unclear, most of the strains selected for low-temperature resistance have been produced by mutation, which cannot satisfy the requirement of production because mutations enhance low temperature tolerance but not to a sufficient degree. Development of genome shuffling offers a way to produce *V. volvacea* strains with improved low-temperature resistance.

2. Methods

2.1. Strains and medium

For this study, we collected 16 *V. volvacea* strains that represented most of the cultivated strains in Southeast Asia (Table 1). Vegetative mycelium were grown on potato dextrose agar (PDA) medium at 32 °C and protoplasts were cultured on regeneration PDA medium (PDA with mannitol).

2.2. Preparation and regeneration of protoplast

Strains were incubated for 3 days at 32 °C in 100 mL of PDA liquid medium for static culture [14]. Cultures were harvested by filter, washed twice with distilled water, and dried with sterile paper. Then 200 mg of mycelium was added with 1 mL aliquot of lywallzyme solution (15 g/L, purchased from The Microbiology Institute of Guangdong, Guangzhou), which contained 0.6 M osmotic stabilizer and was incubated at 32 °C for different time intervals (Table 2). The suspensions were filtered, and centrifuged at 3,400 rpm (1,100*g*) for 10 Min. The obtained protoplasts were collected and washed twice with 0.6 M osmotic stabilizer. The pure protoplasts were then diluted to 1×10^5 cfu/mL with regeneration PDA medium, spread on PDA and regeneration PDA medium respectively and cultured for 72 H at 32 °C. The rates of protoplast regeneration were determined by counting colonies using the following formulas: protoplast regeneration rate (%) $= (A - B)/C \times 100\%$, where A is the total number of colonies counted on PDA medium containing 0.6 M osmotic stabilizer, B is the number of colonies counted on PDA medium without osmotic stabilizer, and C is the number of protoplasts spread [15].

In order to identify the optimum conditions for protoplast regeneration, the protoplast regeneration rates with different mycelium age, osmotic stabilizer, concentration of lywallzyme, hydrolysis temperature, hydrolysis time, and cultural temperature were determined (Table 2).

2.3. Mutagenesis

Protoplasts of V23, V106, and V97 (randomly selected) were mutagenized with either ethylmethylsulfonate (EMS) at different concentrations of 0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 1.75%, and 2% (v/v) at 32 °C for 1 H; ultraviolet (UV) irradiation (15 W, 30 cm) for different treatment intervals of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 Sec [16]; ^{60}Co-γ irradiation (10 Gy/Min) of different doses of 0, 300, 450, 600, 750, and 900 Gy; or electron beam implantation with different doses of 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 Gy (implantation sources were produced by an ion-beam bioengineering instrument devised by Shanghai Academy of Agricultural Sciences) and then spread on regeneration PDA plates. The trials of each strain were done on five paralleled PDA plates. The plates were then incubated at 32 °C for 3 days. The rates of protoplast lethality were determined by counting colonies using the following formulus: protoplast lethality (%) $= (1 - X/Y) \times 100\%$, where X is protoplast regeneration rate after mutation, and Y is protoplast regeneration rate before mutation. The data were the average of the three experimental strains (V23, V106, and V97).

2.4. Protoplast inactivation

The protoplast suspensions of V23, V106, and V97 were inactivated by heat or UV radiation. For heat inactivation, the protoplasts were kept at 50 °C for 1, 2, 3, 4, 5, and 6 Min, respectively; for UV inactivation, protoplasts were placed 30 cm away under a 30 W UV lamp for 70, 75, 80, 85, 90, 95, 100, 105, 110, and 115 Sec, respectively. After serial dilution, the inactivated protoplast suspensions were regenerated by regeneration PDA medium. The plates were then incubated at 32 °C for 72 H. When 100% of protoplasts were inactive, the time interval of treatment was deemed

TABLE 1 *Starting strains and the source*

Strain number	*Strain name*	*Storage time of mycelia at 0 °C (H)*	*Storage time of fruiting body at 10 °C (h)*	*Source of strains*
1	V23	15.6	16.0	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
2	V9715	12.6	12.5	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
3	V9	14.8	15.2	Sanzhen Biological S.&T. Co., Sangming, China
4	V106	15.2	15.8	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
5	V97	15.3	15.8	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
6	V874	13.6	14.2	Institute of Vegetables, Guangdong Academy of Agricultural Sciences
7	V5-2	12.8	13.3	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
8	VW	11.5	10.6	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
9	VG	12.6	11.1	Institute of Vegetables, Guangdong Academy of Agricultural Sciences
10	VT-1	10.2	11.3	Taiwan China
11	VT-2	10.4	12.2	Taiwan China
12	V14	11.3	12.0	The Chinese University of Hong Kong
13	NO3	9.5	11.0	Tha Wung, Thailand
14	VB2	13.2	13.6	Qingpu District, Shanghai
15	V5	13.5	15.5	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences
16	V28	15.3	16.0	Edible Fungi Institute, Shanghai Academy of Agricultural Sciences

TABLE 2 *Different treatment condition of protoplast regeneration*

Factor	*Treatment*
Mycelia age	36, 48, 60, 72, 84, and 96 H
Osmotic stabilizers for generation	Sucrose, sorbitol, mannitol, KCl, NaCl, and $MgSO_4$
The concentration of lywallzyme	0.5%, 1%, 1.5%, 2%, 2.5%, 3%
Hydrolysis temperature	28, 30, 32, 34, and 36 °C
Hydrolysis time	1.5, 2, 2.5, 3, 3.5, and 4 H
Temperature of regeneration	28, 30, 32, 34, and 36 °C
Osmotic stabilizers for regeneration	Sucrose, sorbitol, mannitol, KCl, NaCl, and $MgSO_4$

suitable for protoplast inactivation of all 16 starting strains.
The inactivated protoplasts were then used for fusion.

2.5. Protoplast fusion

Protoplasts of V23 and V97 were fused using a modified method of Dai and Copley [17]. 77(and)-278.2(36)]TJ/T1_0 1 Tf 5.9776 0 0 5.9776 450.855 208.2

mixed with 0.01 M $CaCl_2$. They were then serially diluted and regenerated on PDA plates at 32 °C for 72 H. The protoplast fusion rate was calculated as follows: $a = [(b - c)/d] \times 100\%$, where a is protoplast fusion rate; b is the total colony number of fusion protoplasts on solid regeneration PDA; c is the colony number of inactivated parental protoplasts on solid regeneration PDA; and d is the colony number of parental protoplasts on solid regeneration PDA.

In order to identify the best conditions for protoplast fusion, the protoplast fusion rates were determined when PEG concentration was 25%, 30%, 35%, 40%, and 45% (m/v); temperature was 28, 30, 32, 34, and 36 °C; and pH was 6.5, 7, 7.5, 8, 8.5, and 9, respectively.

2.6. Genome shuffling

The protoplasts of heat- and UV-inactivated mutation strains were mixed at equal ratios, incubated at 32 °C for 72 H, and then spread on PDA regeneration plates. Afterward, these plates were treated at 0 °C for 28 H, and then cultivated at 32 °C for 3 days to obtain the first-round shuffled protoplasts. The shuffled protoplasts were cultured continuously for five generations under low-temperature treatment at 0 °C for 28 H, those of which maintained normal growth rate, colony color, and morphology were selected out as genetically stable F_1 shuffled protoplasts. The F_1 protoplasts were collected and mixed for a second round of genome shuffling. The shuffled protoplasts in the second round were selected at 0 °C for 32 H. For four rounds of selection, the shuffled protoplasts in each round were screened by a prolonged 4 H of low-temperature treatment compared with the treatment time of the last round. Each generation of shuffled protoplasts was defined as F_1, F_2, F_3, and F_4, respectively.

2.7. Low-temperature test of shuffled fruiting body

The genome shuffled strains were cultured according to the method described [1]. After harvested, the fruiting bodies were kept at 10 °C [18], and were then slit open to observe whether they liquefied or not every 2 H.

2.8. Random amplified polymorphic DNA amplification and data analysis

Random amplified polymorphic DNA (RAPD) amplification was performed in a volume of 15 μL 1× PCR buffer (TaKaRa, Dalian) containing 90 ng template DNA, 0.6 μM each primer (Table 3), 75 μM each dNTP, 1.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa, Dalian) . The PCR parameters were 2 Min at 94 °C followed by 1 Min at 94 °C, 1 Min at 36 °C, and 1 Min at 72 °C for 40 cycles, followed by a final extension of 10 Min at 72 °C. The amplified bands with different intensities and locations were detected and analyzed by the Quantity One 4.1 (BioRad, Shanghai) software [19, 20].

3. Results

3.1. Preparation and regeneration of *V. volvacea* protoplasts

Preparation of protoplasts is the most important step for successful protoplast mutagenesis, fusion, regeneration, as well as genome shuffling. Many factors affect protoplast formation, for example, age of mycelium, cell wall hydrolytic enzymes, stabilizer, and hydrolytic process including hydrolytic time, temperature, pH, and aeration. In order to obtain protoplasts with high quantity, quality, and activity, the most significant factors such as mycelium age (Fig. 1a), enzyme concentration (Fig. 1b), hydrolytic time (Fig. 1c), hydrolytic temperature (Fig. 1d), stabilizer (Fig. 1e), and temperature of protoplast regeneration (Fig. 1f) were analyzed by testing the yield of protoplasts and the regeneration rate. As can be seen from Fig. 1a, when the bacteria age was 60 H, protoplast yield reached the highest, 3.56×10^7 individual/mL. Protoplast regeneration rate reached a peak as 2.56%, while the bacteria age was 84 H. Therefore, fungus mycelium at 60 H was used in preparation of protoplasts. The best enzyme concentration was determined as 1.5% (m/v) in protoplast preparation while protoplast yield reached maximum, 2.51 × 107 individual/mL (from Fig. 1b). Figure 1c shows that protoplast yield was up to 3.56 × 107 individual/mL after enzymolysis for 3 H. After being hydrolyzed for 2 H, protoplast regeneration rate was up to 2.17%. Considering the protoplast yield and regeneration rate, optimal time and reaction temperature for enzyme hydrolysis was determined as at 32 °C and 3 H, respectively (Fig. 1d). As shown in Fig. 1e, different osmotic stabilizer had different effect on protoplast yield and regeneration rate. When potassium chloride, sodium chloride, and magnesium sulfate were used, protoplasts cannot regenerate. The optimum temperature for both preparation and regeneration of protoplasts was 32 °C. At the same concentration (0.6 M), mannitol was the best choice compared with other osmotic stabilizers such as sucrose, sorbitol, KCl, NaCl, and $MgSO_4$.

3.2. Mutagenic dose effect on lethality

The relationship between mutagen doses and lethality rate of protoplasts was shown in Fig. 2. Protoplasts of *V. volvacea* were mutated by UV irradiation, EMS, electron beam implantation, and ^{60}Co-γ irradiation, respectively. Lethality of the mutated protoplasts was assayed by comparison with untreated protoplasts and used to establish a dose–effect curve.

Mutation lethality increased as the mutagen dose went up. As a result of different mutation mechanisms and injuries, different growth conditions appeared after the protoplasts were treated by each of the four mutagens respectively. It favored generating protoplasts with forward mutation when the lethality was within 70%–80%. The dose–effect curve showed that 40 Sec of UV irradiation caused a lethality of 70.27%, and 1% (v/v) EMS caused a lethality of up to 71.20% [21]. When the doses of electron beam implantation and 60^{Co}-γ irradiation were 600 and 750 Gy, respectively, the lethality was

TABLE 3 *Primers used in RAPD*

Primer number	*Sequence*	*Primer number*	*Sequence*	*Primer number*	*Sequence*
1	TCGGCGATAG	13	[redacted]ACCGACA	25	AGGTGACCGT
2	TTGGCACGGG	14	GAAGCCAGCC	26	GGAGGGTGTT
3	GTGTGCCCC[redacted]	15	GGACCTGCTC	27	CATTCGAC[redacted]
4	GTAGACCCG[redacted]	16	CAGGCCCTTC	28	GACGAC[redacted]
5	CCACAGCAGT	17	AGTCAGCCAC	29	ACAGTAGCGG
6	GTGAGG[redacted]	18	AATCGC[redacted]	30	GGGATC[redacted]
7	GTCGCC[redacted]	19	AGGGGTCTTG	31	TTGCAGGCAG
8	TTGGCACGGG	20	GAAACGGGTG	32	GTGTCGCGAG
9	GTGTGCCCC[redacted]	21	GTGACGTAGG	33	AGATTCC[redacted]
10	GAGAGCCAAC	22			

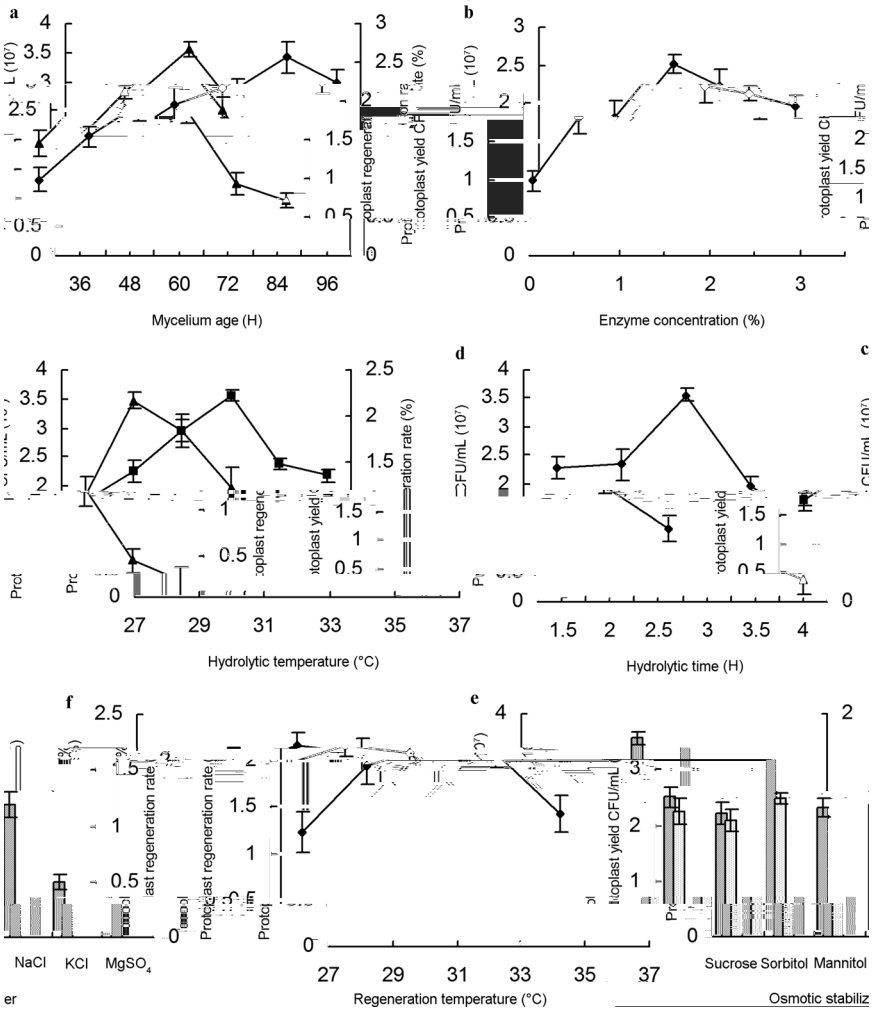

FIG. 1 *Different factors affecting the production yield and regeneration of V. volvacea protoplasts. (a) Mycelia age; (b) enzyme concentration; (c) hydrolytic time; (d) hydrolytic temperature; (e) stabilizer; and (f) temperature of protoplast regeneration.*

The shuffled strains VF_1, VF_2, and VF_3 and the starting strain (V23) were cultured to test their low-temperature storage time and commodity characteristics. The harvested fruiting bodies were stored at 10 °C and timed until they liquefied. The storage times of VF_1, VF_2, and VF_3 were 20, 28, and 28 H, respectively, significantly longer than that of V23 (Fig. 5).

3.7. RAPD analysis

To confirm genome shuffling, an RAPD polymorphism analysis was carried out using parental mutant strains and shuffled strains. The parental mutant strains (1–16) showed different DNA patterns although some of them seemed similar. For example, parental mutant strain 2 was similar to parental mutant strain 5 and parental mutant strain 12 was similar to parental mutant strain 14. However, the band (with yellow circle) appeared in strain 5 but was not present in strain 2. Similarly, the band (with yellow circle) appeared in strain 12 but was not present in strain 14. According to the RAPD profiles, there were apparent differences between the RAPD profiles of parental mutant strains (1–16) and the three shuffled strains VF_1, VF_2, and VF_3 (17–19) (Fig. 6). Unique bands were marked with red circles. It showed that genetic information was transferred from the parental strains to the shuffled strains by genome shuffling.

4. Discussion

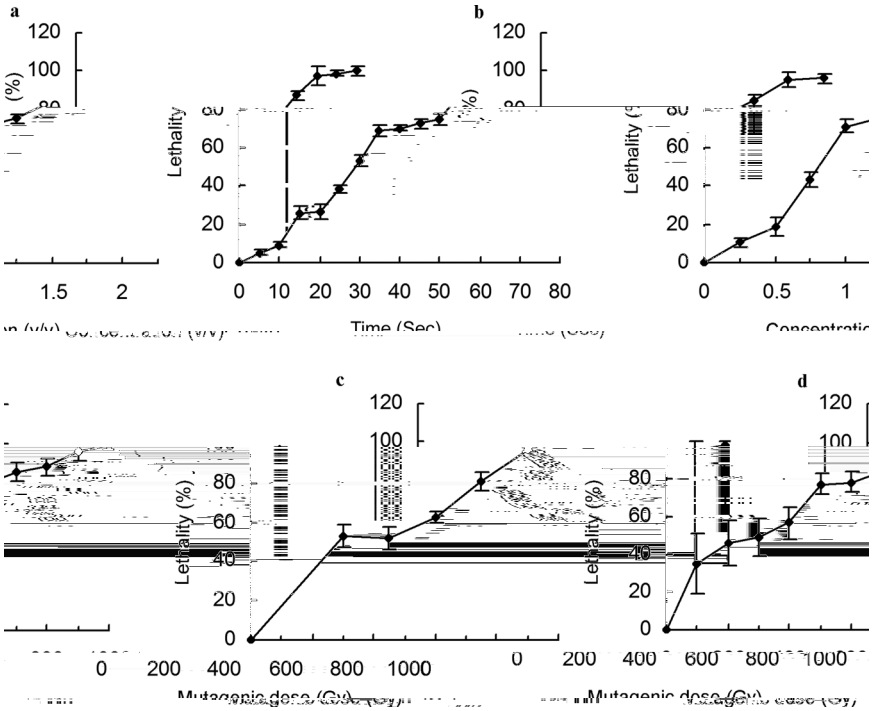

FIG. 2 *Dose effect of different mutagens on protoplasts of V. volvacea. (a) UV irradiation; (b) EMS; (c) electron beam implantation; and (d) ^{60}Co-γ irradiation.*

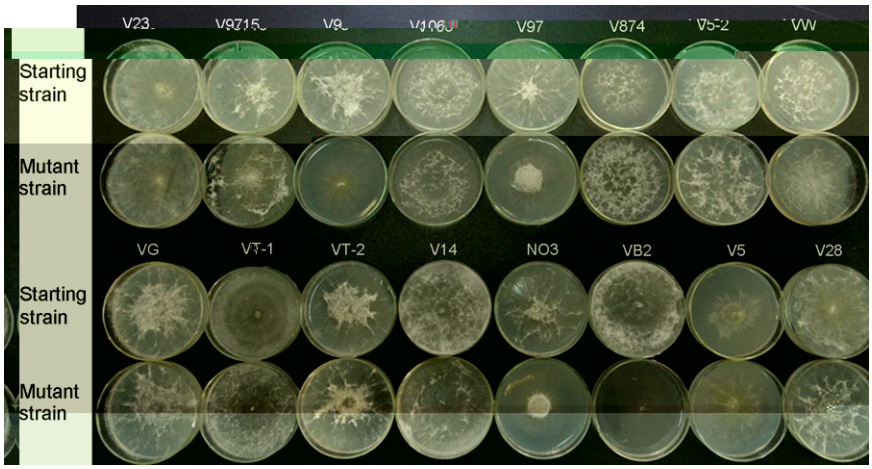

FIG. 3 *The selected 16 mutated strains and their starting strains.*

study, genome shuffling was first applied in producing strains of *V. volvacea* with enhanced low-temperature resistance.

Genome shuffling is a technology for strain improvement based on protoplast fusion, combining the advantage of multi-parental crossing allowed by DNA shuffling with the recombination of entire genomes normally associated with conventional breeding [10]. Traditional mutagenesis strategies have negative results such as insensitivity and output decrease with other disadvantages of high cost, low efficiency of mutation, and accumulation of negative mutations during repeated mutation cycles. Genome shuffling was first successfully applied for rapid enhancement of tylosin production in *S. fradiae*. Since then, this method has been widely used for strain improvement of bacteria [23], actinomycetes, and lower fungi, but not edible fungi because it needs a long cultivation period and a lot of screening work [24–29].

Genome shuffling is evolution at molecular level over the whole genome by DNA recombination. Multiple favorable phenotypes can occur simultaneously and can be used in a broader range of species trait to optimize the recombination [30, 31]. Similarly, the reason for improved low temperature resistance of the mutant strains is not just a point mutation of a

TABLE 4 *The condition of mutant strains of V. volvacea with low-temperature resistance*

Strain number	Strain name	Mutant strain number			
		UV	EMS	Electron beam implantation	^{60}Co-γ
1	V23	5*	6	5	5
2	V9715	3	5	5	5*
3	V9	2	2*	1	2
4	V106	3*	2	2	2
5	V97	2*	1	2	2
6	V874	3	3	4*	2
7	V5-2	3	2	3	3*
8	VW	3	3	2*	1
9	VG	4	3	3	4*
10	VT-1	3	5	2*	2
11	VT-2	5*	3	2	3
12	V14	3	3*	3	4
13	NO3	2*	3	4	3
14	VB2	2*	3	3	2
15	V5	3	3	4	2*
16	V28	5	4*	2	3

*The strains selected as parental strains for genome shuffling.

TABLE 5 *Low-temperature resistance of fusants of genetic stability*

Generation	Fused protoplast number	Selected fused protoplast number at 0 °C
F_1	310	54
F_2	212	40
F_3	244	43
F_4	201	20

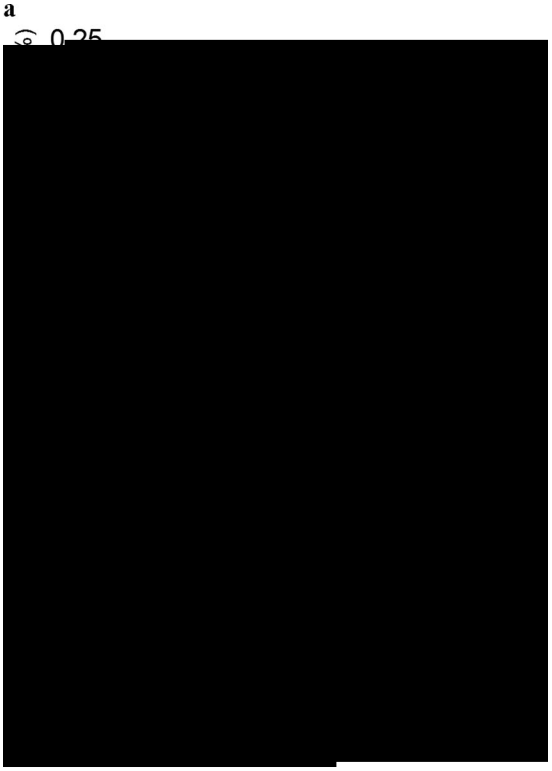

FIG. 4 *Different factors affecting the fusion rate of protoplasts (a) PEG concentration; (b) temperature; and (c) pH.*

gene; it must be the recombination of multiple gene fragments. In this study, the genome sequence of the mutant strains has changed from RFPD result, although the recombination location remains unknown. To provide more detailed evidence to explain the phenomenon at molecular level is an important direction for future study.

As starting material, mycelium of *V. volvacea* with stable characters favored mutant screening by avoiding characteristics separation along with meiosis and genetics recombination. Besides, it was convenient to culture mycelium for breeding without the limitation of season. Mycelium is composed of polycells connected by cell walls, and so it is difficult to determine which cells have been mutated. After separated by enzymolysis, protoplasts are easily screened according to the mutant

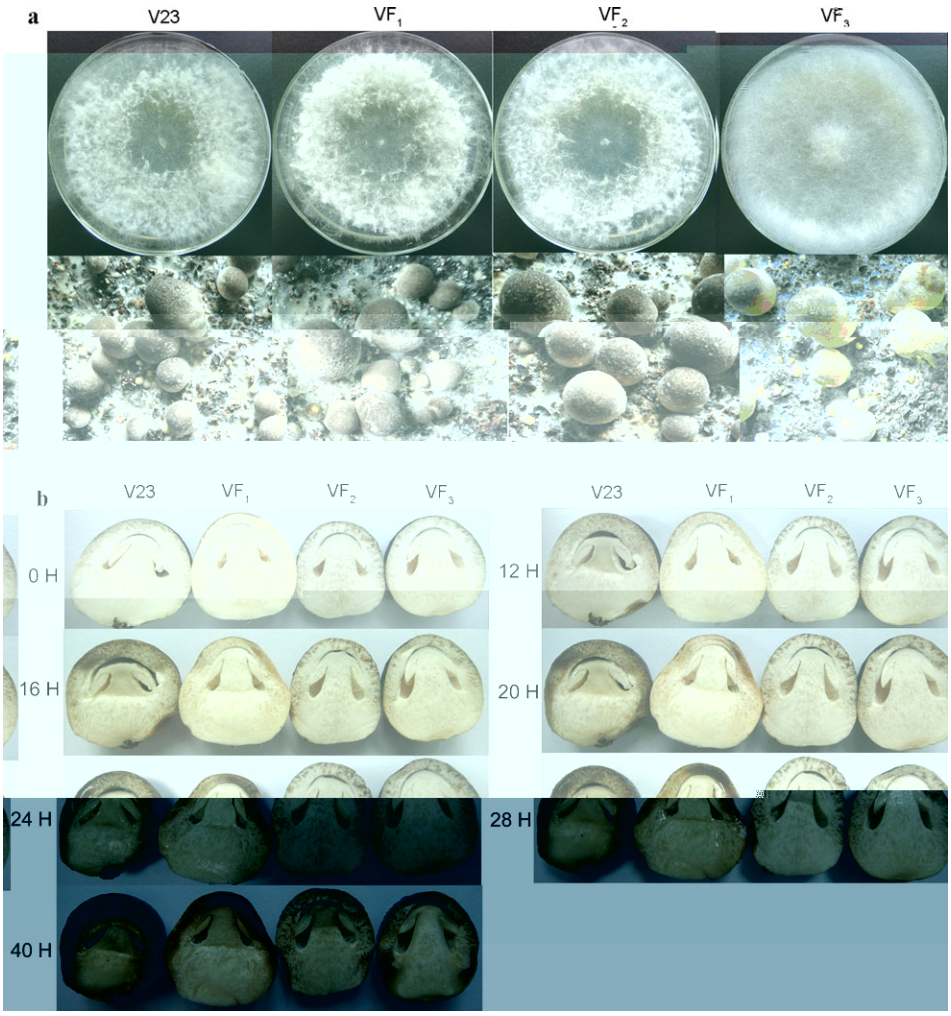

FIG. 5

The two development stages and low-temperature testing of strains V23, VF1, VF2, and VF3. (a) Aerial mycelia and fruiting bodies of V23, VF1, VF2, and VF3; (b) longitudinal sections of 10 °C-treated V23, VF1, VF2, and VF3, respectively, after 0, 12, 16, 20, 24, 28, and 40 H.

trait. Protoplasts, without cell wall surrounded, are sensitive to their environment, therefore, by mutation treatments, more mutation of the genes can be obtained [32].

In this study, protoplasts were mutated by UV irradiation, EMS, electron beam implantation, and ^{60}Co-γ irradiation. Different mutagenic mechanisms of the four methods cause different mutated sites on genes. UV irradiation induces pyrimidine dimers by crosslinking of two neighboring pyrimidines, especially of thymine. Nucleobases can be alkalized by EMS treatment, which results in substitutions during base pairing. High-energy radiation of electron beam implantation and ^{60}Co-γ irradiation can activate the inner electrons and trigger atomic ionization or combination, leading to covalent bond rupture of genes. Compared with ^{60}Co-γ irradiation, a unit dose of electron beam implantation causes more DNA double-strand breaks, resulting a higher mutation rate and a wider mutation spectrum [33–35]. Thus, a fixed dose of the mutagen could induce mutation in specific sites and stably damage DNA. In this study, four methods of mutation could generate mutations in different genes of *V. volvacea* with wide mutation spectrum.

Parental library construction is a key step of genome shuffling. The 16 starting strains of *V. volvacea* were collected from different regions and countries, including most commonly used strains in production. These strains contain the most complete information of the whole genome of *V. volvacea*, constituting a large parental pool with population diversity. The starting strains with abundant genetic information and multiple mutant strategies contribute to obtaining a mutant strain library rich in genetic information and low-temperature resistance genes. After four rounds of genome shuffling and selection under low temperature, large low-temperature-related genes were accumulated by recombination, leading to the production of a high low-temperature-resistant strain (Fig. 7).

As an important factor of fungal protoplast isolation and regeneration, osmotic stabilizer has an important role in the formation and regeneration of protoplasts. Within a

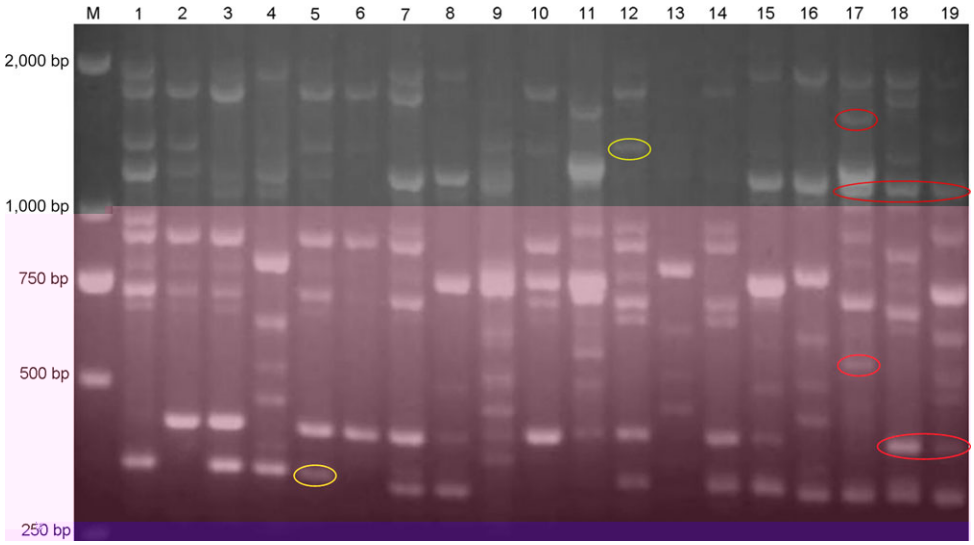

FIG. 6 *RAPD profiles of the shuffled and parental mutant strains. Lanes 1–16, 16 parental mutant strains; lanes 17–19, VF1, VF2, and VF3.*

certain range of concentrations of osmotic stabilizer, as the concentration increased from low to high, protoplast yield and regeneration rates changed significantly. The highest regeneration rate of protoplasts was obtained at 0.6 M. It showed in preliminary experiments that the concentration of mannitol solution (0.6 M) was close to cell osmotic pressure. Therefore, it is believed that when the concentration of the stabilizer is at 0.6 M, the cells suffer less impact and can well maintain their physiological state [22].

It is crucial to recognize heterothallic from homothallic fusion during protoplast fusion. Usually, auxotrophic markers are used in selecting heterothallic fusion, however, it is difficult to get auxotrophic strains of *V. volvacea* [36]. In this study, parents-inactivated marker was successfully applied in screening for heterothallic fusion of *V. volvacea* by genetic combination of different treated protoplasts (heat and UV irradiation), which were inactive in different position. Thus, it could improve the efficiency of screening for recombinant fusion and low-temperature resistant fusants by enhancing the selective pressure of low temperature, and thereby avoiding complex molecular genetic markers.

From the result of low-temperature testing of shuffled fruiting bodies, it showed that the control V23 fruiting body liquefied when kept at 10 °C for 16 H, but the shuffled fruiting bodies of strains VF_1, VF_2, and VF_3 maintained good commodity qualities, and could be stored fresh for 20, 28, and 28 H at 10 °C, respectively. Thus, the shuffled fruiting bodies were superior to that of V23, which had the highest low-temperature resistance among the parental strains.

Molecular markers can reflect the genetic variation in DNA, based on the alteration of individual nucleotide sequences. RAPD polymorphisms between the parents and shuffled strains were significantly different, indicating that the genetic information of the shuffled strains had been changed. As can be seen from the Fig. 6, there are more abundant DNA bands in the three shuffled strains than in parental mutant strains. It was interesting that the RAPD patterns of the shuffled strains were not simply made up of any two parental strains but generated new unique DNA bands. It suggested that the shuffled strains might include multiple parental DNA.

The most interesting issue of this study is what change has taken place in the genome of the cold-resistant fungi. There may be some changes in the genome of fungi, leading to efficiency of certain metabolic pathways being increased or reduced, one or more signal transduction pathway being activated or deactivated, or some anti-freeze protection agents, such as some proteins, polysaccharides, and so on, being generated. In short, it is not the contribution of such a simple change as a single gene mutation to generate cold-tolerant fungi. Cold resistance of fungi can be enhanced by more complex genetic changes. In another way, genes recombination caused multiple genes to change their expressions. These changes might lead to alter the amount of materials in cytoplasm, such as KCl, NaCl, trehalose, or chaotropic compounds [37–39]. The character of cytoplasm became different, so, low-temperature resistance of the cell has been improved. In future research, the amounts of material in cytoplasm will be detected, which might explain the antifreeze mechanism of mushrooms [10, 40].

Up to the present, there has been no report on genome shuffling applied in higher fungi. In this study, we successfully bred three genetically stable strains with enhanced low-temperature resistance by genome shuffling. It demonstrates that genome shuffling can improve the desired phenotype of the higher fungi strain within a short period, which lays a solid base for both the molecular study of recombinant fusions of higher eukaryotes and the application in food industry.

5. Acknowledgements

This work was financially supported by the Zhejiang Provincial Natural Science Foundation of China (grant no. LQ12C02001),

the Shanghai Agricultural Science Committee Key Foundation of China (grant no. 2014), the China Postdoctoral Science Foundation (grant no. 2013M531197), the Shanghai Agricultural Commission Youth Talents Program (2015 (1-30)) and (2014 (1-15)), the Shanghai Agricultural Commission Program (2015-5-6), and the Shanghai Academy of Agricultural Sciences Run Program (ZP17).

6. References

[1] Chang, S. T. (1993) pp. 3–17, Hong Kong: The Chinese University Press.
[2] Chen, S., Ge, W., and Buswell, J. A. (2004) FEMS Microbiol. Lett. 230, 171–176.
[3] Yang, X. (1988) pp. 408–418, Beijing: Agricultural Press.
[4] Chang, S. T. (1978) pp. 573–605, New York: Academic Press.
[5] Han, Y., Chen, M., Cao, H., Pan, Y. (2004) Mycosystema 23, 417–422.
[6] Li, Y., Zhao, M., Ji, Z. (2004) J. Nanjing Agric. Univ. 27, 58–61.
[7] Liu, Z., Zhang, K., Lin, J., and Guo, L. (2011) Sci. Hortic. 130, 18–24.
[8] Guo, L., Lin, J., Xiong, S., and Chen, S. (2005) Acta Microbiol. Sin. 45, 39–43.
[9] Liu, X., Liao, S., and Chen, Z. (2011) Food Sci. 32, 260–264.
[10] Zhang, Y. X., Perry, K., Vinci, V. A., Powell, K., Stemmer, W. P., and del Cardayré, S. B. (2002) Nature 415, 644–646.
[11] Gong, J., Zheng, H., Wu, Z., Chen, T., and Zhao, X. (2009) Biotechnol. Adv. 27, 996–1005.
[12] John, R. P., Gangadharan, D., and Nampoothiri, K. M. (2008) Bioresour. Technol. 99, 8008–8015.
[13] Zhang, W., and Geng, A. (2012) Biotechnol. Biofuels 5, 46.
[14] Li, G., and Li, B. (1999) Mycosystema 18, 79–88.
[15] Cui, Z., and Luo, X. (2003) Mycosystema 22, 498–501.
[16] Xu, Z., Li, G., and Li, B. (2004) Acta Sci. Nat. Univ. Sunyatseni 43, 84–87.
[17] Dai, M., and Copley, S. D. (2004) Appl. Environ. Microbiol. 70, 2391–2397.
[18] Wang, F., Gong, X., Gao, J., and Liu, R. (1990) Edible Fungi 4, 35–37.
[19] Chandra, M., Kalra, A., Sangwan, N. S., Gaurav, S. S., Darokar, M. P., and Sangwan, R. S. (2009) Bioresour. Technol. 100, 1659–1662.
[20] Wang, P., Liu, Y., Yin, Y. G., Jin, H. J., Wang, S. X., Xu, F., Zhao, S., and Geng, X. L. (2011) Int. J. Biol. Sci. 7, 209–220.
[21] Luo, Y., Li, J.G., and Yi, L. (1998) J. Sichuan Normal Univ. (Nat. Sci.) 21, 581–586
[22] Cray, J. A., Russell, J. T., Timson, D. J., Singhal, R. S., and Hallsworth, J. E. (2013) Environ. Microbiol. 15, 287–296
[23] Zhao, J., Li, Y., Zhang, C., Yao, Z., Zhang, L., Bie, X., Lu, F., and Lu, Z. (2012) J. Ind. Microbiol. Biotechnol. 39, 889–896.
[24] Gao, X., Zhao, H., Zhang, G., He, K., and Jin, Y. (2012) Curr. Microbiol. 65, 128–132.
[25] Lv, X., Jin, Y., Li, Y., Zhang, H., and Liang, X. (2013) Appl. Microbiol. Biotechnol. 97, 641–648.