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Serine Protease Inhibitors Expressed in the Process of Budding of Tunicates as Revealed by EST Analysis[1]

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To identify genes expressed during budding of the tunicate *Polyandrocarpa misakiensis*, we isolated and sequenced 624 clones from a directionally constructed cDNA library to prepare a catalog of expressed sequence tags (ESTs). A total of 233 ESTs matched genes of known sequence in the SwissProt database. About 24% out of them showed high similarity to ribosomal proteins, twice the value (12%) of pre-budding animals. ESTs involved in the respiratory chain also appeared with significant redundancy, suggesting that tunicate budding is accompanied by the enhancement of energy conversion as well as protein synthesis. Serine protease inhibitor (serpin) afforded another striking example of a gene that was highly expressed in the process of budding. The deduced amino acid sequences of five serpin cDNAs all had two consensus signatures of the Kazal's type of secretory protease inhibitor, one of which had an active site for trypsin and the other for elastase. In line with this, recombinant GST-fusion protein showed both trypsin and elastase inhibitor activities. In accordance with the EST analysis, the hemolymph taken from the budding stage showed the highest activity of trypsin inhibitor. We discuss a possible role that *Polyandrocarpa* serpins may play in bud development by counteracting trypsin-like serine protease, which could facilitate dedifferentiation of formative tissues.

Key words: budding, expressed sequence tags, serine protease inhibitor, tunicate.

Systematic cDNA analysis began as a complementary division of human genome project (*1, 2*). Partial DNA sequences obtained are referred to as expressed sequence tags (ESTs) (*1*). ESTs have been found useful for mapping the human genome, identifying open reading frames in genome sequences, and searching for new human genes (*2*). It is also useful for creating gene expression profiles of particular cells and tissues (*3, 4*). By counting the frequency of gene appearance, it is easy to characterize the differentiation state of both embryonic and somatic cells, indicating that EST analysis could provide a novel, potent methodology in molecular developmental biology.

Tunicates are a small group of primitive chordates. Their embryogenesis, morphology, physiology, and molecular biology are interesting from the viewpoint of evolution toward vertebrates [for review of developmental biology (*5*)]. Tunicates are also known as the highest organisms that propagate by means of asexual reproduction. Budding of *Polyandrocarpa misakiensis* involves extraordinary outgrowth of the body wall (*6*). A bud of 6-7 mm in length arises from around the basal margin of a parent of about 10 mm long (*7*). It consists of outer and inner epithelial vesicles, between which mesenchymal cells are located (for review, Refs. *8* and *9*). After separation from the parent, the bud begins to reform a small but complete miniature of the parent exclusively from the inner epithelium (*9*).

In *P. misakiensis*, a parent animal successively gives off about 40 buds over several months (*10*), indicating that during its life span it loses a vast amount of somatic cells owing to budding. It is reasonable to assume that a comparable number of cells is promptly recruited, as the parent maintains its morphological integrity even after successive budding. It is interesting to ask what is the motive force of tunicate budding that is accompanied by a large amount of cell loss and recruitment, and this leads to the general question of how cell and tissue homeostasis becomes possible in post-embryonic life.

As mentioned above, the inner epithelium of a bud is the major formative tissue. It is a pigmented, differentiated tissue, but it is able to transdifferentiate into many ectodermal and endodermal tissues and organs (*11, 12*). Thus, another interesting question concerning *Polyandrocarpa* budding is how differentiation of the multipotent, inner epithelium is regulated, in other words, how particular somatic cells maintain a comparable developmental plasticity to embryonic cells.

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Abbreviations: AMC, 7-amino-4-methylcoumarin; EST, expressed sequence tag; GST, glutathione S-transferase; MCA, -4-methylcoumaryl-7-amide; serpin, serine protease inhibitor.

The goal of our project is to construct a catalog of genes that are expressed in the process of bud formation of *P. misakiensis*. In the present study, cDNA was randomly selected and partially sequenced. The gene expression profile was compared with that of juvenile, pre-budding animals. Special attention was paid to serine protease inhibitors, as there is a strong possibility that they may counteract dedifferentiation-promoting factors such as serine protease in the process of tunicate budding (Ohashi *et al.*, submitted). Based on our findings, we discuss the possible function of genes that are expressed during budding.

MATERIALS AND METHODS

Animals—*P. misakiensis* (*6*) was used. Animals were raised in culture boxes set in the Uranouchi Inlet near the Usa Marine Research Center, Kochi University. Of four asexual *Polyandrocarpa* strains maintained, the *white spot* strain was used in this study (*cf.*, Ref. *13*). Animals of the budding stage were cut into anterior and posterior halves with razor blades. The anterior halves were frozen in liquid nitrogen and stored at −80°C until use.

Preparation of cDNA Library—Frozen samples (1.4 g) were thawed and homogenized in guanidinium thiocyanate, and poly(A)$^+$ RNA was collected using a Quick Prep Micro mRNA Purification Kit (Pharmacia, USA). cDNAs were prepared and inserted directionally into λ ziplox vectors at *Sal*I/*Not*I sites, according to the manufacture's instruction manual (Gibco Life Tech., USA). For packaging, a λ DNA *in vitro* packaging kit (Amersham, USA) was used.

Plaque PCR—*Escherichia coli* (Y1090r$^-$) cultured in LB liquid medium containing 0.2% maltose and 10 mM $MgSO_4$ was transfected with λ ziplox phage. Single plaques that appeared on NZYM top agarose were lifted and suspended in 100 μl of SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin, pH 7.5). This plaque suspension was used as template for the polymerase chain reaction (PCR), with 5′-CACTATAGGGAAAGCTGGTACGC-3′ as primer F and 5′-CCCAGTCACGACGTTGTAAAACG-3′ as primer R. PCR was run at 95°C for 2 min for denaturation, followed by 30 cycles of 95°C for 0.5 min, 53°C for 1 min, and 72°C for 1.5 min. Reaction products were examined by agarose gel electrophoresis and those longer than 600 bp were used for further analysis. They contained a total of 180 bp of phage origin at their ends, indicating that inserts longer than 420 bp had been selected.

Subcloning—PCR products were subcloned into TA cloning vector (Invitrogen, USA). Ligation was done for at least 3 h at 16°C. After color selection for β-galactosidase, plasmids were purified from cloned, ampicillin-resistant bacteria. Aliquots of plasmids were digested with *Eco*RI for 2 h to confirm that the insert DNA was of appropriate size.

DNA Sequencing and Homology Search—Both PCR products and plasmid DNA were used as templates for sequencing. For the sequence reaction, a Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham, USA) was used. The reaction products were applied to a DNA sequencer 373A (Applied Biosystems, USA). A search for homologous sequences in the database was constructed *via* the Internet using the BLASTX program. In case where no or low similarity was found, the search was repeated using the BLASTN program.

Preparation of Recombinant Serine Protease Inhibitor—A cDNA fragment of *Polyandrocarpa* serine protease inhibitor 1 (P-serpin 1) covering the coding region from Met1 to Cys135 was amplified by PCR using 5′-ATGGACG-GAATTGTAATGTTT-3′ and 5′-TCGCGAGCGGCCGCC-CTTTTTTTTTTTTTTT-3′ as primers. The PCR fragment was ligated with *Bam*HI linker (5′-GGCCGCGGATCC-3′), digested with *Bam*HI and *Not*I, and inserted into pGEX-4T-2 (Pharmacia, USA) digested with the same restriction enzymes. This plasmid was amplified in the primary bacterial host, DH5α, and then transferred to BL21. Glutathione S-transferase-P-serpin fusion protein was induced by isopropylthio-β-D-galactoside (IPTG) and purified, using a Bulk GST purification kit (Pharmacia, USA). The purity of recombinant protein was confirmed by SDS-PAGE. Protein amount was measured by the method of Lowry *et al.* (*14*).

Preparation of Hemolymph—Growing buds, developing buds, and adult animals were harvested separately. Seawater around the body was wiped off, then the body was squeezed to collect the hemolymph in a microcentrifuge tube. Immediately after centrifugation, the supernatant was examined for its trypsin-inhibitory activity.

Measurement of Protease and Protease-Inhibitory Activities—Aliquots (10 μl) of authentic trypsin (10 μg/ml), chymotrypsin (10 μg/ml), or elastase (50 μg/ml) were diluted with 240 μl of 50 mM phosphate buffer (pH 7.0). Synthetic substrates for trypsin, Boc-Gln-Ala-Arg-4-methylcoumaryl-7-amide (MCA), for chymotrypsin, Suc-Ala-Ala-Pro-Phe-MCA, and for elastase, Suc-Ala-Ala-Ala-MCA, all products of Peptide Institute, Osaka, were dissolved in dimethylsulfoxide as 10 mM stock solution. This was added to the above enzyme solutions mentioned in a final concentration of 0.1 mM. Proteolytic digestion was performed for 1 h at 35°C. Tunicate hemolymph or recombinant protein (5 μl) was added to the enzyme-substrate solution, and its serine protease inhibitor activity was measured.

Immediately after the digestion, the reaction mixture (50 μl) was applied to a high performance liquid chromatography (HPLC) system (Jasco 801SC, 880-02, 880-PU, 875-UV, 821-FP Japan Spectroscopic Corporation, Japan) equipped with a 5C18 reverse phase column (Nakalai Tesque, Japan). The column was eluted with 70% methanol in 0.1% acetic acid at a flow rate of 1 ml/min. Free 7- amino-4-methylcoumarin (AMC) released from the synthetic peptide was monitored with excitation at 380 nm and emission at 460 nm. As described elsewhere (Ohashi *et al.*, submitted), the concentration of AMC was directly proportional to the logarithm of fluorescence intensity, at least in the range from 50 nM to 20 μM. One biological unit (B.U) indicates the enzymatic activity that releases 100 nM AMC per hour at 35°C.

RESULTS

To prepare a catalog of genes expressed in budding *Polyandrocarpa*, a cDNA library was constructed using λ ziplox as a vector, into which poly(A)$^+$ cDNAs were inserted directionally. Out of 1,460 clones selected randomly, 624 had PCR products longer than 600 bp when amplified using phage-specific sequence primers. Finally, 483 clones were successfully sequenced. The similarity search using the

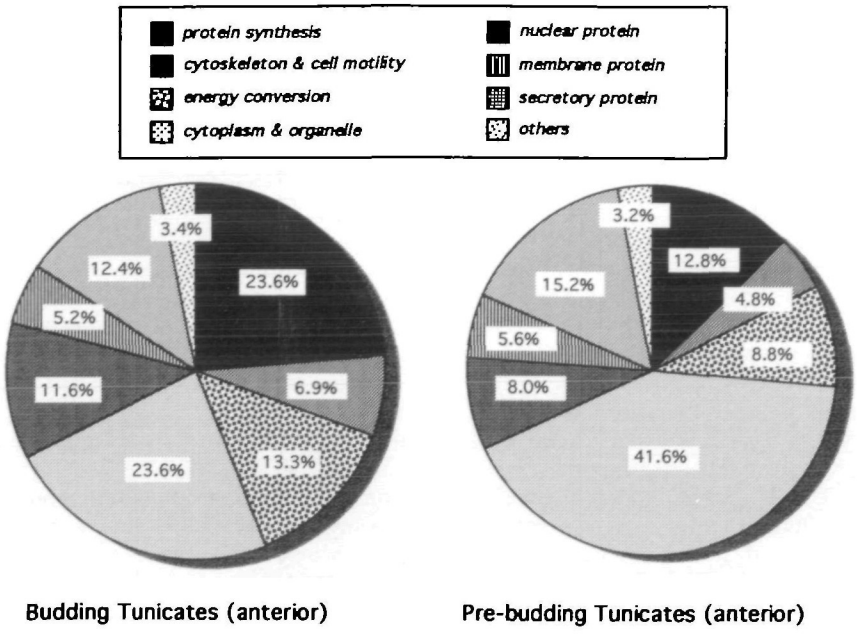

Fig. 1. **EST frequencies of budding and pre-budding tunicates.** ESTs were classified into eight categories: protein synthesis, cytoskeleton and cell motility, energy conversion, other cytoplasmic components, nuclear protein, membrane protein, secretory protein, and others. Note that ESTs related to protein synthesis are about twice as frequent in budding animals as in pre-budding animals.

TABLE I. **EST classification based on possible functions.**

EST number	Putative identification	Frequency
	PROTEIN SYNTHESIS	**55**
Pm01063	Elongation factor 1-α	2
Pm00365	Eukaryotic initiation factor 2γ	1
Pm01150	Eukaryotic initiation factor 4γ	2
Pm00730	Eukaryotic initiation factor 5	1
Pm00114	60S ribosomal protein L1	1
Pm00952	60S ribosomal protein L3	1
Pm00456	60S ribosomal protein L6	1
Pm00878	60S ribosomal protein L7A	1
Pm00868	60S ribosomal protein L13/L13A	5
Pm00261	60S ribosomal protein L18A	1
Pm01031	60S ribosomal protein L19	1
Pm00636	60S ribosomal protein L24	2
Pm00128	60S ribosomal protein L26	1
Pm00431	60S ribosomal protein L27	1
Pm00784	60S ribosomal protein L30	1
Pm00888	60S ribosomal protein L31	2
Pm00942	60S ribosomal protein L35/L35A	3
Pm01004	60S acidic ribosomal protein P0	2
Pm00262	60S acidic ribosomal protein P1	2
Pm00131	60S acidic ribosomal protein P2	2
Pm00859	40S ribosomal protein S2	2
Pm00621	40S ribosomal protein S7	2
Pm00783	40S ribosomal protein S8	2
Pm00658	40S ribosomal protein S10	1
Pm01432	40S ribosomal protein S12	2
Pm00606	40S ribosomal protein S13	1
Pm00129	40S ribosomal protein S15	3
Pm00210	40S ribosomal protein S18	2
Pm00727	40S ribosomal protein S19	1
Pm01042	40S ribosomal protein S20	1
Pm01078	40S ribosomal protein S21	1
Pm00608	40S ribosomal protein S23	1
Pm01021	40S ribosomal protein S25	1
Pm00411	40S ribosomal protein S26	1
Pm00828	40S ribosomal protein S27A	1
	CYTOSKELETON & CELL MOTILITY	**16**
Pm01526	Actin, α skeletal muscle	1
Pm00419	Clathrin heavy chain	1
Pm01245	Dynein heavy chain	1
Pm00982	Dynein light chain	1

TABLE I. Continued

EST number	Putative identification	Frequency
Pm00233	Muscle specific protein (calponin)	1
Pm01607	Myosin-like protein	1
Pm01030	Troponin C	1
Pm00640	Tubulin α chain	7
Pm00747	Tubulin β chain	2
	ENERGY CONVERSION	**31**
Pm00569	Aconitate hydratase	1
Pm00769	Acyl-coenzyme A oxidase I, peroxisomal	1
Pm00913	ADP, ATP carrier protein, isoform T2	1
Pm00544	ATP synthase α chain	1
Pm00306	ATP synthase E chain	1
Pm01404	ATP synthase F chain	1
Pm00540	Creatine kinase, M chain	1
Pm01633	Cytochrome C	1
Pm00267	Cytochrome C oxidase polypeptide I	5
Pm00618	Cytochrome C oxidase polypeptide II	5
Pm00276	Cytochrome C oxidase polypeptide III	1
Pm00375	Cytochrome C oxidase polypeptide VA	1
Pm00234	Cytochrome C oxidase polypeptide VB	3
Pm01268	Fluctose-biphosphate aldolase C	2
Pm01228	NADH-ubiquinone oxidoreductase chain 1	1
Pm00229	NADH-ubiquinone oxidoreductase B12	1
Pm01001	NADH-ubiquinone oxidoreductase B15	1
Pm00992	NADH-ubiquinone oxidoreductase B17	1
Pm00516	NADH-ubiquinone oxidoreductase 39 kDa	1
Pm01043	NADH-ubiquinone oxidoreductase 51 kDa	1
	CYTOPLASM & ORGANELLES	**55**
Pm00409	ADP-ribosylation factor 1	3
Pm01332	ADP-ribosylation factor 2	3
Pm00664	Aminoacyl-histidine dipeptidase	1
Pm00945	Cell division control protein 7	1
Pm00567	Cysteine dioxygenase	1
Pm00362	Dipeptidyl-peptidase I precursor	1
Pm00363	DNA repair protein, RecA	1
Pm00648	FAF1 protein	1
Pm00605	G2/mitotic-specific cyclin B	1
Pm01288	G protein γ subunit	1
Pm00113	Gastrotropin	1
Pm00719	Glucosyltransferase-S precursor	1
Pm01255	β-Glucuronidase precursor	2
Pm00366	Glutathione S-transferase	1

TABLE I. Continued

EST number	Putative identification	Frequency
Pm00926	Heat shock protein 83	1
Pm00238	Histidine-rich, metal binding polypeptide	2
Pm00938	Insulin-induced growth response protein	1
Pm00105	Lanosterol synthase	1
Pm00743	Large proline-rich protein bat2	1
Pm01539	Lin-36	1
Pm01210	Methallothionein-IIE	2
Pm00525	Mitochondrial inner membrane protease	1
Pm00660	Mitochondrial stress-70 protein precursor	1
Pm00208	MT-associated RNA-binding protein	1
Pm00237	Nitrogen permease reactivator protein	1
Pm00157	Peptidyl-prolyl *cis-trans* isomerase	1
Pm00570	Phosphatidylinositol 3-kinase 2	2
Pm01082	Pre-mRNA processing protein PRP39	1
Pm01033	Proline-rich peptide P-B	1
Pm00788	Probable protein-tyrosine phosphatase	1
Pm01191	26S protease regulatory subunit 4	3
Pm00447	RNA-binding protein squid	1
Pm00272	Stathmin	3
Pm00367	T-complex protein 1, θ subunit	1
Pm00841	T-complex protein 1, γ subunit	1
Pm00746	Threonyl-tRNA synthase 2	1
Pm00630	Toxin A	2
Pm00501	Ubiquitin	3
Pm00684	Vitellogenin II	1
Pm00147	X-linked inhibitor of apoptosis protein	1
	NUCLEAR PROTEIN	**27**
Pm01109	Cleavage and polyadenylation specificity	3
Pm00951	CREB-binding protein	1
Pm01535	DNA-directed RNA polymerase II	1
Pm01014	GTP-binding nuclear protein RAN/TC4	2
Pm00312	Histone H1	3
Pm01065	Immediate-early protein	1
Pm00672	Nitrogen regulatory protein	1
Pm01020	20 kDa nuclear CAP binding protein	1
Pm00535	Nuclear localization sequence binding protein	2
Pm00458	Nuclear transition protein 2	1
Pm01574	Nucleolar transcription factor 1	1
Pm01023	Octapeptide-repeat protein T2	1
Pm01082	Pre-mRNA processing protein PRP39	1
Pm00612	Protamine	2
Pm01216	Protein HGV2	1
Pm00549	Putative tumor suppressor luca 15	1

TABLE I. Continued

EST number	Putative identification	Frequency
Pm00554	RNA polymerase β subunit	1
Pm00774	Ubiquitin carboxyl-terminal hydrolase	1
Pm00863	Yeast nuclear protein	1
Pm00864	Zinc-binding protein A33	1
	MEMBRANE PROTEIN	**12**
Pm00553	Angiotensin-converting enzyme precursor	1
Pm00733	Ca/calmodulin-responsive adenylate cyclase	1
Pm00714	CD81 antigen	1
Pm00179	Defender against cell death	1
Pm00535	Glutamate (NMDA) receptor subunit ϵ	1
Pm00529	Milk fat globule-EGF factor 8	1
Pm00804	Mycocerosic acid synthase	1
Pm01024	Neurocan core protein precursor	1
Pm00768	Neurogenic locus notch homolog	2
Pm00687	Phospholipase A2 isozyme CM-III	1
Pm01012	Signal recognition particle receptor β	1
	SECRETORY PROTEIN	**29**
Pm00794	Apolipoprotein (A)	1
Pm00712	Bowman-Birk type proteinase inhibitor	1
Pm00858	Coagulation factor V precursor	1
Pm00738	Coagulation factor VIII precursor (ceruloplasmin)	1
Pm00801	Collagen α1 chain	1
Pm00645	Hemagglutinin precursor	1
Pm00762	Integumentary mucin A1 precursor	1
Pm00830	Lectin (TC-14)	1
Pm00631	Leech-derived tryptase inhibitor	2
Pm01022	Low-density lipoprotein receptor-related protein	3
Pm00236	Ovomucoid (elastase inhibitor)	10
Pm00877	Palmitoyl-protein thioesterase precursor	1
Pm01507	Trypsin γ precursor	1
Pm01175	Trypsin δ precursor	3
Pm01260	von Willebrand factor precursor	1
	OTHERS	**8**
Pm01068	Hypothetical 40.7 kDa protein in DAK1-ORC1	1
Pm00126	Hypothetical 50.3 kDa protein in HSC82-GCV2	1
Pm01259	Hypothetical 52.3 kDa protein in MRPL10	1
Pm00728	Hypothetical 65.3 kDa protein in K12H4.7	1
Pm00837	Hypothetical 86.6 kDa protein	1
Pm00641	Hypothetical 208 kDa protein	1
Pm01585	Hypothetical 259 kDa protein	1
Pm00989	Hypothetical protein in BPS2	1

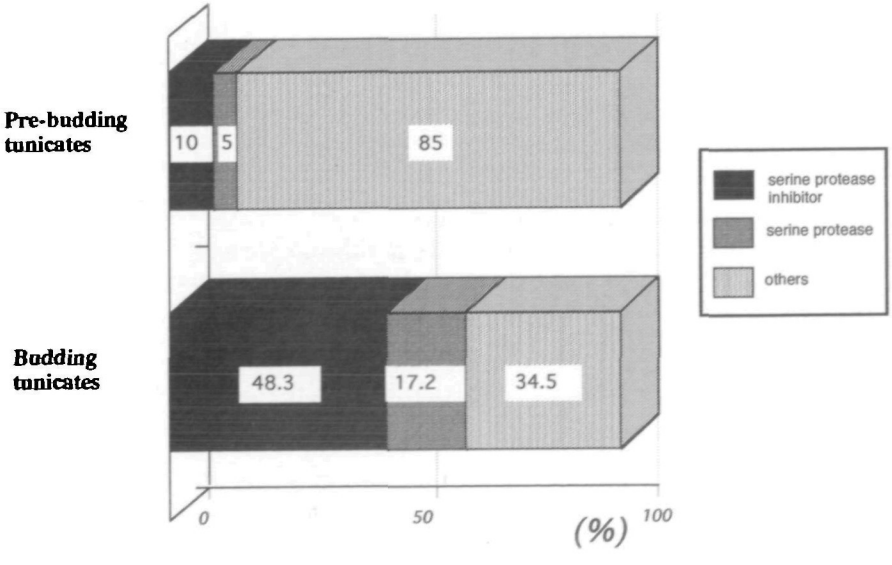

Fig. 2. **Frequency of appearance of serine protease and serine protease inhibitor in the secretory proteins.** Note that genes encoding proteases and protease inhibitors both are highly expressed during the budding stage.

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9-G9   CGATCCAATTTGATTCGTTAAGAAAGTGGACTACAACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGGATCAG
6-F9   CGATCCAATTTGATTCGTTAAGAAAGTGGACTACAACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGGATCAG
7-B8   CGATCCAATTTGATTCGTTAAGAAAGTGGACTACAACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGGATCAG
9-F3   CGATCCAATTTGATTCGTTAAGAAAGTAGACTACGACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGAATCAG
7-A12  CGATCCAATTTGATTCGTTAAGAAAGTAGACTACGACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGAATCAG
       ************************** ****** ********************************** *****

9-G9   ATTCTTGGATGGTCCTTGTGGTTGTGACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGAAGCA
6-F9   ATTCTTGGATGGTCCTTGTGGTTGTGACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGAAGCA
7-B8   ATTCTTGGATGGTCCTTGTGGTTGTGACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGAAGCA
9-F3   ATTCTTGGATGGCCCTTGT------GACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGGAGCG
7-A12  ATTCTTGGATGGCCCTTGT------GACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGGAGCG
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Fig. 3. **Multiple sequence alignment of five ESTs of serpin homologs in *P. misakiensis*.** Note that the upper three ESTs differ from the lower two, indicating at least two kinds of transcripts of *Polyandrocarpa* serpin (P-serpin). Asterisks show consensus.

```
A

   1 CGAATTGAAAAGAGAAAAATTATGGACGGAATTGTGATGTTTCTGATGTTCCTCTGCGCT   60
   1                    M  D  G  I  V  M  F  L  M  F  L  C  A     13

  61 GGCATTGGAATGGCTGAAAATCCTGAACATCTGAAACATCTGAAATACGATTGTCTTGAG  120
  14  G  I  G  M  A  E  N  P  E  H  L  K  H  L  K  Y  D  C  L  E   33

 121 AGAGAACCGAACGGGGTATGCACAATGGAATACGATCCAATTTGCTTCGTTAAGAAAGTG  180
  34  R  E  P  N  G  V  C  T  M  E  Y  D  P  I  C  F  V  K  K  V   53

 181 GACTACAACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGGATCAGATTCTTGGATGGT  240
  54  D  Y  N  N  D  C  L  A  C  E  G  K  K  R  I  R  F  L  D  G   73
                          1

 241 CCTTGTGGTTGTGACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGAAG  300
  74  P  C  G  C  D  D  L  D  S  L  P  T  P  T  K  R  K  C  L  K   93

 301 CACGAGACCGAGGGAGCATGCCCGAAAATACTGAAACCTGTCTGTGCACTTGAAAGGAAA  360
  94  H  E  T  E  G  A  C  P  K  I  L  K  P  V  C  A  L  E  R  K  113

 361 ACAGCTTCCAACAAATGCGCCGCTTGCGTCGGACAGTCAAAGATCCGCTATACCGTTGGG  420
 114  T  A  S  N  K  C  A  A  C  V  G  Q  S  K  I  R  Y  T  V  G  133
                                2

 421 GAATGCTAAACACATCAAATGTACTGCCATATGCCTACTTGCTGATGTTTGCGAAGCAAT  480
 134  E  C  *                                                    135

 481 AAAGATTTGAGCAAATGGAAAAAAAAAAAGCAAA                          513

B

   1 AACGAATTGAAAAGAGAAAAATTATGGACGGAATTGTAATGTTTCTGATGTTCCTCTGCGCT   62
   1                    M  D  G  I  V  M  F  L  M  F  L  C  A     13

  63 GGCATTGGAATGGCTGAAAATCCTGAACATCTGAAACATCTGAAATACGATTGTCTTGAG  122
  14  G  I  G  M  A  E  N  P  E  H  L  K  H  L  K  Y  D  C  L  E   33

 123 ATGGAAACGAACGGGATATGCACCATGGAATACGATCCAATTTGCTTCGTTAAGAAAGTA  182
  34  M  E  T  N  G  I  C  T  M  E  Y  D  P  I  C  F  V  K  K  V   53

 183 GACTACGACAACGATTGCTTGGCGTGTGAGGGTAAAAAAAGGATCAGATTCTTGGATGGC  242
  54  D  Y  D  N  D  C  L  A  C  E  G  K  K  R  I  R  F  L  D  G   73
                          1

 243 CCTTGTGACGACTTGGATTCGTTGCCGACACCGACCAAAAGAAAATGCTTGGAGCGTGAA  302
  74  P  C  D  D  L  D  S  L  P  T  P  T  K  R  K  C  L  E  R  E   93

 303 ACCGAGGGAGCATGCCCGAGAATACTGAAACCTGTCTGTGCCCTTGAAAGAAAAACAGCT  362
  94  T  E  G  A  C  P  R  I  L  K  P  V  C  A  L  E  R  K  T  A  113

 363 TCCAACAAATGCTTCGCTTGCTTCGGACAGTCAAAGATCCGCTATACCGTTGGAGAATGC  422
 114  S  N  K  C  F  A  C  F  G  Q  S  K  I  R  Y  T  V  G  E  C  133
                             2

 423 TAAACACACAAATGTTCTGCCATATGCCTACTTGCTGGTGTTTGCGAAGCAATAAAGATT  482
      *

 483 TGAGCAAATGGTTTTTAA                                         500
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Fig. 4. **Nucleotide sequences and deduced amino acid sequences of P-serpin 1 (A) and P-serpin 2 (B).** Single underlines "1" and "2" show two consensus domains of Kazal's type of serpin. Double underlines show the polyadenylation signal. Asterisks indicate a stop codon.

```
A

P-serpin 1      CTMEYDPICFVKKVDYNNDCLAC
P-serpin 2      --+-----------D------
DH, lion        -----F-L-GSDGKV-S-K-SF-
DH, dog         --QD-R-L-GSDGKN-S-K-SF-
DH, cat         -----F-L-GSDGQE-S-K--F-
OM, peafowl     --+-QR-L-GSDNKT-G-K-NF-

B

P-serpin 1      CPKILKPVCALERKTASNKCAAC
P-serpin 2      --R----------------F--
DH, lion        -S-Q---I-GIDH--Y--E-MF-
DH, dog         --RLHQ-I-GTDH--Y--E-MF-
DH, cat         -S-EW--I-GIDH--Y--E-MF-
PSTI-I, rat     --R-YD---GTNGI-YPSE-SL-
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Fig. 5. **Comparison of active sites of P-serpins 1 and 2 with those of other Kazal's serpins.** Elastase inhibitor domain (A) and trypsin inhibitor domain (B) are aligned. The respective active sites are represented by two amino acids (shaded), which are M-[D, E, or Q] in elastase inhibitor and [R or K]-[D, E, H, I, L, Q, or Y] in trypsin inhibitor. DH, double headed protease inhibitor; PSTI, pancreatic secreted trypsin inhibitor.

BLASTX program showed that 233 ESTs matched genes of known sequence with high significance ($p < 0.001$). Further 43 clones showed high similarity to nuclear and mitochondrial ribosomal RNAs in the search using the BLASTN program (not shown). The remaining 207 ESTs had very low or no sequence match to any genes in the database, indicating that they were either novel coding region or non-coding region of genes.

The ESTs with high similarity were classified into several categories based on the putative localization of gene products: cytoplasmic, nuclear, membrane, and secretory types. The cytoplasmic type was subdivided into four groups from the viewpoint of function: protein synthesis (23.6%), cytoskeleton and cell motility (6.9%), energy conversion (13.3%), and others including organelles (23.6%) (Fig. 1). ESTs of protein synthesis consisted exclusively of ribosomal proteins. A total of 30 ribosomal proteins appeared randomly, the only exception being 60S ribosomal protein L13, which hit 5 times (Table I). Cytoskeletal and motility ESTs appeared 15 times. They matched tubulin α chain with exceptionally high frequency. Two major constituents of energy conversion ESTs were cytochrome *c* oxidase polypeptide and ubiquinone oxidoreductase, which are the components of the respiratory chain. Cytochrome *c* oxidase polypeptides I, II, and III constitute the functional core of the enzyme complex that is involved in electron transfer on the mitochondrial inner membrane.

Among other cytoplasmic proteins, stathmin appeared three times (Table I). It is an intracellular phosphoprotein that is thought to act as a second messenger. Ubiquitin

TABLE II. **Serine protease inhibitor activities of P-serpin 1.**

Molar ratio [P-serpin 1]/[trypsin]	Specific activity of trypsin (B.U./nM)	% inhibition of tryptic activity	Molar ratio [P-serpin 1]/[elastase]	Specific activity of elastase (B.U./nM)	% inhibition of elastase activity
0	10.5	—	0	3.2	—
1	5.9	43.8	1	1.3	59.4
4	1.7	83.9	4	0.3	90.6

Authentic proteases were used at the final concentration of 20 nM. Recombinant P-serpin 1 was added to the enzyme solution at the final concentration of 10, 20, 40, or 80 nM. Each experiment was repeated four times, and the mean values are shown in the table.

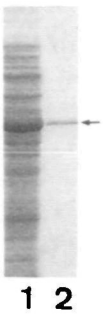

Fig. 6. **SDS-PAGE of recombinant P-serpin.** Bacteria transformed with pGEX-4T/P-serpin 1 were solubilized after IPTG induction. Proteins were resolved by 10% SDS-PAGE. Lane 1, crude lysates of bacteria. Lane 2, a 40-kDa GST/P-serpin 1 fusion protein (arrow) was bound to glutathione-Sepharose 4B and eluted with excess glutathione.

appeared three times. It binds to abnormal or denatured proteins to facilitate enzymatic digestion by ATP-dependent 26S proteasome, of which the regulatory subunit appeared three times (see Table I). A family of ADP-ribosylation factors appeared six times. They transfer ADP ribose to target proteins. For example, chorela toxin inactivates GTPase activity of G-protein by ADP ribosylation. Thus, G-protein remains activated.

In the category of nuclear protein, histone H1 and cleavage and polyadenylation specificity factor each appeared three times (Table I). The latter recognizes the AAUAAA signal of pre-mRNAs and adds a poly(A)$^+$ tail to them.

The ESTs of membrane protein appeared 10 times. Both neurogenic locus Notch homolog and neurocan have epidermal growth factor (EGF)-like domains in their polypeptide chains.

Among secretory proteins, ovomucoids (or elastase inhibitor) were noteworthy. Together with Bowman-Birk type proteinase inhibitor and leech-derived tryptase inhibitor, homologues of serine protease inhibitor (serpin) appeared 13 times in total. Their frequency was about five times that of pre-budding EST (Fig. 2). Trypsin γ and δ precursors appeared 4 times. This frequency of redundancy was also higher than that of pre-budding ESTs (not shown).

Determination of Full Length Serpin Homologues—Figure 3 shows the sequence alignment of five serpin-like ESTs selected randomly from 10 clones. Two cDNA clones, 7-A12 and 9-F3, showed the deletion of six nucleotides in comparison with the other 3 clones. There were also several nucleotide substitution in these 2 clones. These results strongly suggest that the *Polyandrocarpa* serpin-like ESTs found in this study include at least two groups of transcripts that are very similar to each other.

TABLE III. **Trypsin inhibitor activity of *Polyandrocarpa* hemolymph.**

Origins of hemolymph	% inhibition of tryptic activity	% inhibition of chymotryptic activity
Growing bud	96.9	1.0
Developing bud, 1-day	95.0	5.0
Developing bud, 2-day	80.1	0
Adult, prebudding	75.5	0
Adult, budding	84.0	0

Each experiment was repeated five times, and the mean values are shown in the table.

Two clones, 9-G9 and 9-F3, were chosen from their respective groups and their complete nucleotide sequences were determined. In the former, 500 nucleotides were determined. The deduced open reading frame (ORF) was 405 bp in length, encoding 135 amino acids (Fig. 4A). The latter was 6 nucleotides and 2 amino acids shorter than the former (Fig. 4B). In both cases, the deduced amino acid sequences had Kozak's consensus motif around the initiation codon. A polyadenylation signal was found in the 3′ non-coding region. *Polyandrocarpa* serpin homologue (P-serpin) had two tandem repeats of a unique sequence that was characterized by four cysteine residues (Figs. 4 and 5). It was consistent with the consensus signature [C-X(7)-C-X(6)-Y-X(3)-C-X(2,3)-C] of Kazal's type of secretory serpin. The first motif of P-serpin began with C-T-M-E, in which the unique sequence consisting of M-E is known to be the active site for elastase (Fig. 5A). On the other hand, the second motif began with C-P-R-I, in which the sequence R-I is known to be the active site for trypsin (Fig. 5B).

Protease Inhibitory Activity of Recombinant P-Serpin—Recombinant GST/P-serpin 1 fusion protein (40 kDa) was bound to the affinity column and eluted with glutathione (Fig. 6). It was added to authentic trypsin or elastase in the presence of synthetic substrates. In a molar ratio of 1:1 (inhibitor/enzyme), P-serpin 1 inhibited the tryptic activity by 43.8% and the elastase activity by 59.4% (Table II). On increasing the molar ratio to 4:1, the inhibition reached about 90%. GST alone lacked such protease-inhibitory activity (not shown). These data showed that recombinant P-serpin 1 had the serine protease inhibitor activity, but no conclusion could be drawn regarding the binding stoichiometry. An appreciable amount of the recombinant protein seemed to take the wrong secondary structure owing to incorrect disulfide bridging among six half-cystines (four in the consensus signature and two outside it). Protein refolding is needed for the determination of stoichiometry.

Endogenous Serpin Activity during Budding—We examined whether protease inhibitor activity changes during budding in accordance with EST data. Hemolymphs were

extracted from growing buds, developing buds, and adult animals and added *in vitro* to authentic trypsin. They consistently showed high activity of trypsin inhibitor, and the highest activity was seen in the hemolymph of growing buds (Table III). This inhibitory activity decreased to some extent when buds entered the developmental phase. The activity remained at the same level in the pre-budding phase of adult animals, but increased again in the hemolymph of budding animals. In contrast with its dramatic trypsin-inhibitory activity, the hemolymph had no apparent effect on chymotrypsin (Table III), showing the high specificity of secretory serpin of *Polyandrocarpa* for trypsin.

DISCUSSION

Putative Number of Polyandrocarpa Genes—The genome has been estimated to contain 4,000 genes in bacteria (*E. coli*), 6,000 in yeast, 15,000 in nematode, and ~100,000 in human (for review, Ref. *15*). In the process of evolution from ancestral primitive chordates to jawed pisces, the genome may have duplicated twice (*16*), and this speculation is supported by the discovery of four Hox gene clusters (*17*). It is reasonable, therefore, to assume that tunicates, a group of primitive chordates, should have about one fourth of vertebrate genome size, leading to the assumption of 20,000-25,000 genes per tunicate genome. In mammals, about 650 different ESTs (0.65% of 100,000 genes) have been identified in two respective cell lines, HepG2 (*3*) and F9 (*4*). More than 2,500 (>2.5%) genes have been found in human brain (*2*). Based on these previous EST works, we assumed that 5% of total genes should be analyzed for characterizing particular tissues. In the case of tunicates, the number was estimated as 1,000-1,200 genes.

In the present study, we aimed to discover budding-specific ESTs of *P. misakiensis*. The anterior half of the adult animal was chosen as a source of mRNAs. It contains growing buds as well as several organs (tissues) such as body wall (epidermis and atrial epithelium) (*12*), dorsal ganglion (nerve cell and ciliated gland cell) (*18*), pharynx (pharyngeal epithelium), endostyle (thyroglandular cell), and oral siphon (body muscle cell) (Sugino *et al.*, in preparation). We then compared the EST catalog from the budding stage with that from the pre-budding stage. At first, we estimated that more than 10,000 ESTs should be investigated to characterize the gene expression profile of budding animals. Fortunately, the difference between these two ESTs appeared very soon.

Difference between ESTs from Budding Stage and Pre-Budding Stage—In the present study, a total of 30 ribosomal protein ESTs appeared with low frequency of redundancy, the only exception being 60S ribosomal protein L13, confirming that the random selection of phage plaques was successful in this work. These accounted for 24% of all ESTs from the budding stage. In mouse embryonal F9 cells, as many as 53% of ESTs are related to transcription and translation, of which about 36% are ribosomal proteins (*4*). In hepatocellular carcinoma, HepG2, about 15% are assigned to ribosomal proteins (*3*). The expression frequency (24%) of ribosomal proteins in budding tunicates is intermediate between these two values and about twice as high as that of ESTs from the pre-budding stage. This is the first, although indirect, evidence for the enhancement of metabolic activity during budding of tunicates.

In *P. misakiensis*, energy conversion ESTs appeared more frequently during budding than in pre-budding animals. The former contained a variety of components of respiratory chain such as cytochrome *c* oxidase and NADH-ubiquinone oxidoreductase. Cytochrome *c* oxidase subunits I, II, and III are encoded by the mitochondrial genome, and the others by the nuclear genome (*19*). Our results, therefore, suggest that both mitochondrial and nuclear genes essential for electron transfer are activated during budding.

As mentioned, budding of *P. misakiensis* removes a large amount of cells from the parent, which should be compensated for by complementary cell growth. In this study, three kinds of cell growth-related genes were identified. Insulin-induced growth-response protein and G2 mitotic-specific cyclin B play roles in G1/S and G2/M traverse of the cell cycle, respectively. Cell division control protein 7 encodes a serine/threonine kinase. Previous studies have shown that both DNA synthesis and mitosis in *P. misakiensis* occur over the whole area of protruding buds (*8, 20*).

Serine Protease Inhibitors Induced in the Process of Budding—One of the most intriguing genes expressed during *Polyandrocarpa* budding was a homolog of serine protease inhibitors (serpin). As mentioned, ESTs of ribosomal proteins were picked up with low frequency of redundancy, suggesting strongly that the extremely high frequency of EST of *Polyandrocarpa* serpin (P-serpin) reflects correctly the number of transcripts of the P-serpin gene.

Bovine pancreas tissue contains two types of serpin. One is a basic, non-secretory polypeptide named Kunitz's inhibitor (*21*). It interacts not only with trypsin but also with chymotrypsin and kallikrein. The other is a secretory polypeptide, referred to as Kazal's inhibitor (*22*), that is characterized by a peculiar consensus signature with conserved half-cystines (*23*). In the present study, *Polyandrocarpa* hemolymph did not show chymotrypsin-inhibitory activity but inhibited trypsin.

In the solitary tunicate *Halocynthia roretzi*, two trypsin inhibitors have been isolated from the hemolymph (*24*), neither of which shows any apparent homology in amino acid sequence with well-defined protease inhibitors (*25*). In the present study, P-serpins 1 and 2 were sequenced fully. They showed homology to ovomucoid (*26-28*), double-headed protease inhibitor (*29*), pancreatic secretory trypsin inhibitor (*30*), and acrosin inhibitor, all of which belong to Kazal's inhibitor family. Double-headed protease inhibitor isolated from mammalian submandibular glands has two functional domains, one serving as trypsin inhibitor, the other as elastase inhibitor (*31*). As shown in the present study, P-serpins 1 and 2 both contained two tandem repeats of Kazal's motif-like double-headed protease inhibitor, although in the second domain tyrosine was substituted by alanine. Their primary domain could be an elastase inhibitor and the second one a trypsin inhibitor.

Possible Role of P-Serpin in Tunicate Budding—Several kinds of biological function have been ascribed to Kazal's type of protease inhibitors. They include egg allergen (*32*), mitogen of endothelial cells (*33*), preservation of the gastrointestinal mucous layer from excessive digestion (*34*), and the stimulation of cell motility of the human

colonic cell line HT29 (*34*).

Recently, we have found using the differential display technique that retinoic acid (RA) induces serine protease gene expression in mesenchymal cells of *P. misakiensis* (Ohashi *et al.*, submitted). RA can trigger the formation of the secondary bud axis *via* transdifferentiation of multipotent, atrial epithelium (*11, 12, 35, 36*). In avian retina, when pigmented epithelial cells transdifferentiate *in vitro* into lens cells (*37*), pP344, a gene product having a serpin-like domain, decreases rapidly in amount (*38, 39*). The present study showed that in *P. misakiensis* the trypsin-inhibitory activity was highest in the hemolymph of growing buds and decreased during bud development. We favor the proposition that, like pP344, P-serpin may counteract endogenous trypsin-like polypeptides that would promote transdifferentiation of regenerative tissues.

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