source is (2.7 ± 0.3) W/cm^2. That is to say, the effective propagation distance of 2.7 W/cm^2 is 4.5 cm while the value of the linear and the nonlinear theory is 5.8 cm and 4.3 cm respectively. From fig. 2, the efficiency factor is $91.2\%\pm1.5\%$ for the transmitted intensity with 11.2 W/cm^2 at the distance of 2.5 cm. The theoretical value is 90.5%. These experimental results coincide quite well with the theory. It shows that the effective propagation distance and the efficiency factor can be used to describe the propagation of the finite amplitude sound wave in biological tissues quantitatively.

3 Conclusion

The theoretical analysis and the experimental results of the finite amplitude ultrasound wave in biological tissues show that the efficiency factor and effective propagation distance decrease while the attenuation coefficient increases due to the existence of the nonlinear effects. The reason why the ultrasound is used successfully and widely in medicine is that the ultrasound can penetrate into the tissues of the human body. Yet, the nonlinear effects of the ultrasound at biomedical ultrasound frequencies and intensities increase the attenuation of the ultrasound and limit the amount of the received ultrasound intensity as well as shorten the effective propagation distance of the ultrasound. Therefore, these nonlinear phenomena have great influence on the accuracy of diagnosis and on the efficiency of the therapy in biomedical ultrasound. Further researches into these problems are of important value to the development of the nonlinear acoustics and to the practical application of the ultrasound in biomedicine.

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Expression of self-incompatibility ribonucleases of Antirrhinum in *Escherichia coli*

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Abstract Self-incompatibility is an intraspecific reproductive barrier to prevent self-fertilization in the flowering plants. In many species, self-incompatibility is controlled by a single *S* locus with multiple alleles. So far, the only gene known in the *S* locus of the Solanaceae, Scrophulariaceae and Rosaceae encodes a class of ribonucleases, called self-incompatibility ribonucleases (S RNases), which have been shown to mediate stylar expression of self-incompatible reaction. As the first step to investigate their three-dimensional structure, we successfully expressed three biologically active S RNases of Antirrihnum (S_2, S_4 and S_5) in *Escherichia coli* (*E. coli*). Their functional expressions caused no detrimental effect on host bacteria growth and provided a basis for a large scale preparation of S RNase proteins. Possible reasons for non-lethality of S RNases on *E. coli* are discussed.

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Keywords: S ribonucleases, Antirrhinum, *Escherichia coli.*

Self-incompatibility is an intraspecific reproductive barrier to prevent self-fertilization and found widely in the flowering plants. In many species, self-incompatibility is determined by a single locus with multiple alleles, known as the *S* locus. So far, the only gene known in the *S* locus of the Solanaceae, Scrophulariaceae and Rosaceae encodes a class of ribonucleases, called self-incompatibility ribonucleases (S RNases). It has been shown that S RNases are expressed in the style to control self-incompatibility with no role in the pollen expression of self-incompatibility. The latter (pollen component) could be a gene encoding a ribonuclease inhibitor[1]. Genes homologous to S RNase genes have been identified ubiquitously ranging from virus to mammals and belong to a superfamily (S superfamily). Apart from the involvement of S RNases in self-incompatibility, no clear role has been defined for other members of the superfamily. They could be involved in defenses against pathogens and RNA metabolisms[1].

No crystal structure has been obtained for any S RNase protein from angiosperms. Clearly, determination of their three-dimensional structure aids greatly to the understanding of how S RNases function during self-incompatible reaction. As the first step towards this goal, the present authors successfully expressed three biologically active S RNases of Antirrhinum (S_2, S_4 and S_5) characterized previously [2]. Two S RNases from *Petunia inflata* were expressed using baculovirus[3]. This note reports for the first time the functional expression of three Antirrhinum S RNases in *E. coli*.

1 Materials and methods

(i) Isolation and cloning of Antirrhinum S RNase genes without signal peptide sequences. Total stylar RNA isolation from Antirrhinum lines containing S_2, S_4 or S_5, the first-strand cDNA synthesis and rapid amplification of cDNA ends (RACE) were performed as described previously[2]. S RNase-gene specific primers were as follows: S_2: Y118, 5′-CCTTTCCAATTTTTGTTCTAC-3′ and G1280, 5′-GCTTGCCCCTTTCTCAAG-3′; S_4: Y117, 5′-CCTGAACTGTTGTTCAA-3′ and G1224, 5′-ACCACCCACCTCCAAATC-3′ and S_5: Y116, 5′-TCTTTCAAGTTATTGTTTTA-3′ and G1481, 5′-ACAGTAGGAATTTTGGG-3′ . The 5′ primers (Y118, Y117 and Y116) were located just after the ends of the respective signal peptide sequences and the 3′ primers (G1280, G1224 and G1481) positioned after the stop codons of S RNase gene open reading frames (ORFs). Amplified PCR (polymerase chain reaction) products of cDNA were purified using Wizard PCR Preps Kit (Promega) before being cloned into pGEM-T (Promega), resulting in pGEM-S2, pGEM-S4 and pGEM-S5. In order to avoid PCR artifacts, cDNA sequences of these clones were further determined by DNA sequence analysis on an ABI 377 automatic sequencer (PE Applied Biosystems). The results confirmed that their sequences are the same as that published previously[2]。The cDNA containing the S RNase genes were released from the vectors by a double restriction enzyme digest with NcoI/SalI and subcloned into the *Nco* I /*Xho* I sites of the expression vector pET-15b (Novagen). The resulting plasmids, pET-S2, pET-S4 and pET-S5, were transformed into *E. coli* BL21 (Novagen).

(ii) Expression and analysis of S RNases in *E. coli.* To induce expression of S RNase genes, pET-S2, pET-S4 and pET-S5 were transformed into *E. coli* strain BL21(DE3) (Novagen) containing a gene encoding T_7RNA polymerase. When the growth of the positive clones in liquid LB broth reaches 0.6 at OD_{600}, 1 mmol/L of isopropyl-β-D-thiogalactoside (IPTG) was added for indcution. Total protein extracts of *E. coli* were prepared and analyzed by 12％SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Coomassie brilliant blue staining of proteins and assay of ribonulcease activity were mainly carried out as described before[4]. Briefly, proteins separated by SDS-PAGE containing rRNA of 0.3 mg/mL (Type III, Sigma) were renatured after extensive rinses with 10 mmol/L Tris-HCl (pH 7.4) in 25% (*v*/*v*) iso-propanol. RNase activity was detected *in situ* after staining with toluidine blue-O (Sigma). Bright bands on a light blue background of RNA indicated the positions of proteins with ribonuclease activity.

2 Results and discussion

(i) Cloning of S RNase genes into an *E . coli* expressional vector. In order to express

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Antirrhinum S RNases *in vitro*, we cloned the cDNAs encoding three S RNase genes of Antirrhinum (S_2, S_4 and S_5) into an *E. coli* expressional vector, pET-15b. S RNases are secreted extracellular proteins and therefore contain signal peptides. Before being cloned into the expressional vector, the signal peptide sequences of the three S RNase genes were removed. S_2, S_4 and S_5 cDNA fragments without signal peptide sequences were amplified from total stylar RNAs by RACE using three pairs of gene-specific primers (see Materials and methods). The cDNA fragments were first cloned into pGEM-T, resulting in pGEM-S2, -S4 and -S5 (fig. 1). The 5′ primers of the S RNases genes were positioned in a way so that the ORF of the cDNA is the same as that produced from ATG within the NcoI site of pGEM-T, and 5 extra amino acids were introduced during this cloning (fig. 1). The predicted molecular weights of the cDNA encoded polypeptides are 24.1, 23.8 and 23.9 ku, respectively. The *Nco*I/*Sal*I DNA inserters from pGEM-S2, -S4 and -S5 were subcloned into the *Nco*I/*Xho*I sites of pET-15b, generating pET-S2, -S4 and -S5 (fig. 1). The cloning junctions and the cDNA inserters of both pGEM and pET clones were confirmed by DNA sequence analysis (data not shown).

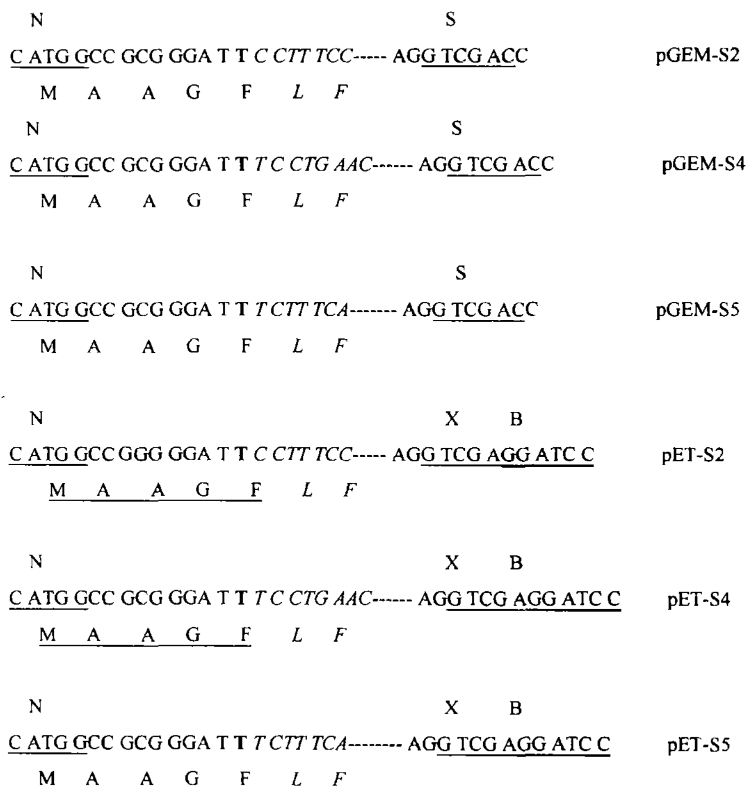

Fig. 1. 5′ end sequences of S RNases genes of Antirrhinum and expressional constructs. Amino acids encoded by the S RNase genes are indicated in italic and those introduced during cloning underlined. Dotted lines show the omitted S RNase sequences and the multiple cloning sites of the vectors. The rest of the vectors are not shown here. N, *Nco* I ; S, *Sal* I ; X, *Xho* I ; B, *Bam*H I . The detailed cloning steps are described in the text.

(ii) Functional expressions of S RNase genes in *E. coli*. The expressional vector contains the promoter of T_7RNA polymerase to drive target gene expression. Foreign protein expression can only be achieved in the presence of T_7RNA polymerase. In addition, the protein expression is inducible because the promoter sequence also contains *lac* operator which can be induced by the addition of IPTG. The inducibility of target protein expression avoids the possibility of its toxic effect on host strains during the cloning process. pET-S2, -S4 and -S5 were transformed into *E. coli* strain BL21(DE3) containing a gene expressing T_7RNA polymerase, respectively. When the growth of the bacteria reached 0.6 at

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O.D$_{600}$, 1 mmol/L of IPTG was added into the culture. After three hours of induction, the expressions of three S RNases were analyzed by SDS-PAGE. The results showed that the three genes were all expressed and produced polypeptides with molecular weights similar to those predicted from the cDNA sequences (fig. 2). No corresponding polypeptides were produced without IPTG induction for the S RNase gene clones and the vector alone (fig. 2). With the increase of time of IPTG induction, the expression of the S RNase genes were also increased accordingly (data not shown).

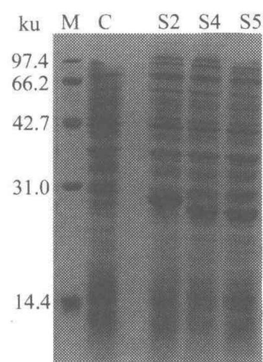

Fig. 2. *In vitro* expression of S RNases. Lane M, protein molecular weight standards. Numbers on the left show the protein sizes in ku. C, vector control; S2, pET-S2; S4: pET-S4; S5, pET-S5. S RNase polypeptides are indicated by arrows.

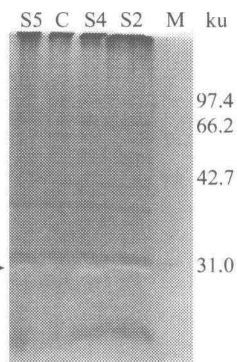

Fig. 3. Activity assay of S RNases expressed in *E. coli*. The arrow indicates the bright bands where the S RNase polypeptides were positioned. The other illustrations are the same as in fig. 2.

To test if the expressed S RNase polypeptides possess biological activity, the proteins separated by SDS-PAGE assayed *in situ* for ribonuclease activity. The results showed that the bright bands appeared in the positions where three S RNases were located after staining with toluidine blue-O, indicating that the Antirrhinum S RNases expressed in *E. coli* are biologically active (fig. 3).

To determine if the expression of an S RNase inhibits the growth of *E. coli*, the bacterial cultures containing pET-S2, -S4 or -S5 were measured for their growth at O.D$_{600}$ every 30 min after the IPTG induction. No difference was detected between them and the bacterial culture containing only the vector, showing that S RNases did not inhibit the growth of *E. coli* (data not shown). This result could be due to the following reasons: (i) although S RNases exhibit ribonuclease activity after being renatured *in vitro*, it is possible that S RNase can not be folded properly within the cytoplasm of *E. coli* and thus no inhibition is expected; (ii) *E. coli* contains a gene, *RNaseI*, homologous to S RNase genes. When *RNaseI* was expressed in the cytoplasm of *E. coli*, no growth inhibition was observed. It was suggested that an inhibitor of *RNaseI* could be present in *E. coli*[5], providing a possibility that such an inhibitor might also be capable of inhibiting the activity of S RNases. These possibilities clearly need further investigations. The successful expression of three Antirrhinum S RNases in *E. coli* provided a basis for a large scale preparation of them and eventually solving their crystal structures.

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