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Antimicrobial activity of γ-thionin-like soybean SE60 in *E. coli* and tobacco plants

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ABSTRACT

The SE60, a low molecular weight, sulfur-rich protein in soybean, is known to be homologous to wheat γ-purothionin. To elucidate the functional role of SE60, we expressed *SE60* cDNA in *Escherichia coli* and in tobacco plants. A single protein band was detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) after anti-FLAG affinity purification of the protein from transformed *E. coli*. While the control *E. coli* cells harboring pFLAG-1 showed standard growth with Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, *E. coli* cells expressing the SE60 fusion protein did not grow at all, suggesting that SE60 has toxic effects on *E. coli* growth. Genomic integration and the expression of transgene in the transgenic tobacco plants were confirmed by Southern and Northern blot analysis, respectively. The transgenic plants demonstrated enhanced resistance against the pathogen *Pseudomonas syringae*. Taken together, these results strongly suggest that SE60 has antimicrobial activity and play a role in the defense mechanism in soybean plants.

Plants are exposed to various pests and pathogens that attack them mechanically and chemically. They, on the other hand, develop defense mechanisms to protect themselves from environmental stresses and pathogens by producing a variety of small biological active peptides such as lipid transfer proteins, puroindolines, thionins, plant defensins, hevein-like peptides, knottin-like peptides, glycine-rich peptides, and snakins, which play an important role in protecting them from the invading bacteria and fungi [1].

Plant thionins are small, cysteine-rich proteins, and approximately 100 individual thionins have been identified in more than 15 different plant species thus far [2]. Although the 2 thionin families, namely, α/β-thionins and γ-thionins, share a common name, they have quite distinct three-dimensional structures. γ-Thionins are composed of 45–47 amino acids residues, and their cysteine residues are highly conserved. Some of the amino acids are positively charged, which confers cationic properties to them [3,4]. However, the other amino acid residues are extremely variable, as demonstrated by *in silico* studies. Hence, it can be concluded that the primary structure homology between γ-thionins is not sufficient to determine their general biological function [5]. Recently, three-dimensional structures of several γ-thionins have been studied in detail, both by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [4,6–8]. γ-Thionins seem to play a variety of roles in nature, such as antibacterial and/or antifungal activity [9,10], the ability to inhibit mammalian cell growth by membrane permeabilization [11], and the ability to inhibit insect α-amylase and proteinases [12,13]. However, a few reports show that some γ-thionins neither have antimicrobial activity nor inhibit digestive enzyme activity. For example, crambin, γ-thionin protein isolated from *Crambe abyssinica* seeds, does not have any antimicrobial activity or enzyme inhibitory activity, and it is responsible for sweet taste in plant seeds [14]. While the functions of α/β-thionins have been widely examined *in vitro*, those of γ-thionins have been relatively less studied.

In a previous study, we obtained a novel cDNA clone, namely, *SE60*, from soybean seeds, which belongs to the γ-thionin family, and characterized it at a molecular level [15,16]. Since γ-thionins isolated from different plant species show different and unique activities, we performed functional analyses of *SE60* to determine its function in soybean seeds. Here, we report that the expression of SE60 protein inhibits *Escherichia coli* growth and confers enhanced resistance to transgenic tobacco plants against the pathogen, *Pseudomonas syringae*. We discuss the possible role of the γ-thionin protein SE60 during the maturation of soybean seeds.

Materials and methods

Bacterial strains and plant materials. Escherichia coli HB101 cells were used for the expression of SE60 fusion protein using the *E. coli* expression vector pFLAG-1 (International Biotechnologies, Inc.), and *E. coli* K802 cells were used for amplifying SE60 using the plant

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binary vector pGA482 [17]. The *P. syringae* pv. *tabaci* strain Pt113, a kind gift from Prof. Hyung Suk Baik, Pusan National University, was used for analyzing the *in vivo* pathogen defense of transgenic tobacco plants. The *Agrobacterium tumefaciens* strain LBA4404 was used for the genetic transformation of tobacco cells from *Nicotiana tabacum* 'Xanthi' plants.

Construction of a pFSE60 E. coli expression vector and induction of the fusion protein in E. coli. The nucleotide sequences of the PCR primer set for amplifying the coding region of the mature SE60 protein is in the Supplementary data. The recombinant plasmid containing the *FLAG-SE60* gene was named as pFSE60. The detailed methods for the induction and purification of the fusion protein are also provided in the Supplementary data.

Measurement of the E. coli growth rate. Five hundred microliters of an overnight culture of *E. coli* HB101 cells harboring pFSE60 was added to 50 ml of LB medium with or without 0.5 mM IPTG at 37 °C and the mixture was shaken at 200 rpm. The absorbance of the solution was measured at 600 nm at 1 h interval. The growth ability of the *E. coli* cells transformed with pFSE60 was analyzed by culturing them on LB agar plates with or without 0.5 mM IPTG.

Construction of a plant expression vector. In order to express SE60 protein in plants, the plasmid p212, which contained the cauliflower mosaic virus (CaMV) 35S promoter [18], and the plasmid p13, which contained the terminator region of the tomato proteinase inhibitor I gene (TI-I) [19] were used. The detailed process for generating *CaMV::SE60* chimeric gene was described in the Supplementary data.

Tobacco plant transformation. The leaf disk transformation procedure [20] was used to produce transgenic tobacco plants. Plants regenerated on the Murashige and Skoog (MS) medium [21] were transferred into soil and grown at 26 °C in a growth chamber under a 16 h/8 h light–dark cycle, with a light intensity of 100 μmol m^{-2} s^{-1}.

Bioassay of transgenic tobacco plants against P. syringae. The regions on the underside of leaves, which were identified by the markings made on the upperside, were scratched carefully with cotton plugs coated with carborundum and inoculated with 15 μl of an overnight culture of *P. syringae*. After inoculation, the plants were kept at 28 °C for 7 days and photographed.

Results and discussion

SE60, a γ-thionin gene in soybean

In order to isolate genes that are regulated developmentally and/or tissue-specifically, we differentially screened a soybean cDNA library constructed from immature seeds (*Glycine max* L. 'Paldal') [15]. This led us to identify *SE60* cDNA, which encodes a low molecular weight, sulfur-rich protein in soybean seeds. A genomic clone obtained by screening the genomic library (*Glycine max* L. 'Paldal') revealed that the SE60 protein is synthesized as a preprotein with 75 amino acids. Cleavage of the hydrophobic sig-

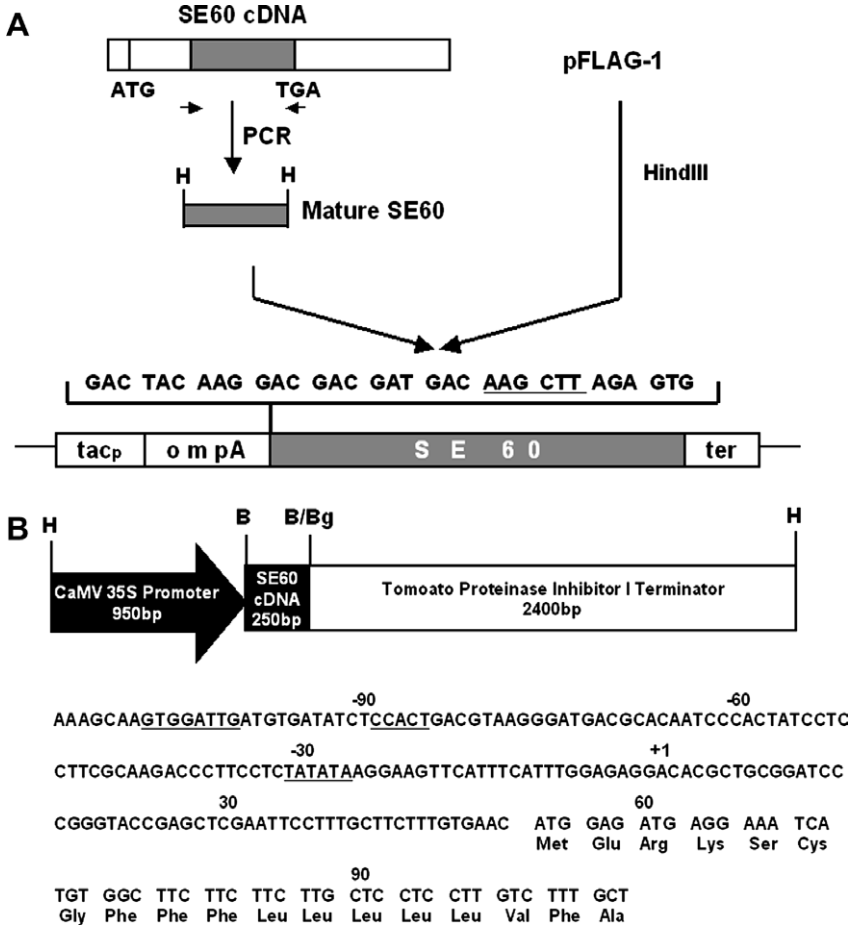

Fig. 1. Construction of the pFSE60 plasmid (A) and the chimeric *CaMV::SE60* gene (B). (A) The *SE60* cDNA was manipulated by PCR so as to contain only the mature SE60 protein region, and the amplified 150 bp DNA was cloned into the HindIII site of pFLAG-1 at the same reading frame along with the FLAG peptide region. (B) The *CaMV::SE60* gene was generated between the *CaMV* 35S promoter and the coding region of *SE60* cDNA, and the nucleotide sequences of this junction region were shown below. The tomato proteinase inhibitor I terminator was used as a transcription terminator. The promoter elements (TATAAA and CCACT) and a sequence (GTGGATTG) homologous to the SV40 enhancer core are underlined. The transcription initiation site is denoted by +1. Symbols: H, HindIII; B, BamHI; Bg, BglII.