

Characterization of a stress-induced, developmentally regulated gene family from soybean

Dring N. Crowell^{1,3}, Maliyakal E. John², David Russell² and Richard M. Amasino¹

¹Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA; ²Agracetus, Inc., 8520 University Green, Middleton, WI 53562, USA; ³Current address: Department of Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, IN 46205, USA

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Abstract

We describe a family of stress-induced, developmentally regulated soybean genes for which cDNAs have been obtained from two different cultivars (*Glycine max* cv. Mandarin and *Glycine max* cv. Williams). The mRNAs corresponding to these cDNAs, called SAM22 and H4, respectively, accumulate predominantly in the roots of soybean seedlings but are present at high levels in the roots and leaves of mature plants. SAM22 accumulation is especially dramatic in senescent leaves. In addition, SAM22 accumulation can be induced in young leaves by wounding or by transpiration-mediated uptake of salicylic acid, methyl viologen, fungal elicitor, hydrogen peroxide or sodium phosphate (pH 6.9). Taken together, these data indicate that the genes corresponding to SAM22 and H4 are induced by various stresses and developmental cues. Southern blot analysis indicates that multiple copies of sequences related to SAM22 exist in the soybean genome. We also show that the nucleotide sequences of the cDNAs corresponding to SAM22 and H4 are 86% identical at the nucleotide level to each other and 70% identical at the amino acid level to the 'disease resistance response proteins' of *Pisum sativum*.

Introduction

Stress responses are important aspects of environmental adaptation in plants. These responses enable plants to survive a range of environmental conditions by giving them a plastic developmental program. Indeed, some stress responses are important features of normal plant develop-

ment. For example, certain dehydration-responsive genes are induced by osmotic stress in various plant tissues and are also expressed in developing embryos, which undergo programmed desiccation [15, 17, 18, 26]. Similarly, the bean chalcone synthase gene, a wound-inducible gene, has been shown to be induced at the point of penetration of young lateral roots through the

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X60043 (SAM22) and X60044 (H4).

cortex and epidermis of the main root [25], presumably due to the wounding that occurs during this normal developmental process.

The characterization of stress-induced, developmentally regulated genes is important to understanding the influence of environmental stimuli on plant development. We have isolated various soybean (*Glycine max* cv. Mandarin) cDNAs for which the accumulation of the corresponding mRNAs is developmentally regulated and responsive to external stimuli [5]. One of these mRNAs (SAM22, or starvation-associated message 22) accumulates approximately 10-fold as soybean suspension cultures enter stationary phase. The accumulation of this mRNA in stationary phase cultures may be due to the stress of phytohormone depletion because subsequent starvation for either cytokinin or auxin causes no further accumulation of SAM22 but the addition of cytokinin to cytokinin-starved cells or auxin to auxin-starved cells causes SAM22 levels to decrease dramatically within 4 hours. This mRNA also accumulates predominantly in the roots of soybean seedlings [5].

We now describe in detail the tissue-specific expression of the SAM22 gene and its homologue from *Glycine max* cv. Williams (H4). Furthermore, we show that SAM22 gene expression is induced in soybean leaves by various stresses. The involvement of stress-induced genes in plant development is discussed.

Materials and methods

Plant growth conditions and induction studies

Soybean plants (*Glycine max* cv. Mandarin, cv. Williams, and cv. Elgin) were grown in Jiffy Mix at 25 °C under continuous light. Tissues (i.e., primary leaves, cotyledons, hypocotyl sections or roots) were harvested at various times after germination by amputation into liquid nitrogen and then stored at -80 °C prior to RNA isolation. Wound inductions were done *in planta* by crushing the primary leaves of eight-day-old soybean plants with steel forceps. Chemical inductions

were done by excising the primary leaves of eight-day-old soybean plants and allowing them to float, such that the cut petiole was immersed, on 200 ml of one of the following solutions: water, 10 mM salicylic acid, 0.1 mM methyl viologen (i.e., paraquat), 200 µg/ml chitosan, 1 mM H₂O₂, or 50 mM sodium phosphate (pH 6.9). The leaves were thus exposed, by transpiration-mediated uptake for various times, to the stresses caused by these compounds. These leaves were then frozen in liquid nitrogen and stored at -80 °C prior to RNA isolation.

RNA isolations and RNA blot analyses

Unless otherwise indicated, nucleic acid manipulations were as described [24]. All RNA blot analyses were performed on total RNA, which was isolated as described [6, 12]. Briefly, 5 µg of total RNA per lane was size-fractionated by 7.2% (v/v) formaldehyde -1.0% (w/v) agarose gel electrophoresis, transferred to nylon membranes by capillary blotting, and hybridized to antisense RNA probes, which were made by T3 RNA polymerase reaction [6, 12, 22]. Hybridizations were as described [22] except that the hybridization buffer lacked both DNA and polyethylene glycol and contained 1 mg/ml of yeast RNA. Quantitation of changes in mRNA levels was done by scanning RNA blots with a Betagen scanner.

Isolation of cDNA clones

The isolation of a SAM22 cDNA clone from a cDNA library made with the mRNA of a cytokinin-starved soybean suspension culture (*G. max* cv. Mandarin) has been described [5].

A second cDNA clone (H4) was isolated from a cDNA library made with the mRNA of root tissue from one-week-old soybean seedlings (*G. max* cv. Williams). Double-stranded, dC-tailed cDNA was synthesized according to the method of D'Alessio [7] and annealed to dG-tailed pBR322 vector DNA (Bethesda Research Labs, Gaithersburg, MD). The resulting plasmid

DNA was used to transform *Escherichia coli* strain RR1. Replica filters of this library were differentially screened by hybridization to ^{32}P -labeled cDNA probes made with the mRNA of root, stem and leaf tissue from one-week-old soybeans. This screen resulted in the identification of 5 cDNA clones that showed strong, specific hybridization to the root cDNA probe. Clone H4 was selected for further study.

DNA isolations and DNA blot analyses

Soybean genomic DNA was prepared as described [22]. This DNA (5 μg per sample) was digested with *Eco* RI or *Hind* III under conditions recommended by the vendor (New England Biolabs, Beverly, MA). Digested soybean DNA samples were then fractionated by 1.0% (w/v) agarose gel electrophoresis (3.75 V/cm), denatured in 0.4 M NaOH for 1 h, blotted onto nylon membranes and annealed to RNA probes as described [22, 28].

DNA sequence analyses

Sequence analyses of cDNAs were done as described [4, 29] by The Cetus Corporation (Emeryville, CA) and by Lofstrand, Inc. (Gaithersburg, MD). All sequences were manipulated using the sequence software of the University of Wisconsin Genetics Computer Group [9]. Homology searches were performed by using the program FASTA to search the database Genpept [21].

Results

Developmental regulation

We have reported the isolation of a soybean (*G. max* cv. Mandarin) cDNA for which the corresponding mRNA (SAM22) is stationary phase-inducible in cultured soybean cells and root-specific in soybean seedlings [5]. We now present a more detailed analysis of the developmental regulation of SAM22 accumulation. Consistent with

previous results, SAM22 accumulates predominantly in the roots of very young soybean seedlings (Fig. 1). However, as these plants mature, SAM22 also accumulates in cotyledonous tissue. This accumulation correlates with the onset of senescence, as judged by the appearance of yellow patches at the fringes of the cotyledons. Furthermore, if these plants are allowed to mature further (i.e., one month), SAM22 abundance increases in primary leaves, especially senescent primary leaves (also judged by the appearance of yellow patches). These results demonstrate that SAM22 accumulation is specific to roots and aging, especially senescent, cotyledons and leaves of vegetative soybean plants. We are currently investigating the possibility that SAM22 also accumulates in floral tissues.

Another cDNA (H4), related to the SAM22 cDNA (see below), was independently isolated from a cDNA library made with the mRNA of root tissue from one-week-old soybean (*G. max* cv. Williams) seedlings. This cDNA was selected following a differential screen of the library because it corresponds to an mRNA present in the roots, but not in the leaves or stems, of soybean seedlings. However, like SAM22, the H4 mRNA

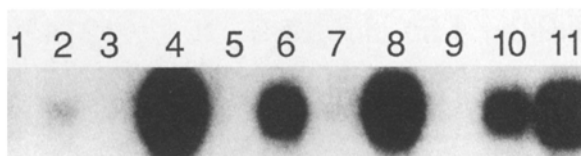


Fig. 1. Developmental regulation of SAM22 accumulation. Total RNA (5 μg per lane) from the soybean (*G. max* cv. Elgin) tissues described below was probed with a ^{32}P -labeled SAM22 RNA probe. Tissues are: lane 1, primary leaves of 5-day-old soybeans (unexpanded primary leaves); lane 2, cotyledons of 5-day-old soybeans; lane 3, hypocotyl sections of 5-day-old soybeans; lane 4, roots of 5-day-old soybeans; lane 5, primary leaves of 8-day-old soybeans (expanded primary leaves); lane 6, cotyledons of 8-day-old soybeans; lane 7, hypocotyl sections of 8-day-old soybeans; lane 8, roots of 8-day-old soybeans; lane 9, primary leaves of 8-day-old soybeans (same as lane 5); lane 10, green primary leaves of one-month-old soybeans (non-senescent); lane 11, yellow primary leaves of one-month-old soybeans (senescent). A markedly different pattern of mRNA accumulation was observed when the same total RNA was analyzed with a control probe corresponding to an iron superoxide dismutase cDNA (data not shown).

accumulates not only in roots, but also in leaves, of mature plants (data not shown).

Stress induction

The accumulation of SAM22 in stationary phase soybean suspension cultures and in roots and senescent tissues of whole soybean plants suggests that this mRNA may accumulate in response to various stresses. To test this hypothesis, we examined various stresses, including three (e.g., salicylic acid, fungal elicitor and wounding) known to induce other stress-responsive genes [11, 20, 25, 30], for their ability to induce the accumulation of SAM22 in primary leaves of eight-day-old soybean plants. As shown in Fig. 2, these leaves do not contain significant amounts of SAM22 if harvested without treatment or if excised and exposed to water for 20 h by transpiration-mediated uptake. However, a 30–50-fold increase in SAM22 abundance is observed if these leaves are excised and exposed to 10 mM salicylic acid or to

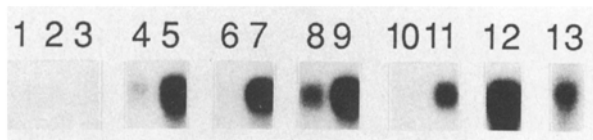


Fig. 2. Induction of SAM22 accumulation in young soybean leaves by stress. Total RNA (5 μ g per lane) from primary leaves of 8-day-old soybean seedlings (*G. max.* cv. Elgin), treated as described below, was probed with a ^{32} P-labeled SAM22 RNA probe. All chemical treatments were done by transpiration-mediated uptake. Treatments are: lane 1, no treatment; lane 2, water (4 h); lane 3, water (20 h); lane 4, 10 mM salicylic acid (4 h); lane 5, 10 mM salicylic acid (20 h); lane 6, 0.1 mM methyl viologen (4 h); lane 7, 0.1 mM methyl viologen (20 h); lane 8, 200 μ g/ml chitosan (4 h); lane 9, 200 μ g/ml chitosan (20 h); lane 10, 1.0 mM H_2O_2 (4 h); lane 11, 1.0 mM H_2O_2 (20 h); lane 12, 50 mM sodium phosphate, pH 6.9 (20 h); lane 13, wounded primary leaves. The inductions shown are not from the same experiment, but controls such as those in lanes 1–3 were done for all experiments. Inductions were done at least twice. No induction of mRNA accumulation was observed when the same total RNA was analyzed with a control probe corresponding to an iron superoxide dismutase cDNA (data not shown).

0.1 mM methyl viologen (i.e., paraquat) for 20 h by transpiration-mediated uptake. Similar increases in SAM22 abundance are observed if leaves are exposed to 200 μ g/ml chitosan (a fungal elicitor) or to 1.0 mM H_2O_2 . Even exposure to 50 mM sodium phosphate (pH 6.9) causes a dramatic increase in the level of SAM22, possibly due to the stress caused by raising extracellular salt and/or pH. Mechanical damage (i.e., wounding) also induces SAM22 accumulation, though wound-induced increases in SAM22 abundance are less dramatic (5–10-fold). These results indicate that various stresses induce the accumulation of SAM22 in young soybean leaves. Surprisingly, transpiration-mediated uptake of 10 μ M or 100 μ M abscisic acid has no effect on SAM22 abundance in this system (data not shown).

Southern analysis

In order to further characterize the SAM22 and H4 genes, we performed Southern blot analyses (Fig. 3) and quantitative slot blot analyses (data not shown) on total soybean DNA. For the quantitative analyses, 5 μ g of total soybean DNA was analyzed alongside 20 pg, 200 pg and 2000 pg of plasmid DNA containing either the SAM22 cDNA or the H4 cDNA. These amounts of plasmid DNA were calculated, based on a haploid soybean genome size of approximately 1×10^9 base pairs, to be equivalent to 1 copy, 10 copies and 100 copies per haploid genome, respectively, when compared to a total DNA sample of 5 μ g. Quantitative slot blot analyses indicated that multiple copies (approximately 10) of the SAM22 gene or related genes exist per haploid soybean genome. However, few cross-hybridizing bands appear in *Eco* RI or *Hind* III digests of total soybean DNA, suggesting that either these sequences exist in tandem arrays or they are dispersed and highly conserved such that different copies produce co-migrating restriction fragments in a Southern blot analysis. Southern blot analyses with SAM22 probes and with H4 probes produced similar results (data not shown).

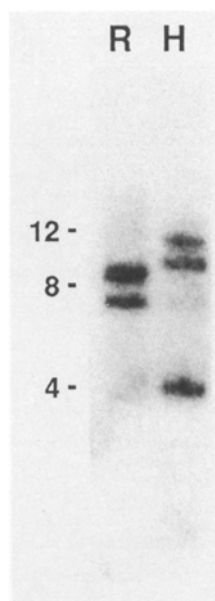


Fig. 3. Southern blot analysis of SAM22-H4 sequences in soybean DNA. Soybean DNA (5 μ g per lane) from *G. max* cv. Williams was digested with either *Eco* RI (R) or *Hind* III (H) and hybridized to a ^{32} P-labeled H4 probe.

Sequence homology

A comparison of SAM22 and H4 cDNA sequences is shown in Fig. 4. These sequences demonstrate that: (1) the SAM22 and H4 cDNAs are 86% identical at the nucleotide level, (2) the majority of the sequence divergence occurs at the 3' end, downstream of the putative translation termination codon, (3) the SAM22 and H4 cDNAs contain open reading frames of 474 bp, which are predicted to encode 16761 Da and 16746 Da proteins, respectively, and (4) the deduced amino acid sequences of the SAM22 and H4 open reading frames are 70% identical to the 'disease resistance response proteins' [8, 10, 11, 23] of *Pisum sativum* (see Fig. 5).

Discussion

In this paper, we describe a soybean gene family for which the corresponding mRNAs exhibit two notable features: accumulation in response to various external stresses and tissue-specific accu-

mulation in the tissues of whole plants (i.e., in roots and senescent tissues). The accumulation of these mRNAs in roots and senescent tissues is consistent with a role in stress physiology since roots undergo wounding during the penetration of young lateral roots through the body of the main root and since senescent tissues undergo programmed cell death. Hence, these stress-induced genes may have an important role in plant development (i.e., in root development and tissue senescence). Future experiments will determine whether root-specific expression of the SAM22-H4 gene family is localized to points of penetration of young lateral roots and whether different members of the SAM22-H4 family contribute differently to the overall pattern of expression of these genes.

The suggestion that stress-induced genes may have a role in plant development is not new. For example, osmotic stress induces the expression of the rice RAB21 gene and the wheat Em genes in leaves and cultured embryos, respectively. These genes are also induced during the later stages of embryo maturation, at which point the embryos undergo programmed desiccation [15, 17, 18, 26]. Hence, these genes may be essential to the development of embryos by enabling them to withstand the dehydration that occurs during their maturation. Furthermore, it has been shown that the bean chalcone synthase gene is highly expressed at points of penetration of young lateral roots through the main root [25]. Expression of genes involved in flavonoid biosynthesis (e.g., chalcone synthase) results in the production of phytoalexins [13], compounds known to protect plants from microbial attack. Hence, wound-induced expression of flavonoid biosynthetic genes may be essential during root development to prevent infections that might otherwise occur at wounds caused by lateral root penetration.

The sequences of the SAM22 and H4 cDNAs are herein shown to correspond to the sequences of the 'disease resistance response protein' genes of *P. sativum* [8, 10, 11, 23]. These genes correspond to mRNAs that have been reported to accumulate to higher levels and/or for longer times when peas are challenged with a pathogen to

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SAM22 AAAGGGCACTCCTCTGCTAAAGAAGCACACAGCAGCAAGCATCTCTCTCAAACCTAGTAGTATTATCTCCATTCCGTTCTACATATAATCATTCATA 100
H4                                         CTTTCTTTTATTATTTCATA 19

      M G V F T F E D E I N S P V A P A T L Y K A L V T D A D N V I P K
SAM22 ATGGGTGTTTTCACATTGAGGATGAAATCAACTCCCCTGTGGCTCCTGCTACTCTTTACAAGGCCCTAGTTACAGATGCCGACACGTCATCCCAAGG 200
H4     ATGGGTATTTTCACATTGAGGATGAAACACCTCCCCTGTGGCTCCTGCTACTCCTTTACAAGGCTCTAGTTACAGATGCCGACACGTCATCCCAAGG 119
      M G I F T F E D E T T S P V A P A T L Y K A L V T D A D N V I P K
                                         20

      A L D S F K S V E N V E G N G G P G T I K K I T F L E D G E T K F V
SAM22 CTCTTGATTCCTTCAAGAGTGTGAAACCTTGAGGGAAATGGTGGCCAGGAACCATCAAGAAGATCACTTTCCTTGAGGATGGAGAAACCAAGTTGT 300
H4     CTGTGCAAGCCCTTCAAGAGTGTGAAACCTTGAGGGGAAACGGTGGCCCTGGAACCATCAAGAAGATCACTTTCCTTGAGGATGGAGAAACCAAGTTGT 219
      A V E A F R S V E N L E G N G G P G T I K K I T F V E D G E S K F V
                                         40                                     60

      L H K I E S I D E A N L G Y S Y S V V G G A A L P D T A E K I T F
SAM22 GCTGCACAAAATAGAAAGCATTGATGAGGCGAACTTGGGATATAGCTACAGCGTGGTTGGGGGTGCTGCATTGCCAGACACGGCGGAGAAAGATCACATTC 400
H4     GTTGCACAAAATAGAAATCAGTTGATGAGGCAAACTTGGGATACAGCTATAGCGTAGTTGGTGGAGTTGGTTGCCAGACACAGTGGAGAAAGATCACATTC 319
      L H K I E S V D E A N L G Y S Y S V V G G V G L P D T V E K I T F
                                         80                                     100

      D S K L V A G P N G G S A G K L T V K Y E T K G D A E P N Q D E L
SAM22 GACTCCAAATGGTGTGCTGGTCCCAATGGAGGGTCTGCTGGGAAGCTCACTGTCAAATACGAAACAAAGGAGATGCTGAGCCCAACCAAGACGAAGTCA 500
H4     GAATGCAAAATGGCTGCTGGCGCAACGAGGGTCTGCTGGGAAGCTAACTGTCAAATACCAACCAAGGAGATGCTGAGCCCAACCAAGACGACCTCA 419
      E C K L A A G A N G G S A G K L T V K Y Q T K G D A Q P N P D D L
                                         120

      K T G K A K A D A L F K A I E A Y L L A H P D Y N *
SAM22 AAATGGGAAAAGCCAAAGGCTGATGCTCTCTTCAAGGCCATTGAGGCTTACCTTTTGGCCCATCCGATTACAACCTAATCC..... 580
H4     AAATGGCAAAGTCAAGTCTGATGCTCTTTTCAAGGCCGTTGAGGCTTACCTTTTGGCCCAATCCTATTACAACCTGATCCAATTCGATCTTCAGTATTCA 519
      K I G K V K S D A L F K A V E A Y L L A N P H Y N *
                                         140

      . . . TCTTCAACTTACTTGT . . . . . CATCACTTT . . . . . CTGCTTGGAGACTTATCCAATCACTCTGTTCTCAACTTAAGT . . . . . T 649
SAM22
H4     GTGATCTGCAAGACCTTGTCTTATATTATATACAAGAGTTTCTTGCTTGGTGGAGTGTATTCAATCACT . . . . . TTGAGTGTGGTGT 608

      GCCTTCCA . . . ATGTATCCAGTCTTCTCTTCTTCTTCTTCTTCTTCCATATAAATTGTGAGA . . . TTAATGATATATCTGTATCTCACTTCATCAAT . . . 739
SAM22
H4     GGCTTCCAATGTATGTAACGAGTGTCTCTTCTTCTTCTTCTTCTTCTTCCACAGAATTGTGAGAGCCAGTTGATCTCTTGTATGTCACTTCATCAAT 708

      . AATAAATTATAAATAAGATGAGCAATCATTACAAAAA 783
SAM22
H4     CAATATGTTATAAATAAATGAGATTGGAAGAA 742

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Fig. 4. Nucleotide sequences of SAM22 and H4 cDNAs and deduced amino acid sequences of SAM22 and H4 protein products. The sequence alignment was generated with the program BESTFIT [9]. Putative polyadenylation signals are underlined.

which they are resistant than when they are challenged with a pathogen to which they are sensitive [11]. Hence, the accumulation of these pathogen-responsive mRNAs reportedly correlates with disease resistance. However, since disease resistance can result from localized hypersensitivity to pathogen attack, it is possible that these genes simply respond locally to the increased stress of a hypersensitive reaction. We believe that the SAM22-H4 genes are probably involved in the physiological changes that occur in response to generalized stress, rather than in

disease resistance *per se*, since stresses unrelated to pathogenicity strongly induce the expression of these genes. However, disease resistance may be one of the physiological consequences of generalized stress responses.

The environmental and developmental regulation of the genes in the SAM22-H4 family mimics that of genes encoding various pathogenesis-related proteins [3, 20, 30]. Consistent with this observation, pathogenesis-related protein genes from parsley [27] and from potato [16] have been shown to be highly homologous to the 'disease

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MGMFTFEDINSEVAPAILLYKALVTDADNVIKHALDSFKSMENVEGNGGP SAM22
MGMFTFEDETTSVAPAILLYKALVTDADNVIKAVEAFRSMENLEGNGGP H4
MGMENVEDETTSVAPAILLYKALVTDADNLTPKVIDAITSIEIVEGNGGA PEADRRRC
MGMENVEDETTSVAPAILLYKALVTDADLTLPKVIDAITSIEIVEGNGGA PEADRRRA

GTIKKLTTFEDGETMEVLHKIESIDHANTGYSYSVGGAAIPDTAEKITE SAM22
GTIKKLTTFEDGESMEVLHKIESVDEANIGYSYSVGGVGLPDTVEKITE H4
GTIKKLTTFEDGETMEVLHKVELVDMANIANYSIVGGVGFDPDTVEKISE PEADRRRC
GTIKKLTTFEDGETMEVLHKVELVDMANIANYSIVGGVGFDPDTVEKISE PEADRRRA

DSKLHAGNGGSSACKLITVKYETKGD-PEENQDELDTCAKADALFKALIEA SAM22
ECKLHAGNGGSSACKLITVKYETKGD-ACENPDLLIKGVKSDALFKAVEA H4
EAKLSAGNGGSSIAKISVKYETKGD-PAFSEEQLKTDKAKGDLFKALIEG PEADRRRC
EAKLSAGNGGSSIAKISVKYETKGD-PAFSEEQLKSDKAKGDLFKALIEG PEADRRRA

YLLAHEDYN SAM22
YLLAHEDYN H4
YCLAHEDYN PEADRRRC
YCLAHEDYN PEADRRRA

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Fig. 5. Amino acid sequence identity between the SAM22 and H4 protein products and two 'disease resistance response proteins' of pea (PEADRRRC and PEADRRRA) [10]. Amino acids that are conserved among all four sequences are boxed.

resistance response protein' genes of pea. (Another homologue of these genes encodes the major pollen allergen of birch [2].) Interestingly, pathogenesis-related proteins have also been implicated in normal developmental processes [14, 19].

The induction of SAM22 accumulation by chitosan, which is a fungal elicitor, and by H_2O_2 is interesting because H_2O_2 is thought to be a second messenger in elicitor-mediated responses [1]. According to this hypothesis, H_2O_2 is produced in response to elicitor outside of the plant cell by an oxygen-dependent reaction. The H_2O_2 then enters the cell and induces various defense mechanisms. Consistent with this hypothesis, we have found that weak SAM22 induction is observed if leaves are vacuum-infiltrated with water and left submerged for 20 h, but no further induction is observed if leaves are exposed to 0.1 mM methyl viologen or 200 μ g/ml chitosan in this way, suggesting that perhaps SAM22 induction by these compounds is an oxygen-dependent process (data not shown).

This paper describes a stress-induced, developmentally regulated gene family from soybean. We are currently working to elucidate the function(s) of the SAM22 and H4 genes and to understand the complex regulation of these genes in response to environmental and developmental signals. In particular, we are interested in identifying putative *cis*-acting promoter sequences that

confer environmental and/or developmental regulation on the SAM22-H4 gene family. This work may lead to the identification of novel *trans*-acting factors that influence the expression of this interesting family of genes.

Acknowledgements

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