A soybean seed protein with carboxylate-binding activity

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Abstract

The seed coat serves as a multifunctional organ with a role in protection and for the supply of nutrients to the embryo sac during development. The composition of the legume seed coat differs from other seed tissues in many ways including its protein composition. An abundant 24 kDa protein (SC24) has been purified and identified from soybean (Glycine max [L.] Merr) seed hulls. The corresponding cDNA and genomic DNA clones for SC24 were isolated and characterized, and expression patterns were determined. The deduced protein sequence of 219 amino acids included an N-terminal signal peptide. Transcripts encoding SC24 were present in the seed coat from 30 days after pollination (DAP) until maturity, but the protein was not detected until the final stages of seed maturation. In mature seeds, most of the SC24 protein was localized to the parenchyma and aleurone layers of the seed coat. The expression of SC24 was also induced in vegetative tissues by pathogen infection and by wounding. The SC24 protein bound to an affinity column containing an isophthalic acid ligand, and was eluted with 7 mM citrate. Polyclonal antibodies raised against recombinant SC24 cross-reacted with the seed coat peroxidase enzyme, suggesting that these two proteins may share an antigenic determinant. Overall, the results indicate that SC24 belongs to a novel class of plant defence proteins with carboxylate-binding activity.

Key words: Citrate binding protein, peroxidase, seed coat, soybean, wound stress.

Introduction

Angiosperm seeds develop from fertilized ovules. The seed coat varies in structure but is essential for the development of seeds (Reiser and Fischer, 1993). At maturity, the seed coat is primarily a protective structure, although its specific role may be varied and complex (Villiers, 1972). Legumes possess highly differentiated seed coats that arise from the inner and outer integuments of the ovule (Corner, 1951; Esau, 1977; Miller et al., 1999). The seed coat plays a vital role in legume seed architecture and is a feature that is used for identification. In addition to its protective function, the seed coat serves as a multifunctional organ and is involved in supplying nutrients to the embryo sac during seed development (Murray, 1987). Seed coats transport, transform, and secrete various metabolites to the embryo (Patrick and Offler, 2001). In the last stages of maturation, there is an immense reduction in the metabolic activity and rapid desiccation of the seed as well as compression of seed coat layers. The mature seed is covered with dead and hard tissues providing the seed with structural protection and mechanical support. It also protects the seed against pathogens, insects, wind, frost, and other stresses (Boesewinkel and Bouman, 1995). In leguminous crops such as soybean, the seed coat also has a significant impact in the agricultural marketplace as a component or by-product of processed seeds.

In mature soybean (Glycine max [L.] Merr.) seed, 4–8% of total seed mass is comprised of seed coat. The composition of the seed coat is very different from that of the embryo, including its soluble protein components. More than 100 proteins have been identified from whole seeds (Herman et al., 2003), and various isoforms of the 11S and 7S storage proteins, β-conglycinin and glycinin, constitute approximately 70% of the total protein (Yaklich, 2001). Most of these seed proteins occur in the embryo and are far less abundant or absent from the seed coat (Gijzen et al., 2001). Protein components identified from extracts of soybean seed coats include proline-rich proteins (Percy et al., 1999), Kunitz and Bowman–Birk trypsin inhibitors (Gijzen et al., 2001; Sessa...
and Wolf, 2001), peroxidase (Gijzen et al., 1993), chitinase (Gijzen et al., 2001), and hydrophobic protein (Gijzen et al., 1999).

The purpose of this study was to identify and characterize a new and unusual 24 kDa protein, SC24, from soybean seed coats. SC24 was purified from seed coats and the corresponding cDNA and genomic clones encoding the protein were isolated. It is shown that SC24 and its corresponding transcript are most abundant in the seed coat at late stages of seed development. The SC24 mRNA is also prevalent in wounded tissues and is induced by infection with the soybean pathogen Phytophthora sojae. Biochemical tests indicate that SC24 has carboxylate-binding activity and that the protein may share antigenic domains with soybean seed coat peroxidase.

Materials and methods

Purification of native SC24 from soybean seed hulls

Soybean seed hulls were obtained from a processing facility (ADM Agri-Industries, Windsor, Ontario). Proteins were extracted from 20 g seed hulls in 150 ml of 60% (v/v) ethanol by gentle shaking for 16 h. This extract was filtered (Miracloth, Calbiochem, La Jolla, CA) and clarified by centrifugation for 20 min at 14 000 g. The supernatant was collected and mixed with 2 vols of acetone, prechilled to −20 °C. Precipitated proteins were collected by centrifugation for 10 min at 14 000 g, and redissolved in 10 ml of 25 mM TRIS–HCl, pH 8.0. A 2.0 ml sample of this concentrated protein extract was separated on a gel filtration column (Superdex-75 16/60, Amersham Biosciences, Baie d’Urfe, Quebec) installed on a liquid chromatography apparatus (FPLC, Amersham Biosciences, Baie d’Urfe, Quebec) and eluted with 20 mM Tricine pH 7.5, 0.1% Triton X-100 in hybridization buffer (0.25 M Na2HPO4 pH 7.2, 1% BSA, 1 mM EDTA, and 7% SDS) with a radiolabelled SC24 gene fragment. The membrane was washed four times for 15 min each at 68 °C in a high stringency wash solution containing 20 mM Na2HPO4 pH 7.2, 1 mM EDTA, and 1% SDS, followed by autoradiography.

Dissection of seed coat

Soybean seeds were soaked in distilled water for 3 h to remove the seed coats. The seed coat tissues were separated by dissection into two fractions. For photography, pieces of tissue were cut and immersed in 3% agarose gel. Cubes containing the tissues were cut from the gel and sectioned using a vibrating blade microtome (Leica VT 1000S), stained with 0.05% (w/v) toluidine blue and observed under an inverted microscope. Pictures were taken with a DXM 1200 Nikon digital camera.

Library screening and DNA sequencing

Construction and screening of soybean cv. Harosoy 63 cDNA and genomic λ libraries were performed according to Gijzen (1997). Positive cDNA clones in phagemid vector (pBK-CMV, Stratagene, La Jolla, CA) were sequenced on both strands by primer walking. Positive genomic DNA clones were plaque purified and subcloned into plasmid vector (pBluescript, Stratagene, La Jolla, CA) for sequence analysis. Automated sequencing of DNA was performed using a kit (ThermoscriptRT-PCR System, Life Technologies). The conditions for PCR were as follows: SC24-8-5'GCGGGATCCCATGTGTTG-3', SC24-9-5'TATTGTTAAACCGTGCTTCTCA-3'. The conditions for PCR were as follows: 94 °C for 12 s, 51 °C for 15 s, and 72 °C for 2 min (35 cycles).
For real time PCR, total RNA (5 μg) was reverse transcribed to cDNA with random hexamers (ThermoScript Reverse Transcriptase Kit, Invitrogen, USA). Real time PCR was performed on a thermocycler (LightCycler, Boehringer Mannheim/Roche Diagnostics, Laval, PQ) using the fluorescent dye, SYBR Green. Cycling conditions were performed using 2 μl of 1:10, 1:100, or 1:1000 (v/v) dilutions of first strand cDNA in a final volume of 20 μl, using buffers and conditions supplied by the manufacturer (FastStart SYBR Green I, Boehringer Mannheim/Roche Diagnostics, PQ). Gene-specific primers and PCR conditions were as described previously. Amplification rate for SC24 cDNA was evaluated from cycle threshold numbers obtained from serial cDNA dilutions. Optimal primer and template concentrations were determined by analysis of the dissociation curves and the amplified products were verified by agarose gel electrophoresis. Amplification profiles monitoring relative amounts of PCR product were visualized on graphs where the log of fluorescence was plotted against the number of cycles. Relative fold change in accumulation of SC24 under a given experimental treatment was standardized against cDNA derived from water-inoculated soybean tissue.

Production and purification of rSC24

Two primers, 5'-AAATCCATGTTGCGATCCTCACT-3' and 5'-GGGGCGCTTCTAAGTAGCTCTCGAGT-3', were designed to produce a 776 bp product from cDNA that included most of the SC24 ORF, but lacked the signal peptide. The PCR product was digested with NcoI and XhoI and cloned into the corresponding sites of an E. coli expression vector (pET-30b, Novagen) to create an rSC24 ORF that included a 5 kDa amino-terminal His-tag. The construct was transformed into E. coli (XL1Blue, Stratagene, La Jolla, CA). Recombinant plasmids were sequence-verified prior to transformation into E. coli strain BL21(DE3) for rSC24 production.

For rSC24 protein production, bacteria from a fresh colony were grown overnight at 37 °C in Luria-Bertani (LB) medium containing 100 μg ml⁻¹ kanamycin. An aliquot of the overnight-grown culture was used to inoculate 25 ml of fresh LB medium containing kanamycin. The culture was grown at 37 °C to an OD₆₀₀ of 0.4–0.6. At this time, isopropylthio-β-D-galactoside was added to a final concentration of 0.4 mM to induce the expression of rSC24. The culture was grown overnight at 20 °C. Bacterial cells were collected by centrifugation, resuspended in lysis buffer (BugBuster, Novagen) containing 100 μg lysozyme and 25 units of Benzonase nuclease ml⁻¹. The cell lysate was clarified by centrifugation at 16 000 g for 20 min at 4 °C. The supernatant containing poly-His tagged rSC24 protein was filtered through 0.2 μm filter (Acrodisc, nylon) and was applied to a chelated Ni²⁺ affinity column (HiTrap chelating HP, Amersham Pharmacia) using FPLC system. The column was washed with 20 mM TRIS pH 7.4 and 500 mM NaCl. The rSC24 was eluted from the Ni²⁺ column using 20 mM TRIS pH 7.4, 500 mM NaCl, and a gradient of imidazole from 50–500 mM. Fractions were collected and analysed by 12.5% SDS–PAGE. Fractions containing rSC24 were pooled together and concentrated using a centrifugal filter device with a 10 kDa molecular weight cut off (Amicon, Millipore).

Antibody generation

A rabbit was bled to collect control serum prior to immunization. The preimmune serum was determined to be free of antibodies that cross-react with soybean proteins. Purified rSC24 with His tag (200 μg) was mixed 1:1 (v/v) with complete Freund’s adjuvant and injected into the rabbit. Booster injections of rSC24 with incomplete Freund’s adjuvant were performed 14 d and 35 d after the first injection. Antiserum to SC24 (α-SC24) was collected on days 24 and 45, and stored at −80 °C.

Western blotting

Frozen plant tissue was ground in extraction buffer (25 mM TRIS–HCl pH 8.0, 1 mM EDTA pH 8.0, 20 mM NaCl, and protease inhibitor cocktail (Roche Applied Sciences) as described in Dhaubhadel et al. (1999) with some modifications. The protein concentration was determined by dye-binding assay (Bradford Coomassie reagent, Bio-Rad Ltd., Mississauga, Canada). The SC24 protein was detected by sequential incubation with α-SC24 and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, each at a dilution of 1:7500, followed by the chemiluminescent detection (ECL system, Amersham Biosciences). For the wounding experiment, proteins (25 μg from each sample) were separated on SDS–PAGE and western blotting was performed as described above. The proteins were detected by using SuperSignal west lenti maximum sensitivity substrate (Pierce Biotechnology Inc., IL) according to the manufacturer’s instructions.

Results

SC24 is an abundant protein in mature soybean seed coats

To purify SC24 from soybean seed hulls, protein extracts were precipitated with acetone and separated by gel filtration chromatography. Shown in Fig. 1 are representative results from such a purification. The SC24 protein is abundant in seed hulls and may be purified to near-homogeneity with relative ease. The purification was assisted by some unusual characteristics of SC24 and another protein that co-eluted with SC24 from the gel-filtration column. First, SC24 eluted relatively late from the gel-filtration column for a protein of its size. The mass of SC24 was estimated to be 24 kDa based upon SDS–PAGE analysis, but the elution volume of the native protein from the gel-filtration column suggested an apparent mass of 11.5 kDa. It is not clear what delays the elution of SC24, but it may be interacting with the agarose- and dextran-based column matrix. Second, the main contaminating protein that co-eluted with SC24 was determined to be the soybean hydrophobic protein (HPS). This protein spontaneously crystallized and precipitated out after chromatography, leaving highly purified SC24 in solution (Fig. 1). Further, analysis of SC24 by peptide microsequencing revealed an amino-terminal sequence identical to that reported for ‘Protein V’ from seed hulls (Gijzen et al., 2001), and similar to a partial sequence reported for a protein released by soybean seeds in hot water (Hirano et al., 1992). Databases of ESTs (http://stalder.agron.iastate.edu/blast/blast.html) were searched for reading frames matching the SC24 amino-terminal peptide sequence to identify transcripts encoding protein. Several corresponding ESTs were identified, but none of these encoded a complete open reading frame (ORF). The EST sequences...
were used to design primers and construct probes for screening a seed coat cDNA library (Gijzen, 1997). Six cDNA clones were isolated, including a transcript that apparently encoded a complete ORF of SC24. This SC24 cDNA was used as a hybridization probe to isolate a 15 841 bp EcoRI genomic fragment containing the SC24 gene.

SC24 binds to polycarboxylates

The cDNA encoding SC24 was 815 bp in length and included 34 bp of 5’ untranslated region (UTR), an ORF of 219 amino acids, and 140 bp of 3’ UTR. The 219 amino acid preprotein consists of a 26 amino acid signal peptide leader sequence that is cleaved during processing (Fig. 2). The search for protein domain architecture did not reveal any known domain within the protein sequence. However, a protein fold recognition server, 3D-PSSM (Kelly et al., 2000), suggested that SC24 may share structural similarity with mouse fibronectin protein with 70% certainty and a PSSM e-value of 0.265. The full-length ORF encoded a preprotein of calculated molecular mass of 24.5 kDa and a mature protein of 21.8 kDa with a pI of 9.3.

The SC24 amino acid sequence (accession number ASS59524) was similar to a citrate binding protein (CBP) isolated from latex of the rubber tree (Rentsch et al., 1995), and to predicted proteins from potato and Angelica dahurica (Fig. 2). Transcripts potentially encoding proteins similar to SC24 were also detected in numerous plant species, including monocots (barley, rice, sugar cane) and dicots (alfalfa, ginseng, poplar, potato) by analysis of EST databases (not shown). Most EST hits originated from cDNA libraries constructed from wounded or pathogen-infected tissues. However, no counterpart to SC24 is present in Arabidopsis. Thus, SC24-like proteins appear to be widely distributed among plants, but apparently are dispensable.

The decision was taken to test whether SC24 could be purified using an affinity column containing an isophthalic acid ligand, since this method had been used to isolate the CBP from latex. The results demonstrate that SC24 binds to this affinity column (Fig. 3). Application of a citrate gradient resulted in the elution of SC24 in the 5–7 mM range. These properties are similar to those described for the CBP, indicating that SC24 and CBP share functional characteristics with regard to their affinity towards polycarboxylates.

SC24 is a low copy gene and contains a single intron

Sequence analysis of the genomic clone revealed that the SC24 gene is encoded within a 1762 bp stretch that contains a single intron of 784 bp and two exons of 417 and 387 bp lengths (Fig. 4A). The SC24 cDNA sequence exactly matched sequences encoded within exons of the genomic clone. The cDNA transcript was nearly full length, missing only 19 bp of the 5’ UTR downstream of the predicted transcription start site. A TATA box was located at −30 from the predicted transcription start site, which is consistent with the distance of the TATA box from the transcription initiation site in many plant genes (Joshi, 1987). No other genes were present within the 15.8 kb genomic clone, as determined by comparison to known EST and protein sequences, and by a gene prediction programme (Burge and Karlin, 1997). To investigate the copy number of SC24, a 32P-labelled cDNA probe was hybridized
to soybean genomic DNA under conditions of high stringency. Hybridization patterns were consistent with that of a single copy gene, since all bands could be accounted for and predicted from analysis of the 15 841 bp EcoRI genomic fragment (Fig. 4D). Furthermore, no evidence of additional SC24-like transcripts could be found by searching the extensive soybean EST databases. The SC24 transcript is abundant in seed coats and is induced by stress

As a first step to determine the expression pattern of the soybean SC24 gene, ESTs were analysed for sequences exhibiting similarities to SC24. Nucleotide sequences corresponding to SC24 were used in BLASTN searches of 309 385 soybean ESTs (http://stadler.agron.iastate.edu/blast/blast.html) originating from more than 80 different cDNA libraries (Shoemaker et al., 2002). This resulted in the identification of 39 matching ESTs from 13 different source cDNA libraries. These data were pooled according to the tissue-type of the source cDNA library (Fig. 5A). The results show that ESTs corresponding to SC24 occurred most frequently in cDNA libraries constructed from wounded cotyledons. Several ESTs matching the SC24 gene sequence were also found in cDNA libraries from plant organs exposed to other stresses, such as elicitor or toxin treatment, or pathogen infection.

Fig. 2. Multiple sequence alignment of deduced amino acid sequences of SC24 with similar protein sequences in the GenBank using the Clustal multiple sequence alignment program. A dark-shaded box indicates identical amino acids while a light-shaded box indicates similar amino acids. Dashes indicate gaps introduced to maximize alignment. The asterisks indicate signal peptide sequence, dashed lines indicate the microsequenced region of the protein, and the solid line indicates the fibronectin domain. Accession numbers for displayed sequences are: SC24 (AAL69983), potato (BAC23048), Angelica (AAG01372), and para rubber tree (T10760).

To study in detail the expression pattern of SC24 in different soybean plant tissues during development, RT-PCR was performed using SC24 gene-specific primers. Results indicate that SC24 transcript accumulation is prevalent in the seed coat from 30 DAP to maturity (Fig. 5B). The SC24 transcript was also detected in the flower buds, flower, leaf, embryo, and pod wall during later stages of development. Analysis by RNA blot hybridization yielded similar results and indicated that SC24 mRNA is most abundant in the seed coat during late stages of seed maturation (not shown). It was also determined that SC24 mRNA may be induced by pathogen infection (Fig. 5C). Transcripts from P. sojae-infected soybean hypocotyls at 3, 6, 12, 24, or 48 h after inoculation with a virulent race of the pathogen were compared by quantitative, real-time RT-PCR using primers specific for SC24. The expression of SC24 was induced within 6 h of infection and peaked at 24 h.

SC24 accumulates late in seed development and in response to wound stress

To produce recombinant SC24 (rSC24), the complete ORF from the SC24 cDNA, without the signal peptide, was cloned into an E. coli expression vector. The rSC24 possessed an N-terminal histidine (His)-tag for purification by Ni²⁺ affinity chromatography. Using these methods,
large amounts of highly purified, soluble rSC24 could be recovered from E. coli cultures suitable for polyclonal antibody production (Fig. 6).

To determine the apparent molecular mass of rSC24, purified protein was analysed by gel filtration chromatography (data not shown). The apparent molecular mass of His-tagged rSC24 was 26 kDa, in close agreement with the deduced molecular mass of 25.9 kDa. However, after cleavage of the 4.1 kDa amino terminal His-tag of rSC24, the apparent molecular mass of the protein was 11.5 kDa, much less than its calculated mass of 21.8 kDa, but identical to the apparent mass of SC24 isolated from seed hulls (Fig. 1). Thus, only after cleavage of its His-tag does rSC24 display an anomalous molecular mass as determined by gel filtration chromatography.

To measure SC24 protein accumulation in different soybean tissues and during seed development, polyclonal antibodies raised against purified rSC24 were used to probe soluble protein extracts by western blotting analysis. Trace amounts of SC24 were detected in protein extracts from flower buds, young pod tissue, and seeds, but not in any other soybean organs. In the seed, SC24 accumulated in seed coat tissues at the late stages of development (Fig. 7A). It was first detected in seed coat at 50 DAP, at a time when the seeds were near maturity. Polyclonal antibodies raised against rSC24 also bound to a 43 kDa protein present in seed coat tissues. The 43 kDa protein was first detected at 30 DAP and continued to accumulate to seed maturity. This cross-reacting protein was identified as the seed coat peroxidase by comparison with purified enzyme purchased from commercial sources. To verify that the 43 kDa signal was not due to endogenous peroxidase activity, the western blotting analysis was performed using alkaline phosphatase conjugated anti-rabbit IgG in place of horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody, and similar results were obtained (data not shown). Surprisingly, the seed coat peroxidase enzyme appears to share a common antigenic domain with SC24, despite the fact that the two proteins are unrelated and do not show any significant similarity in amino acid sequence.

To determine which cell layers of the seed coat contain SC24, two fractions of seed coat tissues that naturally separate from one another when the tissue is sectioned were collected and placed in water (Fig. 7B). Fraction I consisted of the palisade and hourglass cell layers while Fraction II contained the remaining tissues of the seed coat, including the parenchyma and aleurone layers. The results indicate that SC24 is present in parenchyma and aleurone layers, but not in palisade and hourglass cell layers, whereas peroxidase was detected in both fractions (Fig. 7C).

Since SC24 transcripts were abundant in tissues that were subjected to various stresses, the level of SC24 was determined in soybean tissues that were either challenged by pathogen or by wounding. A slight increase in the level of SC24 accumulation was observed after 48 h of P. sojae infection (data not shown). However, a significantly high level of SC24 accumulation was detected in response to wound stress (Fig. 8). After 3 h of wound treatment, the amount of SC24 doubled compared with the control samples. The amount of SC24 protein present in wounded tissues increased to 8.8 and 9.5 times that in unwounded tissues after 24 h and 48 h, respectively, as determined by image intensity analysis (Scion Image Beta program, Scion Corporation).

**Discussion**

Previously, the composition of proteins present in soybean seed hulls was analysed and five major soluble components were identified (Gijzen et al., 2001). A 24 kDa protein (SC24) was also purified and partially sequenced from
extracts of seed hulls. This 24 kDa protein possessed a highly basic pI and was abundant in soybean seed hulls. In this study, the SC24 protein has been characterized further, its citrate binding activity has been demonstrated, the corresponding cDNA and genomic clones have been isolated, and the amount of SC24 mRNA and protein present in various plant tissues during development and in response to stresses have been measured.

The amino acid sequence of SC24 shares similarity with the rubber tree CBP, an abundant protein occurring in the latex, a defensive secretion that is rich in anti-microbial constituents (Rentsch et al., 1995; Subroto et al., 2001). The CBP protein was first identified based upon photoaffinity labelling using analogues of the dicarboxylic acids citrate and malate, in an attempt to identify citrate transport proteins (Rentsch et al., 1995). The rubber tree CBP is considered as a vacuolar protein, possesses a C-terminal extension that is not present in SC24 and related proteins from potato and Angelica.

Plants have developed various responses to environmental stresses by inducing the synthesis of a wide array of defence-related proteins. The expression pattern of SC24 at mRNA level in response to pathogen infection suggests that it may have a role in defence. However, the pattern of SC24 transcript accumulation did not correlate directly with those of protein accumulation in pathogen-infected tissues. Rapid protein turnover and/or degradation may result in lower levels of SC24 accumulation in pathogen challenged tissues. By contrast, a significant increase in SC24 level was detected in response to wound stress. There was slight increase in SC24 accumulation after 3 h of wound treatment, compared with unwounded tissues. The level of SC24 increased dramatically at later time points. The results suggest that SC24 may have a role in the wound repair of damaged tissues and not in the production of wound signals. The appearance of SC24 in seed coat tissues near seed maturity and its localization in the inner layers of seed coat are consistent with a defensive or structural role for the protein.

The SC24 protein displayed unusual properties when subjected to gel filtration chromatography on polydextran media, and was retarded in its elution compared with other proteins of comparable size. Furthermore, SC24 showed affinity towards an isophthalic acid-coupled activated agarose column and could be eluted with increasing concentration of citrate. Citrate is often considered to be an exogenous siderophore, even though it has a simpler chemical composition and weaker iron-binding affinity compared to other siderophores (Yue et al., 2003). Siderophores are crucial for the virulence of various pathogens in animal models of diseases (Ratledge and Dover, 2000) and are a vital factor for the growth and survival of many bacteria and fungi in the soil and in aqueous environment (Guerinot, 1994). It is possible that binding of citrate by SC24 affects the availability of iron to the pathogens which negatively influences their growth and development.

Polyclonal antibodies to rSC24 cross-reacted with the soybean seed coat peroxidase, a surprising result that suggests shared structural and possibly functional characteristics of these two proteins. Higher plant peroxidases are haem-containing oxidoreductases that catalyse the reduction of hydrogen peroxide and oxidation of various hydrogen donors. They function in cell wall biosynthesis.
and defence, and are often induced by wounding or pathogen infection (Campa, 1991; Moerschbacher, 1992; Hiraga et al., 2001). There are no obvious or significant similarities in the aminio acid sequences of SC24 and the seed coat peroxidase that can account for the common antigenic domain(s) that these two proteins must share. However, the three-dimensional structure of soybean seed coat peroxidase has been solved by X-ray crystallography (Henriksen et al., 2001), and may provide a reference for predicting the structure of SC24, once the common antigenic domains have been identified.

Many characteristics of SC24, such as its abundance, stability, expression pattern, and citrate binding activity suggest that this protein may have a role in defence. Past studies have also shown that the seed coat is particularly rich in defence-related proteins and peptides (Gijzen et al., 2001). However, no anti-microbial activity of SC24 could be detected when tested against a variety of fungal, oomycete, and bacterial plant pathogens (not shown). Transgenic Arabidopsis lines expressing SC24 driven by a constitutive promoter (cauliflower mosaic virus 35S) were also created. Transgenic plants expressing the protein were recovered, but did not display any obvious phenotype that differentiates them from control plants (not shown).

To summarize, a major soluble protein present in soybean seed coats, SC24, has been isolated and characterized. The main characteristics of SC24 suggest that it is a defensive protein, thus supporting the biological role of seed coat in seed protection and defence. In addition, soybean seed coat is one of the crucial factors for embryo development and establishment of many agronomic traits such as seed size, lustre, colour, and composition. Therefore, identifying seed coat constituents and characterizing their corresponding genes is important, since soybean is the largest legume crop in the world.

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Fig. 7. Accumulation of SC24 in soybean seed coat. (A) Seed coat proteins (15 µg) were separated by SDS-PAGE and transferred to PVDF membrane by electroblotting. SC24 protein was detected by sequential incubation of the blot with α-SC24 antibody and anti-rabbit IgG conjugated to horseradish peroxidase, followed by the chemiluminescent reaction (ECL system, Amersham Biosciences). The numbers indicate the age of the seed coat in days after pollination. Pure peroxidase protein reacting with α-SC24 antibody is also shown. The numbers on the right indicate sizes of the proteins. MSC, mature seed coat; SH, seed hull; PRX, pure peroxidase protein. (B) A photograph of soybean seed coat cell layers. Seeds were soaked in water and the seed coat was removed and dissected to separate the cell layers in two fractions (I and II). The tissues were cut and immersed in 3% agarose gels and photographed as described in the Materials and methods. HG, hourglass cells; PA, palisade; P, parenchyma; A, aleurone layer. (C) A western blot analysis of SC24 in seed coat cell layers as described previously. The numbers on the right indicate sizes of the proteins (kDa).

Fig. 8. Accumulation of SC24 in wounded and unwounded soybean hypocotyls. Etiolated soybean hypocotyls were wounded as described under the Materials and methods and tissues were collected 3, 24, or 48 h after wounding. Total proteins (25 µg) were separated on SDS-PAGE and transferred to PVDF membrane by electroblotting. SC24 protein was detected by sequential incubation of the blot with α-SC24 antibody and anti-rabbit IgG conjugated to horseradish peroxidase followed by chemiluminescent reaction. C, unwounded control.

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