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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-

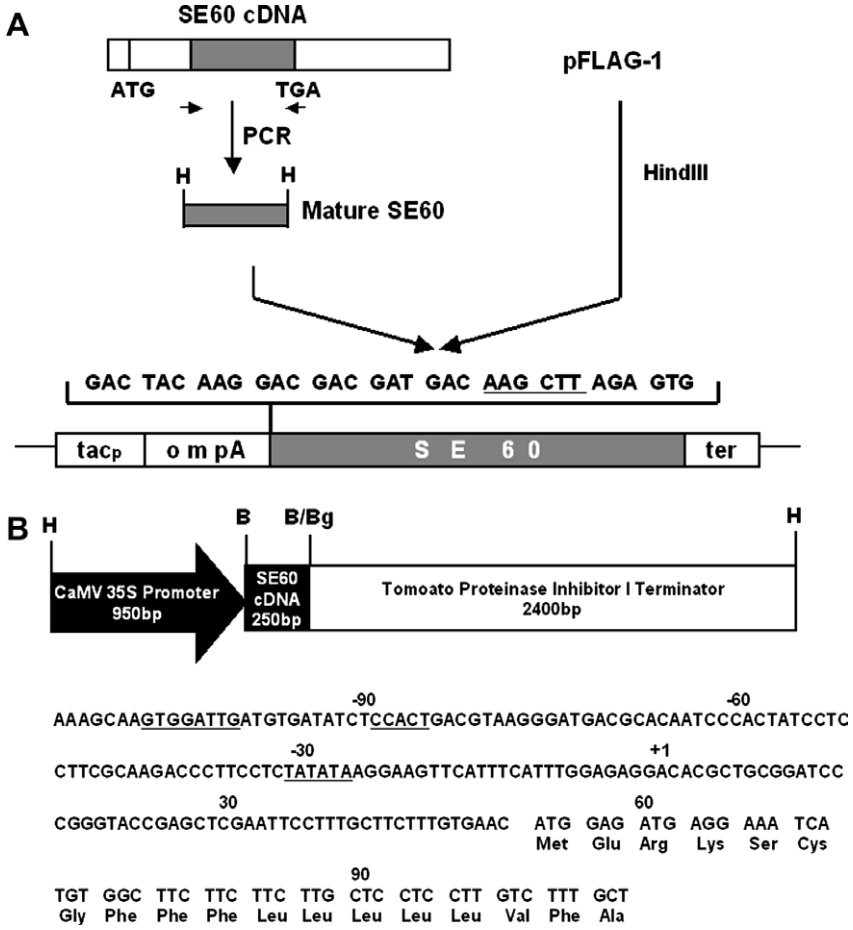

Fig. 1. Construction of the pFSE60 plasmid (A) and the chimeric *CaMV::SE60* gene (B). (A) The *SE60* cDNA was manipulated by PCR so as to contain only the mature SE60 protein region, and the amplified 150 bp DNA was cloned into the HindIII site of pFLAG-1 at the same reading frame along with the FLAG 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.

nal peptide generates the mature protein containing 47 amino acids [16]. Northern blot analysis showed that *SE60* mRNA accumulated in a seed-specific manner and increased as the seeds matured [16]. Since SE60 is a low molecular weight and sulfur-rich protein, we hypothesized that SE60 might possess antimicrobial activity. It is also possible that SE60 might inhibit digestive enzymes of insects, or act as a sulfur-storage protein during seed maturation.

Expression of FLAG-SE60 fusion protein in E. coli cells

To determine the function of SE60 in soybean seeds, we amplified the region containing the mature SE60 protein by PCR by using *SE60* cDNA as a template and the amplified fragment was inserted into the *E. coli* expression vector pFLAG-1 (Fig. 1A). The pFSE60 vector contains the *tac* promoter, which is repressed by the LacI repressor, which in turn is overexpressed from its $lacI^q$ promoter. Therefore, any fusion protein expression can be easily and tightly controlled by IPTG. This feature could minimize toxicity to the *E. coli* cells if the SE60 protein is deleterious to the cells.

The effect of SE60 expression on the growth of *E. coli* cells was measured in LB liquid medium culture with or without 0.5 mM IPTG. The *E. coli* cells with or without the pFLAG-1 vector showed the standard sigmoid growth curve. *E. coli* cells containing pFSE60 showed no growth at all with 0.5 mM IPTG induction (Fig. 2A). However, these cells grew normally in LB medium without IPTG. In addition, *E. coli* growth was analyzed on solid agar media with or without 0.5 mM IPTG (Fig. 2B). *E. coli* cells with or without the pFLAG-1 vector grew normally on the IPTG-containing agar plate. However, *E. coli* cells containing pFSE60 did not grow on the solid medium containing IPTG. These results suggest that SE60 protein might be toxic to the growth of bacteria such as *E. coli*.

It has been proposed that antimicrobial activity of thionins may be the result of pore formation on the cell membrane [10,22,23]. The amphipathic structure of thionins indicates that the toxicity could result from a detergent-like interaction with the lipid bilayers of biological membranes. The hydrophobic domain of the thionins could interact with the hydrophobic aliphatic chains of the membrane lipids, whereas the positively charged basic amino acids of thionins could interact with the negatively charged phosphate groups of the phospholipids [24]. These intermolecular salt bridges seem to form a pore in the cell membrane and result in the disruption of the membrane and ultimately leakage to death [23]. It is possible that SE60 might exert a similar toxic effect on *E. coli* cell membrane. Moreover, due to the presence of the outer membrane protein (OmpA) signal peptide in the FLAG-SE60 fusion protein, which leads the recombinant protein to the periplasmic space, the SE60 protein could have a higher probability of coming in contact with the *E. coli* cell membrane. The SE60 protein localized at the periplasmic space might form a salt bridge in the membrane and finally kill the *E. coli* cells. The amount of the SE60 protein that we could purify from 2500 ml culture medium was very low compared to other cytotoxic proteins such as the *Phytolacca* antiviral protein (PAP), which can be extracted in sufficient amounts from 500 ml of culture [25]. The SE60 protein might have lysed the *E. coli* cells. Therefore, the quantity of purified SE60 fusion protein was insufficient to test its antibacterial property *in vitro*.

In order to confirm the expression of the SE60 fusion protein in *E. coli* cells carrying pFSE60, the cells were induced to synthesize the SE60 fusion protein by adding IPTG when the OD_{600} reached 0.4. After 3 h of incubation, cells were harvested and the total proteins in the medium were extracted by the freeze–thaw method. The SE60 fusion protein was purified from total proteins by anti-FLAG affinity chromatography. A single protein band of approximately 16 kDa was identified in each eluted fraction, in contrast to the expected size of the SE60 fusion protein, which was estimated from the amino acid sequence to be approximately 6 kDa,

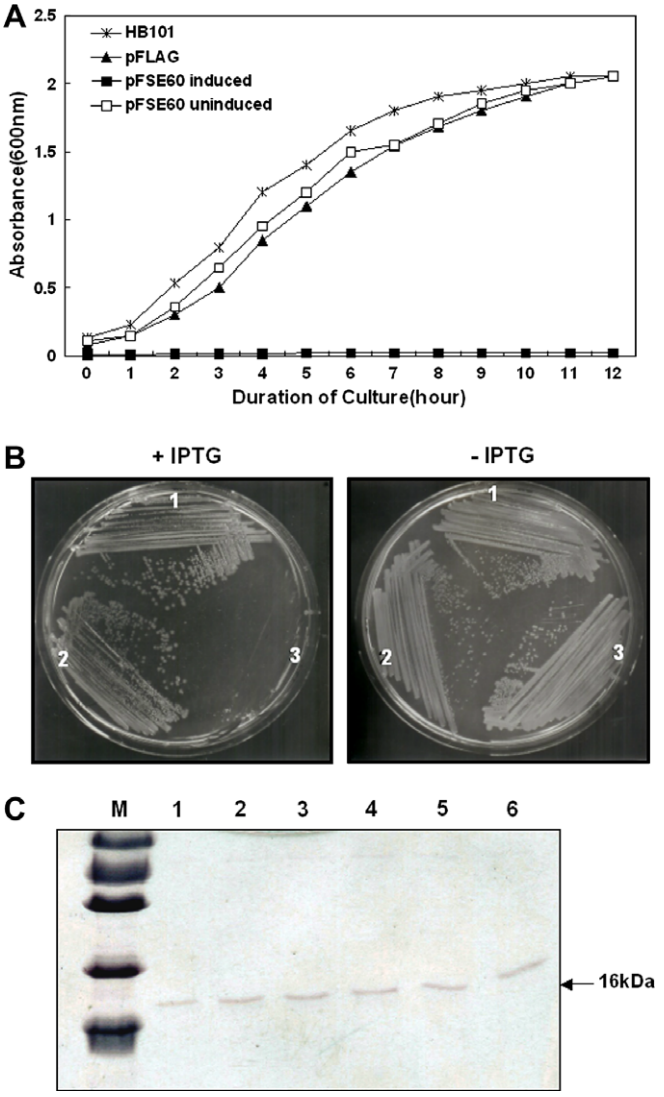

Fig. 2. Expression of the SE60 fusion protein in *E. coli*. *E. coli* cells were grown in LB liquid (A) or solid (B) media with or without 0.5 mM IPTG. The absorbance of the liquid medium containing *E. coli* cells was measured every hour at 600 nm to analyze their growth curve. Symbols in (A): *–*, non-transformed *E. coli* HB101 cells with IPTG; ▲–▲, *E. coli* cells containing pFLAG-1 with IPTG; ■–■, *E. coli* cells containing pFSE60 with IPTG; □–□ *E. coli* cells containing pFSE60 without IPTG. Symbols in (B): 1, non-transformed *E. coli* cells; 2, *E. coli* cells containing pFLAG-1; 3, *E. coli* cells containing pFSE60. (C) FLAG-SE60 fusion protein was purified and electrophoresed on a 15% SDS–PAGE gel. Lanes in (C): lane M, molecular-size markers; lanes 1–6, eluted fraction number of the purified FLAG-SE60 protein.

including the FLAG octapeptide of 1 kDa (Fig. 2C). Such a molecular-size difference observed in the SDS–PAGE gel has also been reported in the case of antifungal proteins in radish seeds [9]. This discrepancy could be attributed to disulfide bridges formed in these proteins, which could lead to unexpected migration on the SDS–PAGE gel [26,27] and/or to the oligomer formation as reported for SPE10, a plant defensin from *Pachyrrhizus erosus* seeds [28].

Ectopic expression of the SE60 protein in tobacco plants

We investigated the function of the SE60 protein in plants by generating a chimeric gene in which the *SE60* cDNA expression would be controlled by *CaMV* 35S promoter. Fig. 1B shows the structure of the *CaMV::SE60* chimeric gene. The 3.5 kb *CaMV::SE60* cDNA fragment was cloned into pGA482 to transform *Agrobacteria*. Finally, tobacco cells were infected with the transformed *Agrobac-*

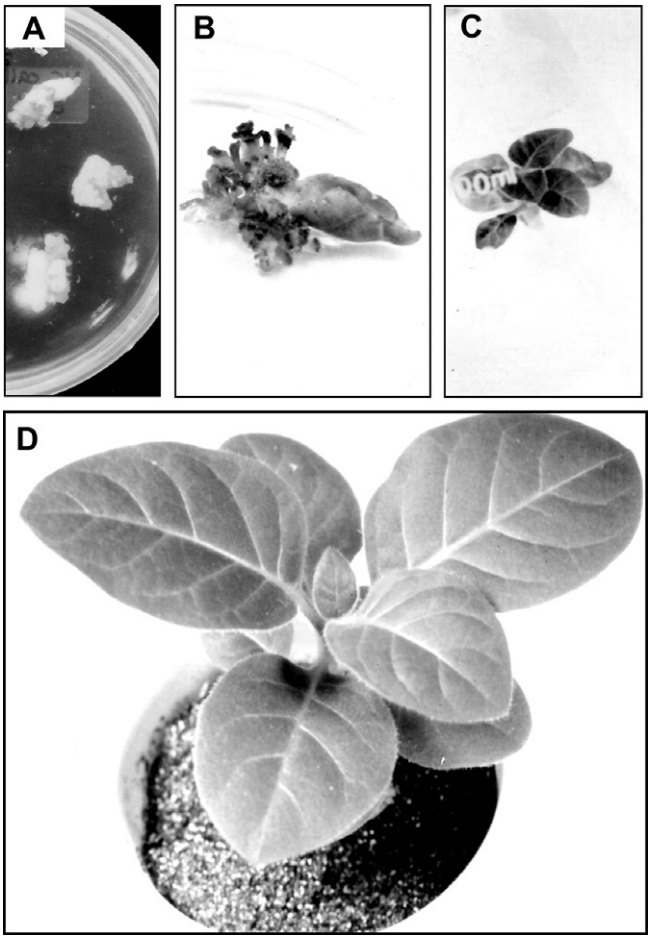

Fig. 3. Tobacco cells were transformed by using *Agrobacterium* cells containing the *CaMV::SE60* chimeric genes, and transgenic adult plants were generated. (A) Transformed calluses on callus-inducing MS agar medium. (B) Shoots induced on shoot-inducing MS agar medium. (C) Shoots transferred to root-inducing medium. (D) Transformed adult tobacco plant.

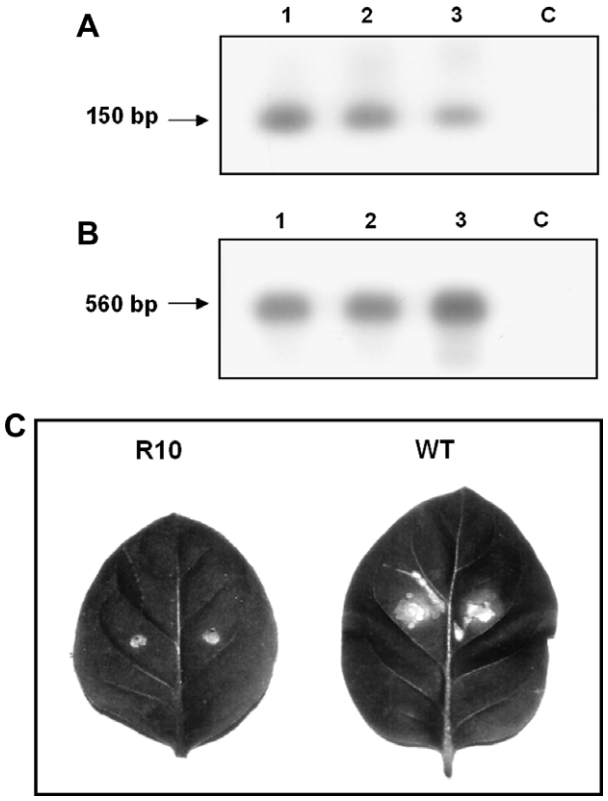

Fig. 4. Expression of the SE60 protein and bioassay of transgenic tobacco plants against *P. syringae*. (A) The incorporation of the *CaMV::SE60* gene in the genome of transgenic tobacco plants was confirmed by PCR amplification, followed by Southern blot analysis with the *SE60* cDNA as a probe. (B) Northern blot analysis for checking transgenic expression with poly(A)$^+$ RNAs isolated from the leaves of transgenic tobacco plants (lanes 1–3) and a non-transgenic tobacco plant (lane C). (C) Lesions on tobacco leaves caused by infection with *P. syringae* at 7 days after pathogen inoculation. Symbols: 1, transgenic line R10; 2, transgenic line R23; 3, transgenic line R25; C, a non-transgenic tobacco plant; R10, transgenic line R10; WT, non-transgenic plant.

teria by the leaf disk method [20], and the transformed tobacco cells were regenerated into whole plants (Fig. 3A–D).

In order to confirm the integration of the chimeric gene into the tobacco genome, genomic DNA was isolated from the leaves of transgenic plants and subjected to PCR amplification with *SE60* cDNA primers. The 150 bp amplified DNAs were electrophoresed on a 1% agarose gel. To ensure that the bands of amplified DNA correspond to the integrated *CaMV::SE60* transgene, the amplified DNAs were transferred onto a nylon membrane and hybridized with *SE60* cDNA probe. We observed that three independent transgenic tobacco plants contained the chimeric gene in their genomes, while the non-transgenic plant did not (Fig. 4A). This indicates that the *CaMV::SE60* transgene was indeed introduced into the genome of kamamycin-resistant tobacco plants.

We also checked the trangene expression by Northern blot analysis with poly(A)$^+$ RNAs. The *SE60* cDNA expression, under the control of *CaMV* 35S promoter, was high in each transgenic tobacco plant but not in the non-transgenic plant. This suggests that the expression of *SE60* in the transgenic tobacco plants is very stable.

Next, we explored the function of SE60 in plants in offering resistance to pathogen infection. We examined this role by inoculating *P. syringae* on the underside of the leaves of the transgenic and non-transgenic tobacco plants after scratching slightly with cotton plugs coated with carborundum. In contrast to the non-transgenic leaves which showed a wide spread of infectious lesion, the leaves of the transgenic plants did not show any symptom of infection (Fig. 4C). We observed the same result in other transgenic lines as well (data not shown). This strongly suggests that the expression of the SE60 protein confers resistance to the transgenic plants against *P. syringae*. The transgenic plants expressing SE60 ectopically showed normal growth like the non-transgenic plants, indicating that the SE60 protein does not affect plant growth. It could be possible that the SE60 protein is transported and stored in cellular compartments in plants, such as in vacuoles. In fact, SE60 protein contains the signal sequence, thus, it is tempting to speculate that transformed tobacco plants could minimize toxic effects to its own cells by compartmentalization of SE60 protein.

Biological function of SE60, a member of γ-thionin family in soybean seeds

Although a few studies have been performed on plant thionins [29], the *in vivo* function of most of them is largely unknown. Several biological functions of thionins have been proposed in plants on the basis of observations made in *in vitro* studies. The findings that purothionins can be reduced *in vitro* by the thioredoxin system of wheat seeds [30] has led to the speculation that purothionins could function as a secondary thiol messenger, with the sulfhydryl form of purothionins being involved in reduction and thereby leading to the activation of fructose-1,6,-biphosphatase. Similarly, purothionins reduced by thioredoxin were shown to block DNA synthesis *in vitro* by inhibiting the enzyme ribonucleotide reductase. This phenome-

non is considered to be the reason for the toxic effect of thionins on mammalian cells undergoing chromosome duplication [31]. In addition, seed-specific thionins might function as storage proteins, particularly for sulfur storage [32]. There are other possible roles of thionins; for example, these proteins can be potential enzyme inhibitors because many enzyme inhibitors found in seeds are cysteine-rich [12,13]. The hypothesis that thionins function in plant defense against pathogens was based on their biocide activities toward plant pathogens *in vitro*. Here, we show that SE60, a member of the γ-thionin family in soybean seeds, confers resistance to transgenic tobacco plants against *P. syringae* and provide the evidence to show that γ-thionin plays a physiological role by exhibiting antimicrobial effects and might function as a defense chemical against invading pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.001.

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