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Transfer of *Ephedra* Genomic DNA to Yeasts by Ion Implantation

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Abstract The genomic DNA from *Ephedra glauca* was randomly transferred to *Saccharomyces cerevisiae* and *Hansenula anomala* by argon and nitrogen ion implantation. Through repeated subculturing and using reversed phase high-performance liquid chromatography analysis to quantify the concentrations of the secondary metabolites, *l*-ephedrine and *d*-pseudoephedrine, 12 recombinant strains of genetically stable yeast were obtained, each using glucose as a carbon source, $NaNO_3$ as a nitrogen source and producing *l*-ephedrine and/or *d*-pseudoephedrine. After culturing in liquid medium for 72 h, extracellular *l*-ephedrine and *d*-pseudoephedrine concentrations of 18.85 and 4.11 mg/L, respectively, were detected. Using *l*-ephedrine and *d*-pseudoephedrine as the target products, the transformation efficiencies of the genomic DNA from *E. glauca* transferred to *S. cerevisiae* and *H. anomala* were 1.15% (1/87) and 2.13% (8/376), respectively. The addition of the amino acid, L-Phe, to culture media substantially changed the amount of *l*-ephedrine and/or *d*-pseudoephedrine produced by the recombined yeasts. However, the change in metabolite production was not consistent among strains, rising in some, while dropping to nondetectable levels in others. After random amplification of polymorphic DNA (RAPD) analysis, four RAPD primers were obtained from the initial 100 RAPD primers, each amplifying different fragments with the recombined yeast Ar_Han0458 genome. Using one primer as polymerase chain reaction primer, the result showed that the recombined yeast Ar_Han0458 genome matched *E. glauca* genomic DNA at 150 bp, indicating a successful transfer of genetic information, facilitated by ion implantation.

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Introduction

l-Ephedrine and *d*-pseudoephedrine are the main alkaloid secondary metabolites of arid and semiarid desert plants belonging to the genus *Ephedra*. This plant group has important medicinal value and derivatives are found in more than 300 currently available ephedrine-containing drugs. The demand for ephedrine on the world market is predicted to be 2,600–3,000 t per year over the next several years. China is the only country extracting ephedrine from uncultivated *Ephedra*; about 200–300 t of plant biomass are needed to produce 1 t of ephedrine [1]. *Ephedra* is the only genus in the family Ephedraceae, a group of woody shrubs. There are about 40 species of *Ephedra* found in various dry regions around the world, where they have important soil–water conservation functions, serve as wind breaks, and help maintain the fragile arid and semiarid desert environments. The seeds of *Ephedra* are difficult to germinate, and very few seedlings are actually produced in the wild. For this reason, lost wild *Ephedra* resources are difficult to restore. Because of the commercial demand for *Ephedra*, residents often harvest wild-growing plants, causing further deterioration of desert environments which may already be under pressure from human encroachment, habitat destruction, and pollution.

Although questions have recently been raised regarding the safety of some over-the-counter products containing ephedrine, demand for the chemical is likely to continue to grow. Commercial production of *l*-ephedrine and *d*-pseudoephedrine using modified microorganisms, therefore, should lessen the impacts to wild *Ephedra* populations and help protect deserts where these plants are found. However, the biosynthesis pathways of *l*-ephedrine and the *d*-pseudoephedrin are complicated and controlled by multiple genes [2–5]. In addition, critical data about the gene related to *l*-ephedrine biosynthesis is lacking. As a result, no strains have been constructed using conventional gene engineering methods. Given the economic significance of ephedrine, construction of a recombined strain of cells able to produce *l*-ephedrine and *d*-pseudoephedrin, using *Ephedra* genomic DNA transformation, would have theoretical and practical applications.

In the mid-1980 s, induced mutation of rice by ion implantation was reported for the first time in Ion Beam Bioengineering Key Lab, Institute of Plasma Physics, Chinese Academy of Sciences [6]. A new highly interdisciplinary field, ion beam bioengineering, was initiated and has commanded increasing interest [7]. Since then, ion implantation as a new genetic modification method has been widely applied in industrial and agricultural fields and has acquired a series of achievements [8, 9]. More recently, breakthroughs have been made to use ion implantation for the direct transfer of exogenous macromolecules into biological cells.

Ion implantation is a relatively new method for creating transgenic organisms [10]. Yu et al. [11] reported the successful GUS and CAT gene transfer into the suspension cells and mature rice embryos following the 20–30-keV argon (Ar)–ion implantation. The activities of GUS and CAT gene in the receptors transferred by ion implantation had been detected. Song et al. [12] transferred genomic DNA of the medicinal plant gingko (*Ginkgo biloba*) to watermelon (*Citrullus lanatus*) by ion implantation. The product bilobalide was detected in the leaves of the transformed watermelon plant, which confirmed the successful transfer of multiple genes. In the current study, ion implantation methods were used to induce DNA macromolecule transformation [13]. The random transfer of *Ephedra glauca* genomic DNA

to *Saccharomyces cerevisiae* and *Hansenula anomala* was investigated. The goals of this study were to obtain a transgenic yeast strain for *l*-ephedrine and *d*-pseudoephedrine production and to provide chemical and molecular evidence of the feasibility and success of genetic transformation.

Materials and Methods

Reagents, Instruments, and Yeast Strains

S. cerevisiae 21882 and *H. anomala* 2340 were obtained from the China General Microbiological Culture Collection Center (Institute of Microbiology, Chinese Academy of Sciences [CAS], Beijing, China).

Reagents used in this investigation were glucose·H_2O, $NaNO_3$, yeast extract, $K_2HPO_4{\cdot}3H_2O$, $MgSO_4{\cdot}7H_2O$, agar, bromothymol blue (BTB), acetonitrile (chromatographically pure), silicate sand, silica gel, cetyl trimethylammonium bromide, and genomic DNA extraction buffer (including RNase), obtained from ABI (Forster, USA). *l*-Ephedrine and *d*-pseudoephedrine standards were supplied by the Xinjiang International Industry Co. (Urumqi, China); the standards met USA Food and Drug Administration criteria. Random amplification of polymorphic DNA (RAPD) primers and *Taq* polymerase were obtained from Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China). An IBB Device 1 Ion Implanter (Institute of Plasma Physics, CAS), Sigma 3-18K centrifuge (Sigma, St. Louis, MO, USA), and TC-512 thermal cycler (Techne, Minneapolis, MN, USA) were used in this study. A Waters 1525 Reversed Phase-High Performance Liquid Chromatograph (RP-HPLC; Waters Corporation, Milford, MA, USA) was used for the analysis of *l*-ephedrine and *d*-pseudoephedrine.

Media and Protection Solution

YPD medium [14]: yeast extract powder 1.0%, peptone 2.0%, glucose·H_2O 2.0%.

BTB agar plate medium: glucose·H_2O 10.0%, $NaNO_3$ 1.0%, yeast extract powder 0.5%, $K_2HPO_4{\cdot}3H_2O$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, adjusted to a pH of 7.0 with 10% NaOH before adding 0.02% BTB and 1.0% agar.

Agar slant medium: glucose·H_2O 10.0%, $NaNO_3$ 1.0%, yeast extract powder 0.5%, $K_2HPO_4{\cdot}3H_2O$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, adjusted to a pH of 7.0 with 10% NaOH before adding 1.0% agar.

Liquid medium: glucose·H_2O 10.0%, $NaNO_3$ 1.0%, yeast extract powder 0.5%, $K_2HPO_4{\cdot}3H_2O$ 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, adjusted to a pH of 7.0 with 10% NaOH.

Wild *E. glauca* Sampling and Preparation of Genomic DNA

The methods used for sampling of wild *E. glauca* and preparation of genomic DNA are described by Shao and Cao [15].

Yeast Cell Film Preparation with the Protection Solution

The inoculum was prepared by transferring a loop of yeast cells from slant medium into YPD liquid medium, followed by culturing on a rotary shaker at 220 r/min for 12 h at 28–30°C. The fermentation broth was diluted to 1.0×10^7 CFU/mL with protection solution

[11], and 0.1 mL was spread in the middle of a sterile 90-mm Petri dish with a sterile glass spatula and air dried under aseptic conditions.

Ion Beam Implanting Yeast Cell Film [16]

The yeast cell films were placed, separately, on the aseptic sample holder in the vacuum target chamber of the ion implantation facility. The films were implanted using argon or nitrogen ions (Ar^+ or N^+) at 15 KeV and a dose of 15×10^{15} ions/cm^2 at a vacuum pressure of 10^{-3} Pa. Some yeast cell films were treated under vacuum conditions, but not implanted by Ar^+ or N^+; these were used as controls.

Transfer of *E. glauca* Genomic DNA and Recombinant Screening

After ion implantation, the treated cells were immediately placed in 2 mL of 400 μg/mL *E. glauca* genomic DNA in TE buffer, statically incubated for 2 h at 28–30°C, and then stirred continuously with a sterile glass spatula for 2 min. The eluent was collected and 0.1 mL was spread evenly on BTB agar plates using a sterile glass spatula; the plates were inverted and cultured for 72 h at 28–30°C. Yeast colonies were selected if they did not produce yellow indicative halos. The yeast cell films not implanted by Ar^+ or N^+ were treated with the same methods. Some yeast cell films implanted by ions were placed in 2 mL of aseptic water, instead of *E. glauca* DNA, and used as controls.

Cultivation of Recombined Yeast and Detection of Ephedrine

The recombined yeast cells grown on BTB indicative plates were inoculated into test tubes, cultured for 72 h at 28–30°C, then inoculated into liquid medium and grown at 28–30°C for 72 h with continuous shaking at 230 r/min. At the end of the incubation period, yeast cells were removed from the culture broth by centrifugation at 8,000×*g* for 10 min. The supernatant was collected and assayed for extracellular *l*-ephedrine and *d*-pseudoephedrine.

The operational specifications of the RP-HPLC were UV2487 double-crossing ultraviolet detectors, detection wavelength (λ) of 210 nm, Kromasil 100-5 C18 column (4.6×150 mm), Breeze 3.30 Chromatograph Data Operation Station, mobile phase 0.02M dihydropotassium phosphate/acetonitrile (95 5 ratio), and a flux of 1.2 mL/min. The sample quantity (injection volume) was 10.0 μL. Standard samples were used for instrument calibration, which occurred one or two times every three to five samples (to reduce potential testing error).

Random Amplification of Polymorphic DNA Analysis

One hundred arbitrary primers, S1 to S20, S61 to S80, S181 to S200, S301 to S320, and S381 to S400, were tested with polymerase chain reaction (PCR) in order to select the best profiles for polymorphism. Reactions were conducted in 10 μL of reaction mixture, containing 1 μL of template DNA, 1 μL of 2.5 μmol/L dNTP, 0.5 μL of 2 mmol/L $MgCl_2$, 4 μL of 2 μmol/L primer, 1 μL of 10× buffer, 0.1 μL of 5 U/μL Taq polymerase, and 2.4 μL of H_2O. Initial denaturation was for 2 min at 93°C, followed by 45 cycles of 2 min at 93°C, 2 min at 34°C, 90 s at 72°C, and a final extension at 72°C for 10 min. PCR products were loaded into agarose gels prepared with 1.5% agarose at 5 V/cm and stained with 0.25 μg/mL ethidium bromide and photographed over UV light.

Results and Discussion

Preliminary Screening in BTB Agar Plate Medium

The N atom on the side chain of the *l*-ephedrine and *d*-pseudoephedrine molecules confers strong alkalinity, resulting in pKa values of 9.58 and 9.74, respectively. BTB is an indicator of pH values ranging from 5.8–7.6 (yellow to blue). Blue halos surrounding the yeast indicated possible secretion of *l*-ephedrine and/or *d*-pseudoephedrine into the medium, since the pKa of *l*-ephedrine or *d*-pseudoephedrine is higher than 7.6. By screening with BTB, 988 successful recombinants were identified among all the treatments (Table 1). No successful recombinants were found in either the vacuum condition nonimplanted yeast cells that had been placed in *E. glauca* genomic DNA, or the control cells that were ion implanted and placed in water.

After screening for positive yeast clones on the BTB agar plate medium, 12 recombinant strains of genetically stable yeasts were obtained after eight subculture cycles (Table 2). When *S. cerevisiae* 21882 was used as the inoculum, only one recombined yeast strain (N_Sce0806) was obtained. The remaining 11 yeast strains (Ar_Han0406, Ar_Han0451, Ar_Han0458, N_Han1016, N_Han1025, N_Han1159, N_Han1179, N_Han2010, N_Han3101, N_Han3140, and N_Han3161) were obtained when *H. anomala* 2340 was used as the inoculum. These 12 strains remained stable in the T_{11} generation and were still producing *l*-ephedrine and/or *d*-pseudoephedrine, (based on RP-HPLC results).

Impacts on *l*-Ephedrine and *d*-Pseudoephedrine Production

Eight of the 12 recombined strains in the T_8 generation (Ar_Han0406, Ar_Han0458, N_Sce0806, N_Han1016, N_Han1159, N_Han2010, N_Han3101, and N_Han3161) produced *l*-ephedrine, and two strains (Ar_Han0451 and N_Han3140) produced *d*-pseudoephedrine. N_Han1025 and N_Han1179 produced both *l*-ephedrine and *d*-pseudoephedrine (Table 2).

When the recombined strains were cultured in the liquid medium augmented with 5 mg/mL L-Phe under the same conditions, *l*-ephedrine and/or *d*-pseudoephedrine concentrations were altered, suggesting different regulatory mechanisms (Table 2). With the addition of L-Phe, biosynthesis of *l*-ephedrine by Ar_Han0406, N_Han1179, N_Han2010, and N_Han3101 was apparently inhibited, as none was detected using RP-HPLC. The concentration of *d*-pseudoephedrine produced by these four strains, on the other hand, increased. Conversely, biosynthesis of *d*-pseudoephedrine by Ar_Han0451 was inhibited by L-Phe while the concentration of *l*-ephedrine produced by this strain was elevated by a substantial amount. In N_Sce0806, inclusion of L-Phe in the culture medium stimulated

Table 1 Number of positive yeast clones obtained using N^+ and Ar^+ implantation.

Yeast strains	Positive yeast clones	
	N^+	Ar^+
S. cerevisiae	87 (1)[a]	67 (0)
H. anomala	376 (8)	458 (3)

[a] Number (in bracket) of stably inherited positive yeast clones

Table 2 Concentration of *l*-ephedrine and/or *d*-pseudoephedrine in extracellular solutions of T_8 generation recombined yeasts cultured for 72 h.

Strain no.	Not add. L-Phe		Add. L-Phe	
	l-Ephedrine (mg/L)	*d*-Pseudoephedrine (mg/L)	*l*-Ephedrine (mg/L)	*d*-Pseudoephedrine (mg/L)
Ar_Han0406	2.35	nd	nd	13.60
Ar_Han0451	nd	4.11	19.04	nd
Ar_Han0458	11.87	nd	0.16	nd
N_Sce0806	4.06	nd	15.84	nd
N_Han1016	3.46	nd	3.04	6.72
N_Han1025	8.35	1.32	3.44	11.04
N_Han1159	2.44	nd	0.72	10.08
N_Han1179	18.85	1.47	nd	36.24
N_Han2010	0.21	nd	nd	3.92
N_Han3101	5.02	nd	nd	1.84
N_Han3140	nd	2.88	4.32	0.48
N_Han3161	3.47	nd	4.16	0.40
S. cerevisiae 21882	nd	nd	nd	nd
H. anomala 2340	nd	nd	nd	nd
Liquid medium	nd	nd	nd	nd

nd no detection

higher production of *l*-ephedrine but had no apparent effect on *d*-pseudoephedrine production, which remained below detection.

There are six types of alkaloids in the medicinal plant, *Ephedra*, represented by three pairs of isomers [1]. The results of RP-HPLC revealed some unknown peaks in the recombinant samples, as shown at the retention times (RTs) of 9.320, 9.820 (Fig. 1), and 12.235 min (Fig. 2). The peaks may represent isomers of *l*-ephedrine and/or *d*-

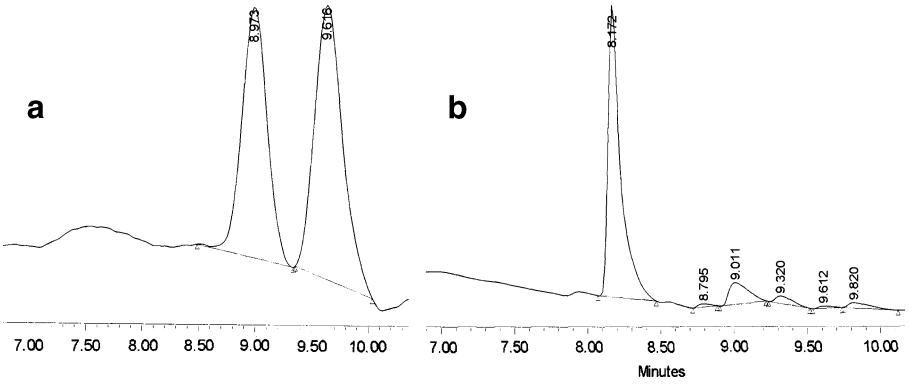

Fig. 1 RP-HPLC analysis of *l*-ephedrine and *d*-pseudoephedrine in the extracellular solution of strain N_Han1179 of T_8 generation recombined yeasts. **a** Standard samples: *l*-ephedrine (RT=8.973 min), *d*-pseudoephedrine (RT=9.616 min). **b** Extracellular solution of strain N_Han1179, cultured for 72 h of T_8 generation yeasts: *l*-ephedrine (RT=9.011 min), *d*-pseudoephedrine (RT=9.612 min)

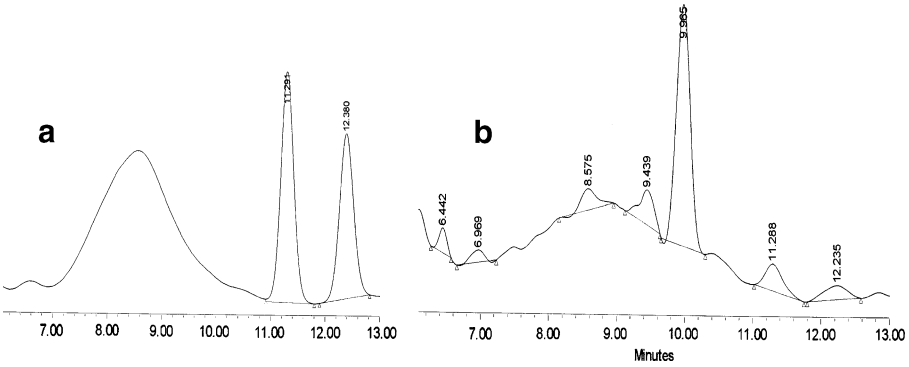

Fig. 2 RP-HPLC analysis of *l*-ephedrine and *d*-pseudoephedrine in the extracellular solution of strain Ar_Han0458 of T_8 generation recombined yeasts. **a** Standard samples: *l*-ephedrine (RT=11.291 min), *d*-pseudoephedrine (RT=12.380 min). **b** Extracellular solution of strain Ar_Han0458 cultured for 72 h of T_8 generation yeasts: *l*-ephedrine (RT=11.288 min)

pseudoephedrine. The results are strongly indicative of a successful transfer of exogenous genomic DNA to yeast.

The Transformation Efficiency of *E. glauca* Genomic DNA Transfer to Yeast

After assaying for extracellular *l*-ephedrine and *d*-pseudoephedrine, the transformation efficiency stability values of *E. glauca* genomic DNA, transferred into *S. cerevisiae* 21882 and *H. anomala* 2346 using N^+ implantation, were 1.15% (1/87) and 2.13% (8/376), respectively (Table 1). These data suggest that the conservation of expression patterns in *S. cerevisiae* 21882 is higher than in *H. anomala* 2346, and the restorative ability of the *S. cerevisiae* 21882 genome is also greater.

During ion implantation, the nonbioactive Ar^+ does not react with target tissue and can escape from the target surface as Ar gas. Therefore, when using ion implantation-mediated DNA macromolecule transformation, the argon ion, which is chemically more stable, is better choice for engraving. Other studies have identified argon as the preferred ion for implantation because of its chemical properties which are favorable to a high transformation efficiency of exogenous DNA and the stability of recombined offspring [17]. In the current study, however, bioactive N^+ was more efficient than Ar^+ for implantation. It is possible that the sedimentation of implanted N^+ augmented the biosynthesis of nitrogenous substances (including ephedrine, pseudoephedrine, and their aromatic amino acid precursors). For the biosynthesis of alkaloid and nitrogenous substances using transformation-mediated exogenous DNA via ion implantation, therefore, N^+ implantation may be the preferred option due to higher transformation efficiency and greater recombinant stability.

RAPD Analysis of the Recombined Yeast

One hundred RAPD primers were initially tested for PCR in order to select the best profiles for polymorphism of strain Ar_Han0458. In the same PCR mixture, the reaction was amplified with *H. anomala* 2346 genomic DNA, Ar_Han0458 genomic DNA, and *E. glauca* genomic DNA. After RAPD analysis, four RAPD primers were obtained from the initial 100 RAPD primers, each one amplifying different fragments.

The Ar_Han0458 fragment amplified with primer S313 was identified by electrophoresis and sequence analysis, which was performed by the Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China), indicating a length of 439 bp. Similarity searches on nucleotide sequences were carried out using BLAST at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The 150-bp fragment of the 5′-flanking region did not match any sequence in the database. The 3′-flanking region of the fragment was found to align with the *S. cerevisiae* genome. BLAST comparison of the 3′-flanking region nucleotide sequence noted an 88% identity with the *S. cerevisiae* genome. The multiple sequence was done using the multiple sequence alignment editor (Fig. 3).

PCR primers were designed from the 150-bp fragment sequence of the 5′-flanking region. *H. anomala* 2346 genomic DNA, Ar_Han0458 genomic DNA, and *E. glauca*

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439_bp          ATGAAGATGCTTAGTTAGATAATGAGTTCGATGTGTGTAAATACA     45
S.cerevisiae    ATATTAAAGTTTACTTTGAAGAAATATTCTATTACTACAGAACAA     45

439_bp          AATAAACAATTTAAGAACAATT...TTAAAGTACATTAAGATGAT     87
S.cerevisiae    AATC..TAATTTAGAAATTTTTGCTTTAAAAATAAGTGTTATGTT     88

439_bp          ATTTTATTTTCAAAAAACAAAGAA.......GAAAAAGCCCCCAA    125
S.cerevisiae    ATATTGTATTGTTTGCTTTGAGGAATTTTTTGAGTTATCTTTTTT    133

439_bp          TAATAAATTCGTGTATATAATAATGAAATTTGTTACATAACTTAA    170
S.cerevisiae    TTTTTAGATCTTGCTTA.AAGAATGAC.TATTTTACATAATCTAA    176

439_bp          TCTTGAGTTTTTTGAGAACCACGTAACAAACCAGTTCTTCTAGCA    215
S.cerevisiae    TCTTGGGTCTTTTGAGAACCACGTAACAAACCAGTTCTTCTGGCG    221

439_bp          GCAATCAAACCAGCCTTTTGACCTGGAACAGCACCTCTTGAAATG    260
S.cerevisiae    GCAATCAAACCGGCCTTTTGACCAGAAACAGCACCTCTAGAGATG    266

439_bp          GTTGAAGCTTTACCAATATGTTGATGGTTACCACCACCGTGAGGA    305
S.cerevisiae    GTAGAAGCCTTACCAATATGTTGATGGTTACCACCACCGTGAGGG    311

439_bp          TGGTCAACTGGATTCATAGCAACACCACGAGTTTTTGGCCATGAG    350
S.cerevisiae    TGATCAACTGGATTCATGGCAACACCACGGGTCTTTGGCCAAGAG    356

439_bp          TTACGTTTAACTCTGTATTTGTGGAAGGCTCTACCAGCTTTCAAT    395
S.cerevisiae    TTTCTCTTCAATCTGTACTTGTGGAAAGCACGACCAGCCTTCAAC    401

439_bp          AATGGTTTATCAACACGACCACCACCGGCAATGACACCAATGAC     439
S.cerevisiae    AATGGTTTGTCAACTCTACCACCACCGGCAATGACACCAATGAC     445
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Fig. 3 Multiple alignment of the differential fragment from strain Ar_Han0458 genomic DNA and *S. cerevisiae* nucleotide sequences. Four hundred thirty-nine base pairs amplified with RAPD primer S313

genomic DNA were used to generate DNA bands (Fig. 4). A visual comparison of the banding patterns shows that *E. glauca* and recombined yeast Ar_Han0458 share an identical banding profile at about 150 bp (Fig. 4, lanes 1 and 2). No common band was observed with template *H. anomala* 2346 genomic DNA (Fig. 4, lane 3). The results indicate the 150-bp sequence from *E. glauca* had been successfully transferred. The functions of 150-bp fragment need to be confirmed.

Conclusions

While it may seem improbable that the large genomic DNA of higher plants could be placed into microorganisms, fruitful transfer of genetic material has been achieved on several occasions. In the case of medicinal plants, only multiple genes of genomic DNA, related to biosynthesis of secondary metabolites, have been successfully transferred into microorganisms [2, 18]. When the biosynthesis pathway and its controlling genes are unknown, the generation of engineered strains capable of producing a secondary plant metabolite presents a practical challenge. The results of the current study have shown that ion implantation technology represents a new transgenic method of generating those desired strains.

Previous research [10, 17] demonstrated that ion beam etching results in the formation of microholes and passages in the surface of the cell layer, providing transfer channels for exogenous genes. Due to the accumulated positive charge, the polarity of the cell surface shifts, which reduces electrostatic repulsion and fosters a more favorable environment for the electrically negative exogenous DNA in an aqueous solution, thus facilitating transfer into the cell. The ion-etched pathways accumulate a positive charge and the negatively charged exogenous DNA is actively attracted into the receptor cell. Ion implantation occurs in a vacuum, and water in the receptor cells must be exhausted. When dry receptor cells are

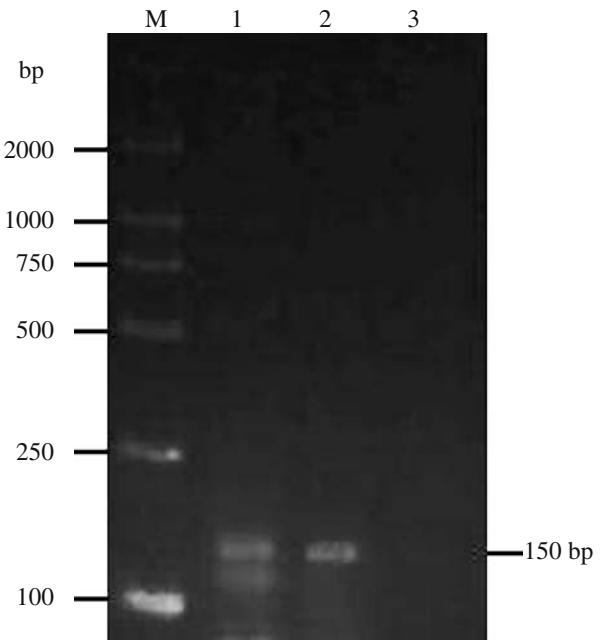

Fig. 4 The fragment in recombined yeast Ar_Han0458 genomic DNA from *Ephedra* genomic DNA. *M* DNA marker DL2000, *lane 1 E. glauca* genomic DNA, *lane 2* recombined yeast Ar_Han0458 genomic DNA, *lane 3* *H. anomala* 2340 genomic DNA

placed in a DNA-containing solution, the probability that exogenous DNA will enter cells is increased due to the effect of absorption and expansion. In addition to an enhancement of passage into the cells, the ion implantation process also causes direct (e.g., DNA breakage) and indirect (e.g., effects of free radicals) damage to the receptor cell chromosomes; this increases the efficiency of exogenous DNA insertion and integration into the host chromosome. Ion beam-induced exogenous DNA transfer is a new method for molecular hybridization between different species.

Historical research has shown that aromatic amino acid biosynthesis is involved in the synthesis of many secondary metabolites in higher plants [19, 20]. The 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthetic enzyme was apparently not subject to feedback inhibition by any of the aromatic amino acids that were produced. However, biosynthesis of aromatic amino acids in yeasts appears to be almost exclusively restricted to the synthesis of L-Phe, L-Tyr, and L-Trp. The three isoenzymes of DAHP synthetic enzymes were suppressed by the three corresponding aromatic amino acids. The biosynthesis pathways of L-Phe and L-Tyr in yeasts are apparently different from those of plants. Although there appears to be little amino acid homology of DAHP enzyme synthesis between plants and yeasts, the dynamic characteristics were, nevertheless, similar. The DAHP enzyme of plants functions well in deficient yeasts, and the deficient host strain exhibits the same DAHP characteristic of not being affected by aromatic amino acid-induced feedback inhibition [19, 20].

The data collected through this study show that the relevant genes, or gene clusters, responsible for the biosynthesis of ephedrine in plants are expressed in yeasts. In addition, the relevant enzyme genes regulating the synthesis of aromatic amino acids in plants are probably transferred and expressed in yeasts. The RAPD analysis also show that some exogenous DNA from *E. glauca* were transferred to yeasts by ion implantation.

The advantage of using ion implantation-induced DNA transformation to create transgenic species is that it does not require prior, intimate knowledge of the genome, and it is not necessary to obtain the target gene sequence. In addition, this process results in higher transformation stability. Regardless of whether the biosynthesis pathway of the secondary metabolite has been clearly defined or the gene related to biosynthesis has been confidently identified, ion implantation can be used as a new transgenic method to obtain easily cultured recombined yeast strains that provide the secondary metabolite. Desirable producing yeast strains can be screened from nonproducing strains using the appropriate method(s). The successful recombinants would be appropriate and valuable for further investigations of genomics, proteomics, and metabonomics, with a goal of securing the genes related to biosynthesis of the natural metabolite, as well as information on genetic regulation.

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