CLONING OF RICE DNA AND IDENTIFICATION OF tRNA GENE CLONES

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ABSTRACT

DNA from 48 hr germinated rice embryos was cut with restriction endonuclease *Bam* H1 and cloned to the *Bam* H1 site on plasmid pBR 322. The clones containing recombinant DNA were selected by their sensitivity to tetracycline and resistance to ampicillin. Using ^{32}P-labelled rice embryo tRNA as a probe two clones were identified to contain tRNA genes by colony hybridization.

INTRODUCTION

THE rapid development of recombinant DNA technology has brought forth a revolution in biology[1,2]. It aids us to have a closer look at the way genes are organized, especially in the complex eucaryotic genomes[3–6]. Although many animal and yeast genes have been studied in detail using recombinant DNA technology, plant genes have seldom been targets for such studies. Germination is an ideal process to study gene expression because it effects a shift in the metabolic status of seeds from a state of dormancy to an active one. An understanding of gene organization and regulation during germination can be accomplished by molecular cloning of DNA from seeds like rice. To study the status of histone, RNA, tRNA and other genes in the rice genome, a general method was developed to clone eucaryotic DNA in a plasmid vector pBR 322. This essentially involves the following steps. The rice embryo and plasmid pBR 322 DNAs were cut with restriction endonuclease *Bam* H1 to generate sticky ends. The plasmid DNA was phosphatased, the DNAs were annealed and joined by T4 phage DNA ligase. The recombinant DNA molecules thus produced were transferred into *E. coli* and colonies containing them were selected by their sensitivity to tetracycline and resistance to ampicillin. Two clones were identified as having tRNA genes by hybridization of the DNA in the clones with ^{32}P-labelled rice tRNAs.

MATERIALS AND METHODS

The two strains of *E. coli* Hb 101 (one that harbours the plasmid pBR 322 and the other that does not) as well as enzymes *Bam* H1 and T4 phage DNA ligase were supplied by Dr. E. S. Srivatsan, University of California at San Diego, La Jolla. Rice DNA was provided by Dr. Elizabeth Zachariah of this laboratory. Trizma base, 2-mercaptoethanol, polyvinylpyrrolidone (PVP) m.w. 360,000, ampicillin, tetracycline, ATP, dithiothreitol (DTT), alkaline phosphatase and sodium dodecylsulphate (SDS) from Sigma Chemical Co., St. Louis; oligo(dT)-cellulose from Collaborative Research, Waltham, Massachusetts; bovine serum albumin (BSA) from Calbiochem, La Jolla; lysine-sepharose 4B and Ficoll, m.w. 400,000, from Pharmacia Fine Chemicals, Uppsala; BA 85 nitrocellulose filters from Schleicher and Schuell, Keene; formamide from Fischer Scientific Co., New York; and bactotryptone and bactoyeast extract from Difco Laboratories, Detroit were used. ^{32}P-Ortho phosphoric acid was from Bhabha Atomic Research Centre, Bombay. All other reagents were of analytical grade.

Luria broth contained 10 g of bactotryptone, 5 g of bactoyeast extract, 0·5 g of NaCl and 2 g of glucose per 1. Luria agar contained 1·4% agar in Luria broth.

Preparation of labelled rice tRNA

Rice seeds were germinated for 18 hr at 30° C in the dark in the presence of ^{32}P-orthophosphoric acid (0·5 mCi/100 seeds) under sterile conditions[7]. Total RNA was prepared according to the method of Palmiter[8] and purified by the method of Bellamy and Ralph[9]. Poly (A$^-$) RNA was separated by using an oligo (dT)-cellulose column[10]. The poly (A$^-$) RNAs were further separated into tRNA, rRNA and others on a lysine-sepharose 4B column[11].

Cloning

Cleaving with Bam *HI*: DNA (2 μg) obtained from 48 hr germinated rice embryos was treated with 8 units of *Bam HI* in a reaction buffer containing 20 mM tris-HCl, pH 7·0, 100 mM NaCl, 7 mM $MgCl_2$ and 2 mM 2-mercaptoethanol, in a total volume of 10 μl at 37° C for 90 min. Plasmid pBR 322 DNA (2·5 μg) was similarly cut with *Bam* H1.

Alkaline phosphatase treatment: The *Bam* H1 treated pBR 322 DNA was deproteinized first by phenol saturated with 10 mM Tris-HCl, pH 7·4, 1 mM EDTA (TE buffer) and then by chloroform. The DNA was ethanol precipitated, dried under vacuum and dissolved in 10 μl of TE buffer. This DNA was treated with 1 unit of *E. coli* alkaline phosphatase

at 37° C for 45 min, deproteinized and precipitated as before.

Ligase reaction : The *Bam* H1 restricted rice DNA was deproteinized and ethanol precipitated. Rice DNA and pBR 322 DNA obtained after the phosphatase reaction were dissolved separately in TE buffer. They were mixed (15 μl) and ligated with 0·1 unit of T4 DNA ligase in a buffer containing 1 mM ATP and 10 mM DTT by incubating at 10–15° C for 24 hr.

Transformation : Ca^{2+} shocked *E. coli* Hb 101 cells (which do not harbour pBR 322 and hence are sensitive to ampicillin and tetracycline) were transformed with recombinant DNA molecules essentially according to Mandel and Higa[12] with some modifications.

Selection of clones and colony hybridization

The colonies which grew on Luria-agar containing ampicillin (30 μg/ml) but not on agar containing tetracycline (30 μg/ml) were selected. The clones obtained were analyzed by colony hybridization according to Grunstein and Wallis[13] using ^{32}P-labelled rice *t*RNA as a probe. The clones were grown on BA 85 nitrocellulose filter circles[13] and the filters were soaked in denaturing solution containing 0·5 M NaOH, 1·5M NaCl for 5 min. They were then soaked in neutralizing solution (1·0 M NaCl, 0·2 M tris-HCl, pH 8·0, 2 × SSC) till the filter pH was around 8·0. The filters were dried at 60° C in a vacuum oven. After incubating with the pre-hybridization mix containing 6 × SSC, 5 × Denhardt's solution[13] (1·0 g Ficoll, 1·0 g PVP and 1·0 g BSA in 1 l of 3 × SSC) and 0·1% SDS for 12 hr at 40° C, the filters were incubated in the hybridization mix containing 6 × SSC, 5 × Denhardt's solution, 10 mM Tris-HCl, pH 7·5, 5 mM EDTA, 0·5% SDS, 50% formamide and ^{32}P-labelled rice *t*RNA for 60 hr at 40° C. The filters were washed with 2 × SSC containing 0·1% SDS, dried and autoradiographed.

Results and Discussion

Plasmid pBR 322 has a molecular weight of 2·7 × 10^6 and possesses resistance to two drugs—ampicillin and tetracycline[14]. *Bam* H1 cuts pBR 322 DNA at the tetracycline resistance gene while the ampicillin resistance gene (β-lactamase) remains intact. This was made use of in identifying the clones obtained by ligating *Bam* H1 restricted rice DNA to the *Bam* H1 site on pBR 322. The restriction and ligation reactions were monitored by electrophoresis on agarose gels.

An aliquot of the transformants grown in Luria broth was plated on an ampicillin-Luria agar plate and 31 colonies were obtained. These were transferred sequentially onto two plates, one containing ampicillin and the other tetracycline. Out of the 31 colonies 27 grew only on ampicillin plates showing that most of the transformants contained recombinant DNA molecules. These recombinant clones are designated as pIR_1 to pIR_{27}. Insert DNA prepared from these clones were analysed by agarose gel electrophoresis and were found to have larger size than control pBR 322 DNA.

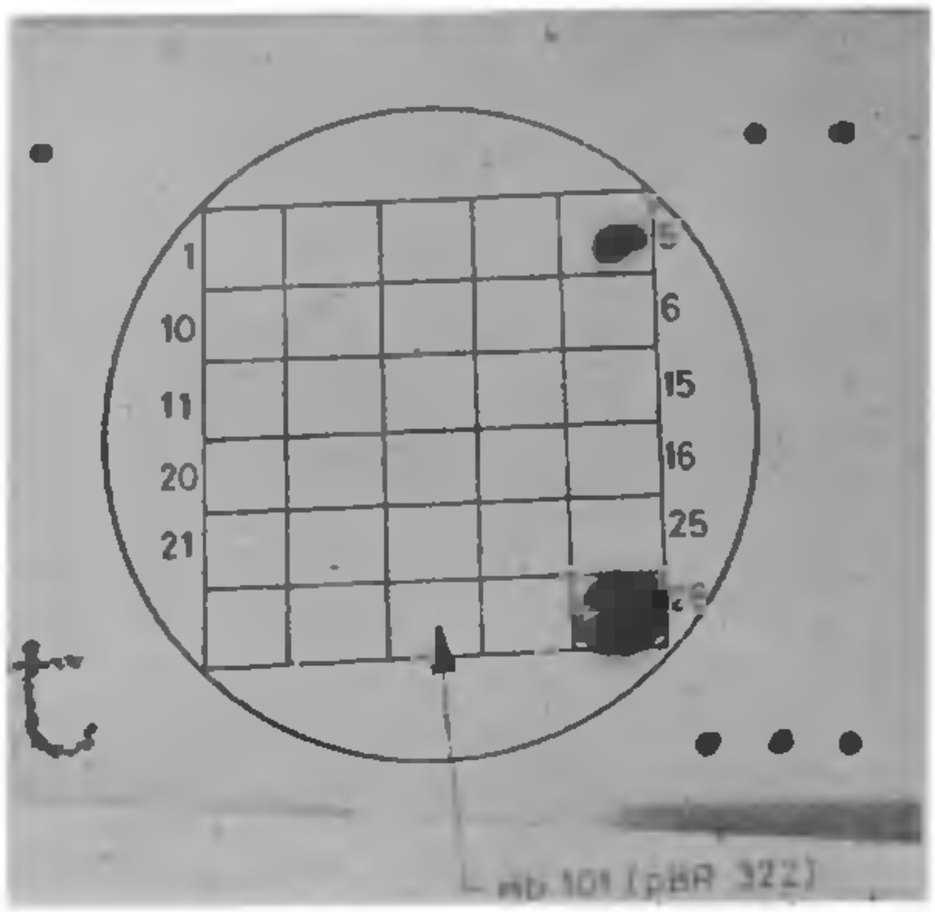

FIG. 1. The clones pIR_{1-27} were transferred to a nitrocellulose filter kept over moist Luria agar containing ampicillin and grown for 18 hr at 37° C. The filter was removed, denatured, neutralized, dried and hybridized with ^{32}P-labelled *t*RNA (6·7 × 10^6 cpm in 18 ml of hybridization mix) as described in the text. *E. coli* Hb 101 (harbouring pBR 322) served as a control. The nitrocellulose filter was washed, dried and autoradiographed. Two spots developed corresponding to the clones pIR_5 and pIR_{26}.

The autoradiographs obtained by exposing X-ray films to nitrocellulose filters after hybridization to ^{32}P-labelled rice *t*RNA showed that two clones hybridized to *t*RNA (Fig. 1). These clones have been identified to be pIR_5 and pIR_{26}. The variation in size and intensity of the spots may be due to the difference in the growth of the colonies and/or size of insert of *tRNA* genes on the plasmid. They provide an excellent system to study the molecular biology of rice *t*RNA, especially the *t*RNA gene organization and regulation. The method reported here is an easy and general one for cloning eucaryotic DNA on to the plasmid vector pBR 322 and a simple way of identifying the clones containing the recombinant DNA molecules. In combination with the rapid sequencing techniques[15,16] available now, it would be possible to make advances in the understanding of *t*RNA genes in plants.

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