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Total DNA of Glycyrrhiza uralensis transformed into Hansenula anomala by ion implantation: Preparing Glycyrrhizic acid in recombined yeasts

JIN Xiang[1] LÜ Jie[1,3] MA Yuan[2] MAO Peihong[1,*]

[1]*Center of Ion Beam Biotechnology*, *College of Physics and Technology*, *Xinjiang University*, *Urumqi 830008*, *China*

[2]*College of Resource and Environment Science*, *Xinjiang University*, *Urumqi 830046*, *China*

[3]*College of Life Science and Pharmacy*, *Nanjing University of Technology*, *Nanjing 210009*, *China*

Abstract Glycyrrhizic acid (GA) in Glycyrrhiza uralensis (G. uralensis) is physiologically active. In this study, the total DNA of wild G. uralensis was randomly transformed into Hansenula anomala by implantation of low-energy Ar^+ and N^+, to produce five recombinant yeast strains relating to biological synthesis of the GA or Glycyrrhetinic acid (GAs). After culturing in liquid medium for 96 h, the resultant GA, 18α-GAs and 18β-Gas were determined by reversed-phase high performance liquid chromatography (RP-HPLC), and the corresponding concentrations were 114.49, 0.56, and 0.81 mg·L^{-1}. After one hundred primers were analyzed with random amplified polymorphic DNA (RAPD), the seven different DNA fragments were produced by the N7059 strain of recombined yeasts, and, the polymerase chain reaction (PCR) verified that one of them came from the genome of G. uralensis, indicating a successful transfer of genetic information by ion implantation.

Key words Ion implantation, total DNA of G. uralensis, Transformation, Recombinant yeast, Glycyrrhizic acid.

1 Introduction

G. uralensis, a perennially deep-rooted herbaceous plant with saline-alkaline tolerance, is of importance for water and soil conservation, soil amelioration and wind blowing-off, and sand fixation of desert and semi -desert. Also, it is a special medicine of cold-/heat-resistant characteristics[1]. Glycyrrhizic acid (GA) in G. uralensis has a physiologically corticotrop-in-like activity, such as anti-aging and anti- inflammation, lowering blood pressure, enhancing physical immunity and physiological functions, alleviating hyper-lipidemia, and maintaining water-electrolyte balance. The high-dose GA can block severely respiratory syndrome (SARS) of virus replication[2,3], accelerate immune recovery of patients with acquired immune deficiency syndrome (AIDS)[4,5], and induce interferon that inhibits cancer cell growth in a manner. In addition, the GA potassium salt, a calorie-free and high-intensity sweetening agent that is 500 times sweeter than sucrose, can be used to produce supplementing food of disease-preventing features. Because of these, however, excessive G. uralensis picking and grubbing has been endangering the plant species, and ecological environment as well.

The GA as a source-dependent product is usually extracted from G. uralensis, but not chemically synthesized due to its complex structure. Thus far, the GA microbial strains are still constructed by conventional genetic engineering, because the GA biologically synthesized by the microbial fermentation is limited by a little underlying DNA information. To protect the ecological environment in arid and semiarid deserts, transforming the genomic DNA of G. uralensis into recombinant strains is suitable, so as to produce the GA via chemical synthesis.

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* Corresponding author. *E-mail address:* phmao@china.com

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The ion implantation into a living body, as a new transgenic method, has an unique mechanism[6]. Song *et al.*[7] transformed the genomic DNA of ginkgo into a watermelon, and detected the ginkgolides in the watermelon leaves, indicating that the ginkgolides can be biologically synthesized and expressed by the genomic DNA of ginkgo in the watermelon. Mao *et al.*[8] and Lu *et al.*[9] reported that the genomic DNA of ephedra was transformed into yeasts by ion implantation, thus producing genetically stable recombinant yeasts for the ephedrine or pseudo-ephedrine synthesizing.

In this study, the total DNA of G. uralensis was transformed into Hansenula anomala with Ar^+ or N^+ implantation[10], and the resultant recombinant yeast strains could produce the GA.

2 Materials and methods

2.1 Reagents and yeast

All reagents were purchased from Sigma (St. Louis, MO, USA) except otherwise stated. Hansenula anomala 2340 was obtained from the Chinese General Microbiological Culture Collection Center.

2.2 Film of yeast cells

The inoculum was prepared by transferring a loop of yeast cells from slant to liquid medium of yeast extract peptone dextrose (YPD)[11], and cultured by a rotary shaker of 220 r/min at 28–30°C for 12 h. On diluting the fermentation broth into 1.0×10^7 CFU/mL (Colony-forming Unit) in protection solution[8], the 0.1 mL solution was spread at the middle of 90-mm petri dish by a glass spatula under sterile condition, and dried in aseptic air.

2.3 Ion implantation

An implanter (Institute of Plasma Physics, Chinese Academy of Sciences) was used for the Ar^+ or N^+ implantation under 10^{-3} Pa as previously described[8,9]. The dried film of yeast cells was implanted by doses of 15×10^{15} ions/cm^2 at 15 keV, and the same film without implantation was used as a control.

2.4 Introduction of genomic DNA for G. uralensis

The total DNA of G. uralensis was extracted by cetyl trimethylammonium bromide (CTAB) method[12]. After ion implantation, the film of yeast cells was immediately soaked in 2-mL TE buffer and total DNA of G. uralensis (400 μg·mL^{-1}) at 28–30°C for 2 h, and eluted by a bent glass rod for 2 min. The 0.1-mL eluant was spread on the YPD agar plate, and cultured at 28–30°C for 72 h. The same sample without ion implantation was used as a negative control.

2.5 Detection of GA in the recombinant yeast

Yeast colonies growing on the YPD agar plate were inoculated aslant, and cultured at 28–30°C for 72 h. The resultant YPD medium was inoculated and the cells were cultured in a 230 r·min^{-1} mechanical shaker at 28–30°C for 96 h. After harvest by 8000-g centrifugation for 10 min, the saponins in the supernatant was determined with Liebermann-Burchard analysis, as described by Wu[13].

The GA and GAs were analyzed on a Waters Breeze System equipped with 1525 binary pump, 2487 detector of dual wavelengths absorbance, 717 plus autosampler (Waters, Milford, MA, USA), and a Kromasil 100-5 C18 column (150×4.6 mm). At the flow rate of 1.2 mL·min^{-1}, the mobile phase with the 10-μL injection volume was 0.2 mol·L^{-1} mixture of ammonium acetate, methanol, and acetonitrile (70: 20:10, v:v:v), and was repeatedly calibrated after every three to five samples.

2.6 RAPD analysis for recombined yeast

In order to select the best profiles of polymorphism, one hundred arbitrary primers for S1 to S20, S61 to S80, S181 to S200, S301 to S320, and S381 to S400 were tested by polymerase chain reaction (PCR). The 10-μL PCR mixture was composed of 1-μL template DNA, 1μL dNTP (2.5 μmol·L^{-1}), 0.5 μL $MgCl_2$ (2 μmol·L^{-1}), 4 μL primer (2 μmol·L^{-1}), 1 μL of 10× buffer, 0.1 μL Taq polymerase (5 U·μL^{-1}), and 2.4 μL H_2O. After initiated at 93°C for 2 min, the PCR was denaturalized with 45 cycles at 93, 34 and 72°C for 2, 2, and 1.5 min, and extended at 72°C for 10 min. The PCR products were loaded into 1.5% agarose gels at 5 V·cm^{-1}, stained with 0.25 μg·mL^{-1} ethidium bromide, and photographed over ultraviolet light.

3 Results

3.1 Detection of saponins in recombinant yeast

Yeasts containing the 494 or 772 strains were induced by the Ar^+ or N^+ implantation, and the total DNA of G. uralensis were transformed. A total of generation 1266 T2 in recombinant yeast strains were obtained. The Liebermann-Burchard analysis for the cultivation liquid of generation T2 and T3 in recombinant yeast strains showed that the 22.91% generation T2 contain saponins, and 27.93% positive generation T2, passed down the saponins to generation T3, indicating that about 81% generation T3 produce saponins. Contrarily, the yeasts without Ar^+ or N^+ implantation under vacuum could not produce saponins. As a control, the total DNA of G. uralensis in 2-mL sterile water, which was immersed in the yeast mycoderma after ion implantation, did not yield saponin-producing strains.

3.2 Saponins of generation T3 in the yeast strains

After the saponins of generation T3 continuously passed down through generation T8 recombinant yeasts, the N7059 strain was stable in the produced five recombinant yeast strains (Table 1). RP-HPLC results showed 114.49 mg·L^{-1} GA, 0.56 mg·L^{-1} 18*α*-Gas, and 0.80 mg·L^{-1} 18*β*-GAs in N7059 strain, respectively, and no 18*β*-GAs existed in A2053 strain. The concentrations of GA and GAs varied with the strains, indicating their difference in biological synthesis and metabolism. The results show that the induced exogenous DNA by ion implantation is a random transformation in recombinant yeasts of genetic diversity. The RP-HPLC analysis showed that the GA was not totally dissociated from an unknown component with a longer retention time (RT) in spite of its higher concentration in N7059 strain (Fig.1), nor was the GAs (Fig.2).

Table 1 The yield of GA and Gas(mg·L^{-1}) in the extracellular generation T8 recombinant yeasts for 96 h.

Strains	GA / mg·L^{-1}	18α-GAs/ mg·L^{-1}	18β-GAs/ mg·L^{-1}
A1027	60.25	0.14	0.11
A2053	65.02	0.03	–
A2163	48.41	0.18	0.20
A2474	25.25	0.42	0.81
N7059	114.49	0.56	0.80
H. anomala 2340	–	–	–
Liquid medium	–	–	–

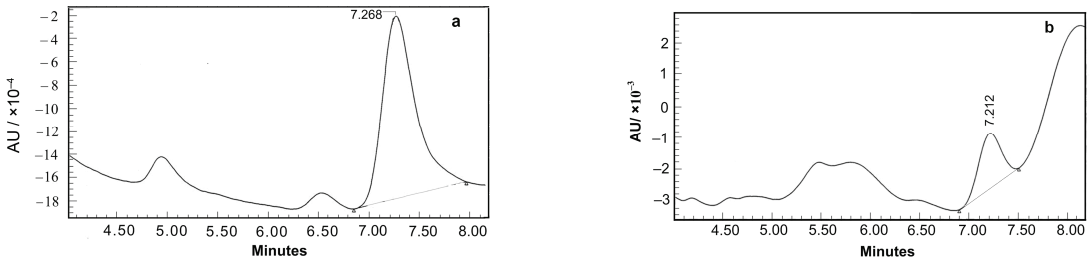

Fig.1 RP-HPLC analysis for GA. the GA standard samples with RT=7.268 min(a); The GA in N7059 strain of generation T8 recombinant yeasts for 96 h, RT = 7.212 min(b).

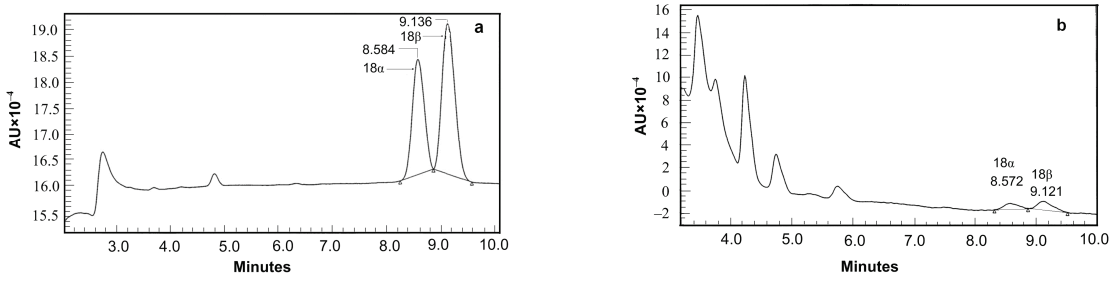

Fig.2 RP-HPLC analysis of GAs. The GAs standard samples with RT=8.584 and 9.136 min for18*α*-GAs and18*β*-GAs(a); The GAs in N7059 strain of generation T8 recombinant yeasts for 96 h, RT = 8.542 and 9.121 min for18*α*-GAs and18*β*-Gas (b), respectively.

3.3 RAPD analysis of the recombined yeast

In order to select the best profiles of N7059 strain polymorphism, one hundred RAPD primers were used for initial PCR, and amplified with the genomic DNA of 2340 in H. anomala, N7059 strain, and G. uralensis, so that the S392 random primer can amplify various fragments. Fig.3 shows that the three kinds of genomic DNA can generate different DNA bands with the primers of S392 and the four RAPD as control (S64, S65, S74, and S76). The genomic DNA of 2340 in H. anomala and N7059 strain were amplified into the identical bands in Lanes 1 and 2, but not that of G. uralensis in Lane 3, indicating that G. uralensis are successfully transformed into the recombined yeast strains by ion implantation, and probably related with biological synthesis of the GA or GAs.

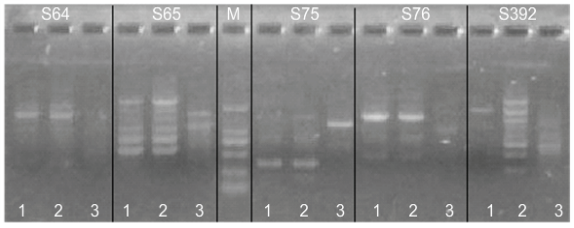

Fig.3 The genomic DNA of N7059 strain from G. uralensis in recombined yeast. RAPD primers (S64, S65, S75, S76 and S392), DNA Marker DL2000 (M); The genomic DNA of 2340 in H. anomala (Lane 1), N7059 strain (Lane 2), G. uralensis (Lane 3).

4 Discussion

The ion implantation forms a channel on the surface of cells due to a highly etching effect on receptor cells, and allows exogenous DNA to enter[13]. The implanted Ar^+ and N^+ greatly accumulate the surface of the negative receptor cells, alleviating the electrostatic repulsion of receptor cells against exogenous DNA in solution. Also, the ion implantation brings direct/ indirect damage to chromosomes in receptor cells, such as fracture, and the effect of free radicals, to increase the recombination and integration between exogenous DNA and receptor cell DNA. The exogenous DNA transformation induced by ion implantation provides a favorable approach for genetic exchange between distant and super-distant species without cloning target DNA in advance.

The total DNA of G. uralensis is successfully transformed into the yeasts by Ar^+ and N^+ implantation, and the stable saponin products is confirmed by the five transformed strains. Relating to the biological synthesis of natural medicines or secondary metabolites, many times larger the genomic DNA of natural medicines than that of yeast may be genetically transferred[8,9,15]. Without biological DNA information of natural medicines, the ion implantation inducing exogenous DNA transformation can also be employed to screen microbial engineering strains, and aptly produce natural medicines. On deeply studying genomics, proteomics, and metabonomics of genetically stable recombinant yeasts, the GA DNA may be biologically synthesized to transfer randomly the total DNA of G. uralensis into recombinant strain yeast.

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