The seed coat-specific expression of a subtilisin-like gene, \textit{SCSI}, from soybean

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Received: 10 January 2000 / Accepted: 22 February 2000

\textbf{Abstract.} A seed coat-specific gene, \textit{SCSI} (\textit{Seed Coat Subtilisins I}), from soybean, \textit{Glycine max} [L.] Merill, has been identified and studied. The gene belongs to a small family of genes with sequence similarity to the subtilisins, which are serine proteases. Northern blot analysis showed that \textit{SCSI} RNA accumulates to maximal levels in seed coats at 12 days post anthesis, preceding the final stages of seed coat differentiation. The \textit{SCSI} RNA was not found in other tissues including embryos, seed pods, flowers, stems, roots or leaves. In-situ hybridization studies confirmed the temporal pattern of expression observed by Northern blot analysis and further revealed a restricted pattern of RNA accumulation in thick-walled parenchyma cells of the seed coats. These cells are important in the apoplastic translocation of nutrients en route to the embryo from the vascular tissues. The tissue-specific subtilisin-like gene may be required for regulating the differentiation of the thick-walled parenchyma cells.

\textbf{Key words:} \textit{Glycine} (seed coat) – Seed coat – Parenchyma (thick walled) – Subtilisin-like gene

\section*{Introduction}

Seed coats are essential for the development of seeds (reviewed in Reiser and Fischer 1993) and play a pivotal role in seed architecture (Corner 1951; Esau 1977), composition and yield (reviewed in Weber et al. 1997). Surprisingly, few seed coat genes have been examined. The inner and outer integuments that differentiate into the seed coats after fertilization have been well studied in the ovule and a variety of regulatory genes essential for ovule development have been identified by mutagenesis (reviewed in Reiser and Fischer 1993). Generally, these genes have not been examined after fertilization, except for the petunia MADS box genes, \textit{FBP7} and \textit{FBP11} (Colombo et al. 1997). In \textit{Arabidopsis}, a mutation (BANYULS) in seed coat pigmentation identified a gene expressed early in seed coat development that may code for a tissue-specific enzyme in the phenylpropanoid pathway (Devic et al. 1999). Other genes have been described during the subsequent stages of seed coat tissue differentiation but these have not been found to be seed coat specific. For instance, the soybean seed coat peroxidase gene, \textit{Ep}, is selectively expressed in the hourglass cells of mature seed coats but it is also expressed at low levels in roots (Gijzen 1997; Gijzen et al. 1999a). Similarly, genes such as the pea transcription factor AP1/AGL9 (Buchner and Boutin 1998) and the fava bean invertase \textit{VF1NV1} (Weber et al. 1995) exhibit strong expression in seed coats and lower levels of expression in other tissues.

In many species, the stages of tissue differentiation within the seed coat following fertilization have not been examined in great detail. Our earlier work with soybean revealed dramatic changes within the first 3 weeks after fertilization (Miller et al. 1999). The thick-walled parenchyma, which is derived from the inner integument, is very prominent during the first week but is rapidly degraded by the second week. The outer integument undergoes a number of complex changes which include the differentiation of the inner layer of thick-walled parenchyma, the vascular tissue which extends from the hilum region, a hypodermis of hourglass cells and the epidermal palisade layer. At maturity, many of the inner layers are crushed by the developing embryo and only the thin-walled parenchyma, hourglass cells and palisade cells remain.

The goal of this study was to isolate and characterize genes that accompany the differentiation of the soybean seed coat tissues after fertilization. The gene, \textit{SCSI}, was specifically expressed in the thick-walled parenchyma cells derived from the outer integument. It is a member of the subtilase superfamily of subtilisin-like serine protease genes. The members of this superfamily are found widely among organisms where they are involved in a variety of
biological processes (Barrett and Rawlings 1995; Siezen and Leunissen 1997). In eukaryotes, subtilisins are important regulatory molecules and govern the processing and maturation of active peptides such as hormones (Barr 1991). Only a small number of plant subtilisin-like genes have been cloned. They code for plasma-membrane proteases, such as tomato SBP50 (Schaller and Ryan 1994) or extracellular proteases such as tomato P69 (Vera et al. 1989), lily LIM9 (Taylor et al. 1997), spruce af70 (Sabala et al. 1997) and melon cucumisin (Yamagata et al. 1994). Although precise functions have not yet been assigned to plant subtilisins, evidence that P69B (Tornero et al. 1996) and SBP 50 (Schaller and Ryan 1994) may be involved in signal transduction indicates the potential for regulatory roles in plants. The discovery of a seed coat-specific subtilisin-like gene raises the possibility for a role in regulating seed coat development.

Materials and methods

Plant materials. *Glycine max* (L.) Merrill cv. Maple Presto plants were grown under fluorescent and incandescent light (300 μmol m −2 s −1) for a 12-h photoperiod in growth cabinets set at 25 °C and 20 °C, respectively, during the light and the dark cycles, with the relative humidity at 80%. To follow seed development flowers were tagged at each plant node on the day of full anthesis (Peterson et al. 1992).

Soybean pods and seeds were collected between 6 and 24 days post anthesis (dpa) at 3-d intervals. The seeds were separated into seed coat and embryo tissues. Leaf, stem and root tissues from 2-week-old seedlings and flowers at full anthesis were also collected.

Construction and screening of a cDNA library. A cDNA library was constructed in Lambda GEM-4 (Promega) from poly(A) − mRNA isolated from seed coats harvested from seeds of four fresh-weight groups (< 50 mg, 50–100 mg, 150–250 mg and > 250 mg) that had been pooled in approximately equal ratios prior to RNA extraction. Total RNA was isolated from the seed coats using Trizol reagent (Gibco BRL) from which poly(A) − RNA was isolated using Oligotex resin (Qiagen). First- and second-strand cDNAs were synthesized using the Riboclonoe cDNA synthesis kit and an XbaI primer-adaptor from Promega, ligated to an EcoRI adaptor and then cloned into a Lambda GEM-4 vector. The soybean seed coat cDNA library was differentially screened with positive and negative seed coat cDNA probes. The positive seed coat probe was a cDNA preparation derived from poly(A) − mRNA isolated from soybean seed coats. The negative seed coat probe was derived from poly(A) − mRNA from seedling, flower bud, leaf, pod and root tissues.

Another seed coat cDNA library was constructed in lambda ZAP Express (Stratagene) from poly(A) − mRNA isolated from soybean (*G. max* [L.] Merr. cv. Harosoy 63) seed coats. The seed coats had been harvested from seeds that were approximately 200 mg in fresh weight. Total RNA was isolated from the seed coats using the phenol/chloroform method described by Wang and Vodkin (1994). The poly(A) − RNA was purified on oligo (dT)-cellulose columns as described in Sambrook et al. (1989). First- and second-strand cDNAs were synthesized using the cDNA synthesis kit and an XhoI linker-primer from Stratagene, ligated to an EcoRI adaptor and then cloned into a lambda ZAP Express vector. This library was used as a source of full-length clones that were not found in the above library.

Construction and screening of a genomic library. A soybean genomic library was constructed in Lambda FixII (Stratagene) from the total DNA isolated from etiolated seedlings of soybean (*G. max* [L.] Merr.) cv. Harovinton. The DNA was partially digested with BglII prior to ligation into the cloning vector. The SCS1 genomic clone was obtained by screening filter lifts of 300,000 plaque-forming units under high stringency.

Analysis of RNA blots. Total RNA from seed coat, embryo and root tissues was isolated according to published methods (Ouellet et al. 1992; Fobert et al. 1994). Approximately 0.5 g plant tissue was frozen in liquid nitrogen and homogenized in 5 ml extraction buffer (1 M Tris-HCl, pH 9.0; 1% SDS) using a mortar and pestle. The sample was extracted twice with equal volumes of phenol: chloroform: isomyl alcohol (25:24:1; by vol.). The RNA was recovered from the aqueous phase by ethanol-precipitation, resuspended in water, and precipitated overnight in 2 M LiCl at 0 °C. The precipitate was collected by centrifugation, washed in 70% ethanol and resuspended in water. The method was modified for isolation of RNA from pods by adding 3 g of pre-hydrated polyvinylpolypyrrolidione to the extraction buffer to remove interfering phenolic compounds in this tissue (Wang and Vodkin 1994). Samples of RNA from leaf, stem and flower tissues were purified by extraction using Tripure Isolation Reagent kit (Boehringer). Approximately 0.5 g plant tissue was frozen in liquid nitrogen and homogenized with 5 ml Tripure reagent (Boehringer) using a mortar and pestle. After 5 min at room temperature the sample was extracted with 1 ml chloroform. Total RNA was recovered from the aqueous phase by isopropanol-precipitation and dissolved in water.

For RNA blot analysis, a sample of 10 μg of total RNA was electrophoretically separated through formaldehyde agarose gels and transferred to nylon membranes (Sambrook et al. 1989). The blots were pre-hybridized in 7% SDS, 10 mM EDTA, 0.5 M phosphate buffer (pH 7.2) for 3 h at 65 °C (Church and Gilbert 1984). Following pre-hybridization, 106 cpn of 32P-labeled probe, prepared by the random-primer method, was added and hybridization was carried out overnight at 65 °C. The membranes were then washed twice at 65 °C for 30 min in 0.1 × SSC, 0.1% SDS.

In-situ hybridization. In-situ RNA hybridizations were performed on *G. max* [L.] cv. Maple Presto seed sections essentially as described in Cox and Goldberg (1988). Seeds for in-situ hybridization were collected at 3-day intervals from pods that were carefully split longitudinally. When possible, the seed was left attached to one pod wall. The seed tissue was cut into approx. 2-mm sections and immediately placed into vials of FAA fixative [3.7% (v/v) formaldehyde, 50% (v/v) ethanol, 5% (v/v) acetic acid. Any seed sections remaining attached to the pod were fixed as a unit. Seed sections were fixed in FAA fixative for 4 h at room temperature, then dehydrated and embedded in Paraplast. In-situ hybridizations on 8- to 10-μm sections were performed as described previously for soybean seed coats (Gijzen et al. 1999a,b) using RNA probes prepared from the entire cDNA clone.

Light microscopy. Seeds were harvested at specific times in development as described above, and fixed in 4% phosphate-buffered glutaraldehyde (25 mM, pH 6.8). Samples were dehydrated through a solvent series, embedded in glycol methacrylate and stained as described previously (Miller et al. 1999). For observation of protein and carbohydrates, a stepwise staining procedure was used. Slides were first stained for carbohydrates using the Periodic Acid Schiff's procedure, then counterstained for protein with Light Green. For structural observations, Toluidine Blue O was used. Slides were photographed using Kodak E100S slide film.

Results

Cloning and analysis of the subtilisin-like gene, SCS1. A soybean (cv. Maple Presto) cDNA library was prepared from RNA extracted from seed coats that were dissected from seeds ranging in size from < 50 mg to 250 mg. SCS1 was one of the seed coat-specific clones identified