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Urochordate whole body regeneration inaugurates a diverse innate immune signaling profile

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Abstract

The phenomenon of whole body regeneration (WBR) from minute soma fragments is a rare event in chordates, confined to the subfamily of botryllid ascidians and is poorly understood on the cellular and molecular levels. We assembled a list of 1326 ESTs from subtracted mRNA, at early stages of *Botrylloides leachi* WBR, and classified them into functional categories. Sixty-seven (15%) ESTs with roles in innate immunity signaling were classified into a broad functional group, a result supported by domain search and RT–PCR reactions. Gene ontology analysis for human homologous to the immune gene category, identified 22 significant entries, of which "peptidase activity" and "protease inhibitor activity", stood out as functioning during WBR. Analyzing expressions of serine protease Bl-TrSP, a representative candidate gene from the "peptidase activity" subgroup, revealed low transcript levels in naïve vasculature with upregulated expression during WBR. This was confirmed by *in situ* hybridization that further elucidated staining restricted to a circulating population of macrophage cells. Furthermore, Bl-TrSP was localized in regeneration niches within vasculature, in regenerating buds, and in buds, during blastogenesis. Functional inhibition of serine protease activity disrupts early remodeling processes of the vasculature microenvironment and hinders WBR. Comparison of genome-wide transcription of WBR with five other developmental processes in ascidians (including metamorphosis, budding and blastogenesis), revealed a broad conservation of immune signaling expressions, suggesting a ubiquitous route of harnessing immune-related genes within a broader range of tunicate developmental context. This, in turn, may have enabled the high diversity of life history traits represented by urochordate ascidians.

Keywords: Innate immunity; Ascidians; *Botrylloides*; Development; Serine protease; Regeneration; EST library; Urochordates

Introduction

Urochordate ascidians, which occupy a key taxonomic position in the origins of the Vertebrata (Delsuc et al., 2006) display, in their larval stage, ubiquitous chordates' morphological traits. Therefore, they are used as a convenient model taxon to address poorly understood evolutionary and biological issues in vertebrates (Rinkevich, 2002; Satoh and Levine, 2005; Manni and Burighel, 2006). One such group is the botryllid ascidians (family Styelidae, subfamily Botryllinae), colonial sea squirts that exhibit a unique capability to reconstruct the entire adult body architecture from isolated, minute vasculature fragments (Oka and Watanabe, 1957, 1959; Rinkevich et al., 1995, 1996; Rinkevich et al., 2007), a phenomenon termed whole body regeneration (WBR).

The model WBR urochordate species *Botrylloides leachi* (Fig. 1A) is a very common shallow water Mediterranean tunicate (Berrill, 1950) that encrusts diverse hard substrata. Each colony can hold thousands of genetically identical modules (zooids; Fig. 1A, arrowheads), each 2–3 mm long, aligned in two parallel rows and embedded within the gelatinous tunic matrix. All zooids within a colony are interconnected by a network of blood vessels, from which sausage-like shaped vascular termini (ampullae) extend toward the colony margins (Fig. 1A, arrows). The blood vessels and the ampullae are very delicate structures with walls one cell thick (Milkman, 1967).

In contrast to normal asexual reproduction cycles (palleal budding) occurring in the normal physiological state of Botryllid colonies, WBR in *B. leachi* is induced in separated blood vessels

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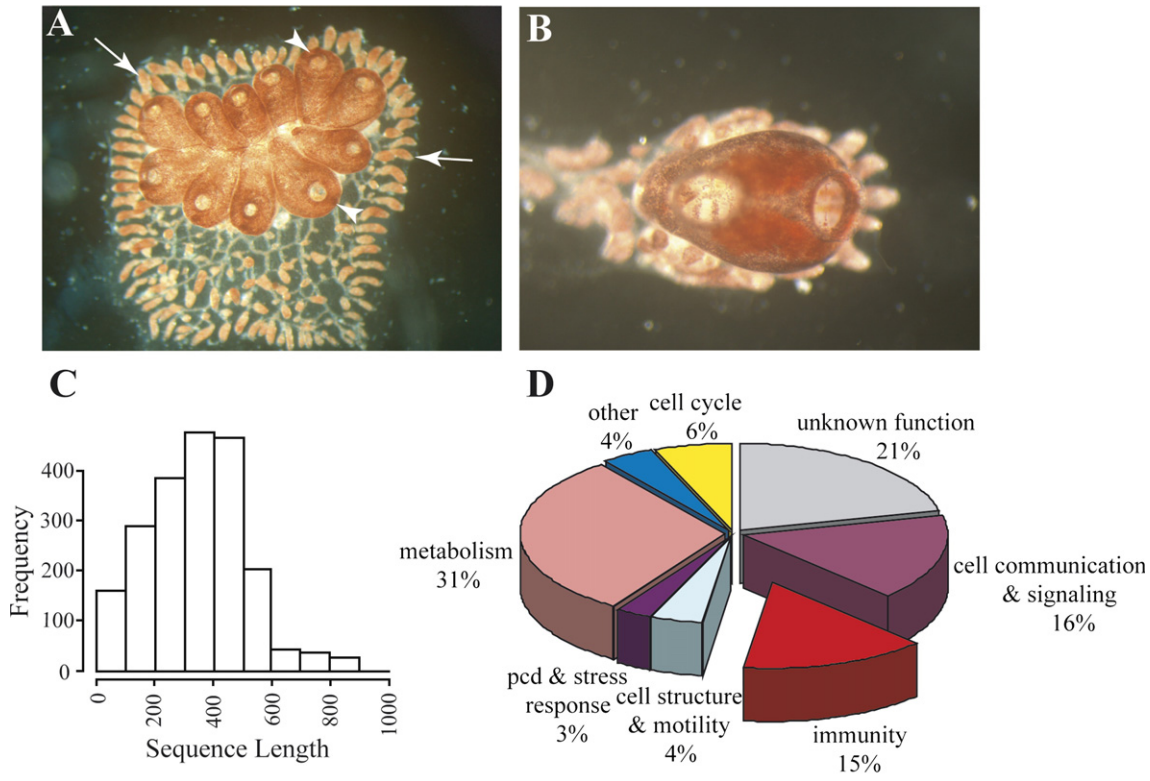

Fig. 1. EST library from early stages of WBR in *Botrylloides leachi*. *Botrylloides leachi* colonies encompassed genetically identical modules-zooids (A, arrowheads) interconnected by a vasculature network with termini-ampullae (A, arrows) extending towards the colony margins. Separated blood vessels regenerated functional adult zooids within 10–14 days through whole body regeneration (B). Distribution of *Botrylloides* subtracted EST library sequence lengths (C). Distribution of 455 SwissProt EST matched (*E*-value ≤ 0.005), by major functional categories.

and develops in areas deprived of their zooids. In fact, even a minute fragment of blood vessel or an ampulla containing approximately 100–200 blood cells can regenerate a new functional organism within 10–14 days from initiation (Fig. 1B), displaying the same idiosyncratic properties as *in situ* regeneration of botryllid colonies from hibernating colonial remnants (Bancroft, 1903; Burighel et al., 1976; Rinkevich et al., 1996). Conversely, intact colonies, or colonial fragments containing a single intact zooid, do not regenerate (Oka and Watanabe, 1959; Milkman, 1967; Rinkevich et al., 1995, 1996). By observing meticulously early phases of WBR, we (Rinkevich et al., 2007) have recently revealed the existence of three distinctive phases in the regeneration process. Staging distinct regeneration niches within vasculature lumens, aggregation of homing haemocytes and blood cell proliferations, led to the construction of discrete regeneration foci. This unconventional botryllid WBR system differs from other regeneration model systems in several fundamental traits, such as epimorphosis without blastema formation, induction of multiple restoration centers by circulating blood cells and concurrent restoration of both soma and germ line (Rinkevich et al., 2007). Therefore, elucidating the molecular cascades of mediators manifested in early steps of botryllid WBR could bring us closer to inferring basic as well as common themes for regeneration.

Innate immunity functions as a primary defense system against a plethora of parasitic forms in all multicellular animals (Fujita, 2002). It is composed of both serum and cell surface components representing, in chorus, a conserved Animalia ancient system (Nonaka, 2001). Innate immunity activates lectins, Toll receptors, complement factors and genes involved in intracellular signal transduction of immune responses. This substitutes the vertebrate's adaptive immunity, in which the major histocompatibility complex (MHC) class I and II genes, T-cell receptors and dimeric immunoglobulin molecules are expressed (Goldsby et al., 2000; Imler and Hoffmann, 2000). A number of recent studies have referred to the contribution of innate immunity components in regulating diverse developmental processes, in addition to their conventional role as a pathogen-purging machinery (Mastellos and Lambris, 2002; Voskoboynik et al., 2004; Godwin and Brockes, 2006; Liu et al., 2007). Although it is well documented that ascidians express diverse innate immune-related genes during immunological challenges (Nonaka et al., 1999; Nonaka, 2001; Khalturin et al., 2003; Raftos et al., 2004; Oren et al., 2007), harnessing of immune-related genes in a tunicate developmental context has rarely been evaluated. Screening of post-larval metamorphosis transcripts in the solitary ascidian *Boltenia villosa* revealed expressions of diverse immunomodulatory agents (Davidson and Swalla, 2002), suggesting that innate-immune molecules in ascidians could indicate a broader range of biological functions, as in higher organisms (Anderson et al., 1993; Ottaviani and Franceschi, 1997; Del Rio-Tsonis et al., 1998; Maeda et al., 2001).

Based on an EST screening analysis for differentially expressed genes during *B. leachi* WBR, we documented all-

embracing immune-related responses, from early stages of WBR and throughout the regeneration process. Gene ontology (GO) analysis performed on the immune-related genes revealed two main groups with characteristic biological processes of protease and protease inhibitory activities. Specifically, we detailed the profile expression of Bl-TrSP, a trypsin-like serine protease, localized in sites of whole body regeneration and in sequential expansion of blastogenic (the cyclical process of asexual reproduction) buds. Afterwards, we demonstrated that the serine protease inhibitor SBTI, previously described as an effective inhibitor of complement associated serine protease (Petersen et al., 2000) and an inhibitor of complement response in ascidians (Roberts et al., 2007), specifically disrupts remodeling processes in the vasculature microenvironment and prevents zooidal regeneration. This implies that innate-immune signaling and serine protease activities are common and dynamic participants in botryllid ascidians' developmental and regeneration cycles.

Materials and methods

Animal husbandry

Colonies of *Botrylloides leachi*, with affixed thin layers of calcareous material, were carefully pealed-off underlying surfaces of stones by industrial razor blades, in shallow waters along the Mediterranean coast of Israel. The colonies were tied individually with fine threads onto 5×7.5 cm glass slides and cultured in 17-l tanks of standing seawater system, as described by Rinkevich and Shapira (1998). Within several days of culture, ampullar contractions and expansions led to complete, or partial, sliding of colonies from the calcareous substrates onto the glass slides. Colonies and their substrates were cleaned weekly with industrial razor blades and fine brushes.

Regeneration assay

Marginal ampullae and fragments of blood vessels were separated from colonies growing on glass slides under a dissecting microscope, using an industrial razorblade and a fine tungsten needle. Next, donor colonies were removed and tied onto other slides. The remaining blood vessel fragments were cut into smaller fragments using a fine tungsten needle and left to regenerate in 17-l tanks (Fig. 1B). Fragments were observed daily under a dissecting microscope and photographed with a Supercam camera (Applitec, Holon, Israel).

Subtraction library

Total RNA was extracted from freshly isolated and regenerating vasculature fragments by EPICENTER MasterPure™ RNA Purification kit. The integrity of the total RNA was verified by agarose gel electrophoresis. First-strand cDNA synthesis was performed on 500 ng from regenerating and freshly isolated total RNA, by Super SMART™ PCR cDNA synthesis kit (Clontech) according to the manufacturer's instructions. cDNA subtraction was done by Clontech PCR-Select™ cDNA Subtraction Kit, according to the manufacturer's instructions. All PCR reactions were done on a Perkin-Elmer GeneAmp PCR System 5700 using Clontech original primers. The amplified fragments were cloned in pTZ57R/T vector (Fermentas; white to blue clones ratio was about 0.8); 2304 individual positive clones were selected and re-cultured in 96-well LB plates with 100 μg/ml ampicillin. Sequencing of the PCR products was performed at the Max Planck Institute for Molecular Genetics, Berlin, Germany.

Sequences processing and assembly

Sequence analyses were performed at the Bioinformatics Support Unit at Ben-Gurion University of the Negev, Israel. Initial sequence processing was done by Sequencher™ program (Gene Codes Corporation). It included trimming of low quality sequence ends, pTZ57R vector sequences and of the Clontech primers (5′-CCCGGGCAGGT-3′, 5′-GCGGCCGAGGT-3′). Further cleaning was done by a dedicated Perl script, which included removal of CDS5 (ACGCGGG) and AC from the 5′ ends and their complementary sequence from the 3′ ends; trimming polyA ends from the 3′ ends and polyT from the 5′ ends, and by rejecting cleaned sequences shorter than 50 bp. Finally, visual inspection was performed to ensure that all remaining sequences were of high quality. The cleaned sequences were assembled into singlets or contigs using CAP3 software (Huang and Madan, 1999).

Blast analysis

Processed sequences (contigs and singlets) longer than 50 bp. were merged into one Fasta file and submitted to a database search using Blastx algorithm. The blast search was employed separately against both, SwissProt and GenBank databases on all unique sequences. Sequences were considered similar if their Blastx *E*-value cutoff was lower than 0.005 (only the top similar match for each sequence was presented). All sequences with *E*-value above this threshold were considered sequences with low similarity.

Gene ontology (GO) enrichment of ESTs

Human database from EBI site (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/) was used for Blastx (Altschul et al., 1990) algorithm. An in-house Perl script was employed to automatically run Blastx algorithm on processed sequences. This process yielded a dataset containing 349 human proteins homologous to differentially expressed *B. leachi* proteins, during early stages of whole body regeneration. This dataset was submitted to GOviz, a software for hypergeometric GO enrichment analysis and graphical visualization for the MultiKnowledge project (http://www.multiknowledge.org). A list of other 19,185 proteins was used as background. The GO term enrichment's *p* values reported herein were corrected for multiple hypothesis testing. *P* values were calculated using hypergeometric distribution and the Bonferroni correction was applied to account for multiple testing. GO terms with *p* values lower than 0.01 were deemed significant.

Analysis of endogenous transcripts by RT–PCR

Total RNA was isolated from fragments of regenerating blood vessels with RNeasy Mini or Midi kits (Qiagen, Valencia CA, USA) as templates. First-strand cDNA was synthesized by first-strand DNA synthesis kit (Fermentas). The PCR amplification was performed using designated sets of primers (Operon; Supplementary Table 1). Cytoplasmic actin primers were added to all samples, acting as reference gene. The PCR reaction was carried out for 30 cycles (95 °C for 1 min, 55–60 °C for 1 min and 72 °C for 1 min) followed by additional 10 min at 72 °C. PCR products were analyzed in 1.5% agarose/EtBr gel alongside a DNA marker.

In situ hybridization

Regenerating blood-vessel fragments were fixed overnight in 4% paraformaldehyde, dehydrated in 70% methanol, embedded in paraffin and cut into 5-μm sections. EST clones were used to obtain sense and antisense DIG-labeled RNA probes that were synthesized using the DIG RNA Labeling Kit (SP6/T7; Roche Molecular Biochemicals, Mannheim, Germany). Hybridization of probes to tissue sections was performed according to Breitschopf et al. (1992) for paraffin-embedded tissue. DIG-labeled RNAs on samples were observed using anti-DIG antibody (Roche). Samples were observed with an Olympus BX50 Upright microscope and photographed with a Supercam camera (Applitec, Holon, Israel).

Serine protease inhibition

Colonies of *B. leachi* were soaked for 24 h in sterilized seawater (SSW) containing 0.04 mg/ml of soybean trypsin inhibitor (SBTI; T-9128; Sigma) and only SSW for control purposes. This serine protease inhibitor is an effective

Table 1
Characterisation of immune relevant transcripts in the *Botrylloides leachi* WBR subtractive library

Sequence no.	GenBank accession number	SwissProt Homologue ID	Gene identity	Organism	*E*-value
1	EL784690	Q6P7Y6_XENTR	Intelectin 1	*X. tropicalis*	1e−61
2	EL784691	Q4U122_CTEID	Intelectin 113	*C. idella*	2e−24
3	EL784692	LECH_CHICK	Hepatic lectin	*G. gallus*	1e−06
4	EL784693	Q8AXR8_ANGJA	C-type lectin 2	*A. japonica*	8e−08
5	EL784694	Q2VWH3_9PERO	Selectin P	*S. chuatsi*	6e−11
6	EL784695	Q2VWH3_9PERO	Selectin P	*S. chuatsi*	1e−05
7	EL784696	Q69HM9_CIOIN	Cortical granule lectin-like	*C. intestinalis*	2e−23
8	EL784697	Q69HM9_CIOIN	Cortical granule lectin-like	*C. intestinalis*	8e−13
9	EL784698	Q5SXE6_MOUSE	Lectin, galactose binding, soluble 9	*M. musculus*	3e−10
10	EL784699	Q9U8W8_TACTR	Techylectin-5A	*T. tridentatus*	9e−05
11	EL784700	Q2QKE2_9TREM	Cysteine protease 3	*P. westermani*	6e−06
12	EL784701	Q6PRC7_IXORI	Legumain-like protease precursor	*I. ricinus*	6e−07
13	EL784702	Q8T3A0_CIOIN	Putative coagulation serine protease	*C. intestinalis*	1e−17
14	EL784703	Q8T3A0_CIOIN	Putative coagulation serine protease	*C. intestinalis*	5e−09
15	EL784704	Q3I1U9_NOMGA	Transmembrane protease serine 2	*N. gabriellae*	2e−18
16	EL784705	YM67_CAEEL	Putative serine protease	*C. elegans*	4e−08
17	EL784706	FACE1_MOUSE	CAAX prenyl protease 1 homolog	*M. musculus*	1e−17
18	EL784707	Q8IU50_CAEEL	ADAMTS-like protease	*C. elegans*	1e−12
19	EL784708	Q6PRC7_IXORI	Legumain-like protease precursor	*I. ricinus*	2e−24
20	EL784709	Q173W0_AEDAE	Serine protease	*A. aegypti*	2e−11
21	EL784710	Q9Y1V3_POLMI	Modular protease precursor	*P. misakiensis*	1e−38
22	EL784711	Q6H320_BOVIN	Glandular kallikrein precursor	*B. taurus*	2e−15
23	EL784712	Q91WG9_MOUSE	Inter-alpha trypsin inhibitor	*M. musculus*	1e−04
24	EL784713	ITIH3_RAT	Inter-alpha-trypsin inhibitor	*R. norvegicus*	4e−12
25	EL784714	Q86GG0_PENJP	Cathepsin C	*P. japonicus*	2e−07
26	EL784715	Q2V9X2_9METZ	Cathepsin L	*H. perleve*	5e−70
27	EL784716	Q6EEA4_PRODO	Cathepsin B	*P. dolloi*	4e−35
28	EL784717	Q95P98_HALRO	Ficolin 3 precursor	*H. roretzi*	4e−17
29	EL784718	Q95PA0_HALRO	Ficolin 1 precursor	*H. roretzi*	1e−19
30	EL784719	Q95P99_HALRO	Ficolin 2 precursor	*H. roretzi*	7e−08
31	EL784720	Q966W1_HALRO	Ficolin 4	*H. roretzi*	5e−36
32	EL784721	Q3LFW5_ORENI	Cytosolic nonspecific dipeptidase	*O. niloticus*	4e−15
33	EL784722	Q48A78_COLP3	Putative Xaa–Pro dipeptidase	*C. psychrerythraea*	1e−52
34	EL784723	Q29112_PIG	Scavenger-receptor protein	*S. scrofa*	1e−14
35	EL784724	Q2F6H5_ANTEL	Cd36/Scavenger receptor class B	*A. elegantissima*	2e−12
36	EL784725	Q6B4I7_RAT	Scavenger receptor class B type 2	*R. norvegicus*	4e−14
37	EL784726	O01654_HALRO	AsMASPa	*H. roretzi*	7e−12
38	EL784727	O01655_HALRO	AsMASPb	*H. roretzi*	5e−31
39	EL784728	Q9BLJ1_CIOIN	Ci-META1	*C. intestinalis*	1e−14
40	EL784729	Q9BLJ2_CIOIN	Ci-META2	*C. intestinalis*	1e−04
41	EL784730	Q2ZJR7_CALSA	Von Willebrand factor	*C. saccharolyticus*	1e−08
42	EL784731	Q8MVQ1_9ASCI	Vwa1 protein	*B. villosa*	1e−06
43	EL784732	Q3US68_MOUSE	Inter-alpha (globulin) inhibitor H5	*M. musculus*	1e−12
44	EL784733	Q8T398_CIOIN	Alpha-2-macroglobulin homologue	*C. intestinalis*	1e−12
45	EL784734	Q69HL6_CIOIN	Polydomain protein-like	*C. intestinalis*	3e−57
46	EL784735	ACET_RABIT	Angiotensin-converting enzyme	*O. cuniculus*	2e−10
47	EL784736	Q6WMS9_BRABE	Interferon gamma-inducible protein	*B. belcheri*	6e−12
48	EL784737	Q6WMS9_BRABE	Interferon gamma-inducible protein	*B. belcheri*	2e−29
49	EL784738	Q9F2B0_THIFE	TapA protein	*T. ferrooxidans*	8e−14
50	EL784739	GRN_RAT	Granulins precursor	*R. norvegicus*	3e−15
51	EL784740	LYG_CHICK	Lysozyme g precursor	*G. gallus*	5e−20
52	EL784741	Q3UYL7_MOUSE	Annexin A7	*M. musculus*	6e−28
53	EL784742	Q17FZ6_AEDAE	Tetraspanin 29fa	*A. aegypti*	0.048
54	EL784743	P79953_XENLA	Oviductin	*X. laevis*	3e−08
55	EL784744	Q75S85_HALRO	Integrin beta Hr1	*H. roretzi*	1e−06
56	EL784745	Q86NA5_CIOIN	Hemocyanin-like protein 2	*C. intestinalis*	2e−14
57	EL784746	TXND5_MOUSE	Thioredoxin-like protein p46	*M. musculus*	6e−14
58	EL784747	Q804W6_FUGRU	Coagulation factor VIII	*F. rubripes*	0.001
59	EL784748	Q9YHC9_XENLA	Polo-like kinase kinase 1	*X. laevis*	9e−05
60	EL784749	BAP31_HUMAN	BCR-associated protein Bap31	*H. sapiens*	5e−05
61	EL784750	Q8MVL2_9ASCI	Thrombospondin-like protein 2	*B. villosa*	2e−13
62	EL784751	Q5XGJ3_XENTR	Properdin factor, complement	*X. tropicalis*	2e−04
63	EL784752	Q28398_ERIEU	Apolipoprotein	*E. europaeus*	4e−51

Table 1 (*continued*)

Sequence no.	GenBank accession number	SwissProt Homologue ID	Gene identity	Organism	*E*-value
64	EL784753	Q1XIU9_CYTJO	Ankyrin precursor	*F. johnsoniae*	8e−05
65	EL784754	Q3TA89_MOUSE	Laminin A-containing protein	*M. musculus*	4e−19
66	EL784755	Q76FN7_TACTR	Toll-like receptor	*T. tridentatus*	5e−09
67	EL784756	Q6P4W6_XENTR	CD82 antigen	*X. tropicalis*	5e−05

inhibitor of complement associated serine protease (Petersen et al., 2000) and an inhibitor of complement response in ascidians (Roberts et al., 2007). The next day, marginal ampullae and fragments of blood vessels were separated from the colonies under a dissecting microscope, using an industrial razorblade and a fine tungsten needle. The donor colonies were completely removed from the glass slides. Regenerating fragments from experimental and control groups were kept in 0.5-l plastic tanks at 20 °C and solutions were changed every other day. Control and experimental fragments were observed daily under a dissecting microscope and photographed with a Supercam camera (Applitec, Holon, Israel).

Results

General library statistics

To isolate candidate genes expressed specifically during early stages of WBR (Phase É; Rinkevich et al., 2007), total RNA was extracted separately from naïve and 2- to 4-day-old regenerating vasculature fragments from subcloned cohorts of *Botrylloides* colonies. cDNAs from naïve fragments were subtracted from regenerating fragments and 2304 clones were randomly isolated and sequenced. The quality of 2092 ESTs (91% of the total ESTs) was found adequate for assembly. The distribution of EST sequence length before assembly ranged between 50 and 900 bp (Fig. 1C) with an average length of 344 bp and standard deviation of 171 bp. These sequences were assembled into 1326 ESTs of which 172 were contigs and 1154 were singlets. Redundancy ([Total number of sequences −Number of unique sequences]/Total number of sequences) was calculated as 36.6%.

Functional categories of ESTs homologues

The BLASTX analysis showed that 34.3% (n=455) of the ESTs had a high degree of similarity to known genes (*E*-value ≤0.005) whereas the remaining 65.7% (871) had low or no similarity (*E*-value >0.005) and were classified as genes with unknown functions. The 455 sequences with significant SwissProt matches were classified into functional categories according to blast results and functions of the matching proteins (Fig. 1D) as follows: 73 (16%) to cell communication and signaling; 19 (4%) to cell structure and motility genes; 28 (6%) to chromatin and cell cycle genes; 11 (3%) PCD (programmed cell death) and stress response; 139 (31%) to metabolism genes; 97 (21%) were novel proteins with unknown function; and 19 (4%) were classified as 'others'. A notable fraction, 67 (15%), of the identified transcripts were classified as being potentially involved in innate immunity. Since urochordate genes are under-represented in protein databases, the distribution pattern revealed here may not be a full representation and genes found with no similarity (n=871) may be unknown members of a functional category.

Analysis of differentially expressed ESTs reveals a comprehensive immune relevant category

Of the 67 unique immune-related genes displayed in Table 1, 9 are contigs and 58 are singlets, all are potentially protein-coding transcripts. Only matches with less than a 2.0e−5 probability of a chance occurrence were classified as significant. The identified transcripts represent a broad functional group with roles in immunity, mainly in complement signaling. In addition to 25 different other immune-related genes (Table 1), the functional group includes 10 lectins (Table 1; seq. 1–10), 12 different proteases (Table 1; seq. 11–22), 2 mannan-binding protein-associated serine protease (MASPs) belonging to the complement lectin pathway (Table 1; seq. 37 and 38), 3 cathepsins (Table 1; seq. 25–27), 4 ficolins (Table 1; seq. 28–31), 3 scavenger receptors (Table 1; seq. 34–36), a single Toll-like receptor (Table 1; seq. 66), 2 von Willebrand factors (Table 1; seq. 41 and 42), 2 dipeptidases (Table 1; seq. 32 and 33), an angiotensin-converting enzyme (Table 1; seq. 46), 2 interferon gamma-inducible proteins (Table 1; seq. 47–48). All 67 ESTs were deposited into GenBank (accession numbers: EL784690–EL784756). The relatively high and comprehensive expression of these immune-related genes in the subtracted library may point to their important functional role during WBR.

A domain search, which was carried out on all sequences, revealed the presence of conserved characteristic domains of immune proteins with high *E*-value (Table 2). The domain search, followed by a literature search, supported gene ontology and predicted function of proteins in immune-related responses. For example, domain search of the lectin family transcripts revealed several characteristic domains including fibrinogen-like domain (Table 2; seq. 1, 2, 7 and 10) important for function recognition (Matsushita et al., 1996; Gokudan et al., 1999; Kairies et al., 2001). A complement control domain termed Sushi/SCR/CCP (Table 2; seq. 5 and 6) found in all C3b/C4b binding proteins (Matsushita et al., 1998) and a Gal-binding domain (Table 2; seq. 9) presented in gallactose-binding lectins. Another important family of recognition molecules expressed in our EST library were the four ficolin transcripts (Table 1; seq. 28–31) with highest similarity to the four different ficolins previously found in the solitary ascidian *Halocynthia roretzi* (Kenjo et al., 2001). Domain search revealed that all ficolins exhibited the characteristic fibrinogen and fibronectin domains (Table 2; seq. 28–31) present in all ascidian and in mammalian ficolins. The function of this conserved domain is expected to be similar to that of C-type lectins in pattern-recognition (Fujita,

Table 2
Domain search of immune relevant transcripts in the *Botrylloides leachi* WBR subtractive library

Sequence no.	GenBank accession number	Gene identity	Domain name	*E*-value	Original database	Original database ID
1	EL784690	Intelectin 1	Fibrinogen C-terminal domain-like	2.6e−34	Superfamily	SSF56496
2	EL784691	Intelectin 113	Fibrinogen C-terminal domain-like	3.90e−15	Superfamily	SSF56496
3	EL784692	Hepatic lectin	C-type lectin (CD209 antigen)	6.60e−07	HMMPanther	PTHR22802
4	EL784693	C-type lectin 2	Lectin_C	2.10e−11	HMMPfam	PF00059
5	EL784694	Selectin P	Sushi/SCR/CCP	2.90e−07	HMMSmart	SM00032
6	EL784695	Selectin P	Sushi/SCR/CCP	7.60e−06	HMMSmart	SM00032
7	EL784696	Cortical granule lectin-like	Fibrinogen C-terminal domain-like	3.50e−11	Superfamily	SSF56496
8	EL784697	Cortical granule lectin-like	NA	seg	seg	seg
9	EL784698	Lectin, galactose binding, soluble 9	Gal-bind_lectin	9.40e−26	HMMPfam	PF00337
10	EL784699	Techylectin-5A	Fibrinogen_C	1.60e−08	HMMPfam	PF00147.8
11	EL784700	Cysteine protease 3	Peptidase_C1	8.30e−07	HMMPfam	PF00112.1
12	EL784701	Legumain-like protease precursor	Peptidase_C13	4.20e−10	HMMPfam	PF01650
13	EL784702	Putative coagulation serine protease	TSP_1	3.30e−10	HMMPfam	PF00090
14	EL784703	Putative coagulation serine protease	Trypsin-like serine proteases	3.80e−08	Superfamily	SSF50494
15	EL784704	Transmembrane protease serine 2	Trypsin-like serine proteases	6.40e−31	Superfamily	SSF50494
16	EL784705	Putative serine protease	Peptidase S28	6.70e−14	HMMPanther	PTHR11010
17	EL784706	CAAX prenyl protease 1 homolog	CAAX prenyl protease STE24	5.20e−19	HMMPanther	PTHR10120
18	EL784707	ADAMTS-like protease	Thrombospondin, type I	2.00e−09	HMMSmart	SM00209
19	EL784708	Legumain-like protease precursor	Peptidase_C13	4.10e−50	HMMPfam	PF01650
20	EL784709	Serine protease	Trypsin-like serine proteases	2.50e−19	Superfamily	SSF50494
21	EL784710	Modular protease precursor	Trypsin-like serine proteases	8.30e−27	Superfamily	SSF50494
22	EL784711	Kallikrein	Trypsin-like serine proteases	2.60e−15	Superfamily	SSF50494
23	EL784712	Inter-alpha trypsin inhibitor, heavy chain 3	Inter-alpha-trypsin inhibitor heavy chain H3	4.20e−06	HMMPanther	PTHR10338
24	EL784713	Inter-alpha-trypsin inhibitor heavy chain H3 precursor	Inter-alpha-trypsin inhibitor heavy chain H3	2.90e−19	HMMPanther	PTHR10338
25	EL784714	Cathepsin C	Peptidase_C1	3.90e−11	HMMPfam	PF00112
26	EL784715	Cathepsin L	Peptidase_C1	1.60e−86	HMMPfam	PF00112
27	EL784716	Cathepsin B	Peptidase_C1	7.50e−28	HMMPfam	PF00112
28	EL784717	Ficolin 3 precursor	Fibrinogen_C	2.80e−28	HMMPfam	PF00147
29	EL784718	Ficolin 1 precursor	Fibrinogen_C	1.70e−20	HMMPfam	PF00147
30	EL784719	Ficolin 2 precursor	Fibrinogen_C	1.40e−09	HMMPfam	PF00147.8
31	EL784720	Ficolin 4	Fibrinogen and fibronectin	4.60e−51	HMMPanther	PTHR19143
32	EL784721	Cytosolic nonspecific dipeptidase	NA	seg	seg	seg
33	EL784722	Putative Xaa–Pro dipeptidase	Dihydropteroate synthetase-like	2.10e−10	Superfamily	SSF51717
34	EL784723	Scavenger-receptor protein	Macrophage scavenger receptor-related	5.60e−22	HMMPanther	PTHR19331
35	EL784724	Cd36/Scavenger receptor class B	Scavenger receptor class B type-2 (SR-B2)	3.50e−16	HMMPanther	PTHR11923
36	EL784725	Scavenger receptor class B type 2	CD36 family	1.50e−12	FPrintScan	PR01609
37	EL784726	AsMASPa	Trypsin-like serine proteases	8.40e−15	Superfamily	SSF50494
38	EL784727	AsMASPb	Serine protease	2.20e−32	HMMPanther	PTHR19355
39	EL784728	Ci-META1	EGF/Laminin	1.60e−07	Superfamily	SSF57196
40	EL784729	Ci-META2	NA	seg	seg	seg
41	EL784730	Von Willebrand factor	vWA-like	2.50e−12	Superfamily	SSF53300
42	EL784731	Vwa1 protein	Complement control module/SCR domain	1.30e−13	Superfamily	SSF57535
43	EL784732	Inter-alpha (globulin) inhibitor H5	Inter-alpha-trypsin inhibitor heavy chain inhibitor H5	8.30e−12	HMMPanther	PTHR10338
44	EL784733	Alpha-2-macroglobulin homologue	Alpha-macroglobulin receptor domain	6.40e−16	Superfamily	SSF49410
45	EL784734	Polydomain protein-like	Complement control module/SCR domain	2.50e−15	Superfamily	SSF57535

Table 2 (*continued*)

Sequence no.	GenBank accession number	Gene identity	Domain name	*E*-value	Original database	Original database ID
46	EL784735	Angiotensin-converting enzyme	Angiotensin-converting enzyme (dipeptidyl carboxypeptidase)	4.30e−18	HMMPanther	PTHR10514
47	EL784736	Interferon gamma-inducible protein	Gamma-interferon inducible lysosomal thiol reductase (GILT)-related	9.90e−09	HMMPanther	PTHR13234
48	EL784737	Interferon gamma-inducible protein 30	Gamma-interferon-inducible lysosomal thiol reductase (GILT)	4.40e−26	HMMPanther	PTHR13234:SF7
49	EL784738	TapA protein	NA	seg	seg	seg
50	EL784739	Granulins precursor	Granulin	3.10e−19	HMMPanther	PTHR12274
51	EL784740	Lysozyme g precursor	Lysozyme-like	1.90e−09	Superfamily	SSF53955
52	EL784741	Annexin A7	Annexin	6.10e−47	HMMPanther	PTHR10502
53	EL784742	Tetraspanin 29fa	Tetraspanin	6.00e−05	HMMPanther	PTHR19282
54	EL784743	Oviductin				
55	EL784744	Integrin beta Hr1	Integrin_b_cyt	6.10e−10	HMMPfam	PF08725.1
56	EL784745	Hemocyanin-like protein 2				
57	EL784746	Thioredoxin-like protein p46	Thioredoxin-like	3.70e−16	Superfamily	SSF52833
58	EL784747	Coagulation factor VIII	NA	seg	seg	seg
59	EL784748	Polo-like kinase kinase 1	NA	seg	seg	seg
60	EL784749	BCR-associated protein Bap31	Bap31	7.70e−06	HMMPfam	PF05529.2
61	EL784750	Thrombospondin-like protein 2	TSP-1 type 1 repeat	1.50e−15	Superfamily	SSF82895
62	EL784751	Properdin factor, complement	TSP-1 type 1 repeat	0.00091	Superfamily	SSF82895
63	EL784752	Apolipoprotein	Kringle	1.70e−34	HMMPfam	PF00051
64	EL784753	Ankyrin precursor	Ankyrin repeat	4.60e−06	Superfamily	SSF48403
65	EL784754	Laminin A-containing protein	Laminin_A	3.40e−28	HMMPfam	PF04916
66	EL784755	Toll-like receptor	Toll-like receptor 1	2.10e−15	HMMPanther	PTHR23154
67	EL784756	CD82 antigen	CD82	2.30e−10	HMMPanther	PTHR19282

2002), discriminating between pathogens and self. Another interesting group is the serine protease family. Six different serine proteases participate in activating the complement system (Matsushita et al., 1998), including two novel MASPs, MASPa and MASPb. Domain search conducted on both MASPa and MASPb library transcripts (Table 2; seq. 37 and 38) revealed a peptidase domain functioning during serine-type proteolysis activity. In addition, all other six serine proteases share the same trypsin-like serine protease (TSP) domain (Table 2; seq. 13–15, 20–22) in their sequence, classifying them as different genuine serine proteases that are expressed during WBR.

Temporal expression of immune-related genes in regeneration

In order to confirm the specificity of the EST library and determine the temporal expression of immune-related transcripts during *Botrylloides* WBR, a series of RT–PCR reactions were performed (Fig. 2) on mRNAs from intact ampullae, on early WBR stages (presumably trapped in our EST library) and on advanced WBR stages, characterized by progressive organogenesis of developing buds (Rinkevich et al., 2007), using transcript-specific primers (Supplementary Table 1). We chose the classes of lectins and proteases for the RT–PCR reactions, since these protein families are highly represented in the EST library and members of these families participate ubiquitously in innate immune responses as described below. Six lectins and four proteases were differentially amplified by RT PCR (Fig. 2), revealing their specific transcript upregulation during WBR, from early stages and throughout advanced stages of regeneration. Thus, innate immune signaling is active from early to advanced developmental stages of WBR with putative functions during the regeneration of zooids in *B. leachi*.

Gene ontology (GO) analysis results

It would be advantageous to assess whether the overall list of genes, specifically the immune-related gene category from the EST dataset, is significantly enriched in any particular GO terms (Altschul et al., 1990) for molecular function. Since, GO annotation is currently unavailable for *B. leachi*, we performed an indirect analysis using the international protein index (IPI). Processed sequences were run against a background of 19,185 proteins from the human database. This yielded an overall dataset containing 349 human proteins homologous to *B. leachi* library sequences (data not shown). Of the 67 immune-related genes in the library, 75% had human homologues by the GO enrichment analysis. This analysis identified 22 significant GO terms for enriched EST transcripts (Fig. 3A). These GO terms are sorted into three major molecular functions with enzyme regulator and catalytic activities being highly represented (74% of transcripts). Among these GO terms, two specific entries for subgroup molecular functions, “peptidase activity” (belonging to the catalytic activity group) and “protease inhibitor activity” (belonging to the enzyme regulator activity group), were found to be highly significant with p values 8.6e−08 and 7.4e−08, respectively (Fig. 3). Fourteen genes in the WBR ESTs dataset exhibited “peptidase activity” (Table 3) and nine genes showed “protease inhibitor activity” (Table 4). We focused on the “peptidase activity” GO term entry by further scoring for peptidase activity, based on domain search. Furthermore, 10 of

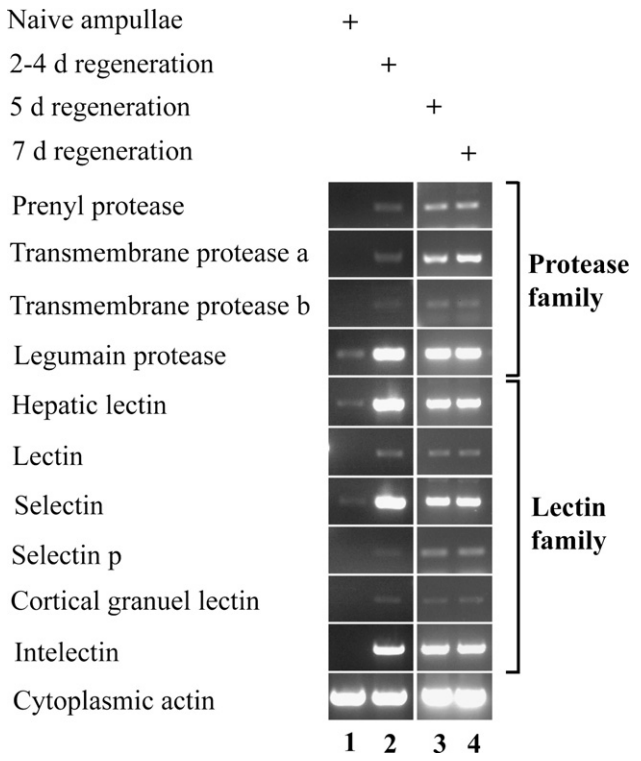

Fig. 2. Temporal expression of immune-related transcripts during WBR. Specific primers (Supplementary Table 1) were used to amplify immune-related transcripts during WBR. Representative transcripts included 4 different proteases and 6 lectin family members. Stages included naïve ampullae and early stages of regeneration (2–4 days) presumably trapped in our EST library. Two additional time points (5 days, 7 days) represented progressive regeneration stages characterized by development and organogenesis. The cytoplasmic actin bands at the bottom served as a positive control. Accession numbers of the above presented genes are as follows: prenyl protease – EL784706, transmembrane protease a –EL784704, transmembrane protease a –EL784743, legumain protease –EL784708, hepatic lectin –EL784692, lectin –EL784693, selectin –EL784695, selectin p –EL784694, cortical granule lectin –EL784696, intelectin –EL784690.

the 14 transcripts within this category exhibited peptidase domains. Genes 1–3 (Table 3; cathepsins L2, F, B precursors) possessed the peptidase_C1 domain, a cysteine-type endopeptidase activity site important for proteolysis. Gene 4 (legumain precursor) revealed a peptidase C13 legumain type domain functioning in legumain and cysteine-type endopeptidase activity during proteolysis processes. Gene 5 (transmembrane protease), gene 6 (plasminogen activator), gene 7 (63 kDa protein) and gene 8 (vitamin K-dependent protein C precursor) all depicted trypsin-like serine and cysteine protease domains characteristic of trypsin like proteases functioning in endopeptidase activity during proteolysis. Gene 9 (CAAX Prenyl protease) possessed the CAAX PRENYL protease domain whereas gene 10 (dipeptidyl peptidase) contained the peptidase C1A and papain C-terminal domains.

Analysis and expression pattern of a Bl-Trypsin-like serine protease (Bl-TrSP) during WBR

As the first step for revealing the roles of the immune-related genes during *Botrylloides* WBR, we chose to analyze the expression of a representative gene from the "peptidase activity" subgroup, the Bl-TrSP (no. 6 in Table 3). We sequenced a 336 bp fragment revealing 66% identity to a modular protease from the budding ascidian *Polyandrocarpa misakiensis* and to other urokinase-type proteases (Fig. 4A). Domain search uncovered a part of a conserved domain (*E*-value: 7e−29) characteristic to trypsin-like serine proteases. To gain insight into the temporal expression pattern of Bl-TrSP during regeneration, total RNA was extracted from regenerating vasculature fragments at different regeneration stages and RT–PCR was performed using sequence-specific primers. Low levels of Bl-TrSP was expressed in naïve ampullae and the expression was upregulated following blood vessel fragmentation (Fig. 4B). The transcript was expressed continuously in PCR products throughout subsequent regeneration phases, for up to day 11. To reveal further spatial activity, the temporary location of Bl-TrSP during the different stages of *Botrylloides* WBR was assessed by *in situ* hybridization, employed on 5-μm-thick paraffin sections. In intact blood vessels, the specific staining was restricted to a circulating population of macrophage cells (Fig. 4C). During early regeneration stage (phase I; Rinkevich et al., 2007), Bl-TrSP-positive macrophage cells were conglomerated in sites of regeneration, confined to the only Bl-TrSP-positive cell type in the regeneration preparative (Fig. 4D). However, as from regeneration phase II (Rinkevich et al., 2007), in addition to macrophage expression, Bl-TrSP was localized in the newly developed regeneration niches and expressed in bud primordia (Fig. 4E). During phase Ø, concurrent with advanced stages of bud's organogenesis (Rinkevich et al., 2007), Bl-TrSP was expressed throughout the developing buds. No staining of Bl-TrSP was detected in blood vessel epithelium, or in other circulating blood cell types. The specific sense probes, which were used as controls, detected no staining pattern (data not shown).

Bl-TrSP is expressed in bud formation during blastogenesis in B. leachi colonies

In botryllid ascidians, all functional zooids in a colony are replaced synchronously in weekly cycles (under 18–20 °C regimen) by an unremitting budding (asexual reproduction) process called blastogenesis. Each generation of buds grow out of the lateral wall of parent zooids. Colonies are typically made of three consecutive blastogenic generations: the adult filtering zooids, the primary and the secondary buds. Thus, botryllid colonies undergo constant astogenic development through blastogenic cycles. To assess possible Bl-TrSP participation during blastogenesis of *B. leachi*, whole colonies were fixed at blastogenic stage C, a representative developmental stage in which both primary and secondary buds are fully present and *in situ* hybridization was performed on 5-μm-thick paraffin sections using Bl-TrSP as a probe. Early-stage spheroid structures of secondary buds were specifically stained (Fig. 4F, arrows). Bl-TrSP staining continued at later stages of bud development and organogenesis, highly marked in the endostylar groove (Fig. 4F, arrowheads), pharynx and prospective

Fig. 3. Gene ontology (GO) enrichment of immune-related transcripts during WBR. Twenty-two significant "molecular function" sub ontology terms were identified. These GO terms were sorted into three major molecular functions with "peptidase activity" (belonging to the catalytic activity group) and "protease inhibitor activity" (belonging to the enzyme regulator activity group), being highly significant with p values 8.6e−08 and 7.4e−08, respectively. P values (red to white color scale) were calculated using the hypergeometric model and corrected for multiple GO term testing.

gill slits (Fig. 4F, pink arrows). This was expressed congruently throughout all the bud structures and the developing buds of the same generation. In adult zooids, staining was confined to the upper mid section of the endostylar groove (Fig. 4G, arrow) and in localized areas within the stomach folds (Fig. 4H, arrows). Specific sense probes that were used as controls detected no staining pattern (data not shown).

The serine protease inhibitor SBTI disrupts vascular microenvironment and prevents WBR

To understand the functional significance of serine protease activity during WBR, peripheral blood vessels separated from *Botrylloides* colonies were left to regenerate in 0.5 l filtered seawater containing the pharmacological serine protease inhibitor SBTI. Control (without SBTI) and experimental fragments were monitored daily for morphological and cellular outcomes. SBTI-treated fragments displayed a deceleration in all morphological characteristics including changes in shape and orientation of blood vessels and localized vessel movements (Fig. 5A) compared to time-matched control fragments. In several cases, vessel movements stopped in an intermediate state without the formation of vessel lacuna as in same-stage controls (n=4/9, Fig. 5B). Circulatory behavior was visible at early stages of regeneration in these experiments, but progressively slowed down until the 10th day when it stopped altogether. In all experimental fragments except one, regenerating buds were never observed even after a 19-day follow-up observation (n=8/9). In the only experiment in which a bud was observed, the bud reached an advanced stage of regeneration but developed malformation and failed to open both siphons. Time-matched control fragments showed normal morphology and dynamics and regenerated buds after 10–11 days (n=5/5, data not shown).

To understand the cellular input of serine protease inhibition and function during WBR, control and SBTI-treated fragments

Table 3
"Peptidase activity" sub ontology group of genes

	Gene ID	Description
1	EL784715	Cathepsin L2 precursor
2	EL784700	Cathepsin F precursor
3	EL784716	Cathepsin B precursor
4	EL784701	Legumain precursor
5	EL784703	Transmembrane protease, serine 7 precursor
6*	EL784710	Plasminogen activator, urokinase
7	EL784709	63 kDa protein
8	EL784726	Vitamin K-dependent protein C precursor
9	EL784706	CAAX prenyl protease 1 homolog
10	EL784714	Dipeptidyl-peptidase 1 precursor
11	EL784698	30 kDa protein
12	EL784752	Plasminogen precursor
13	EL784693	21 kDa protein
14	EL784695	Selectin P

were sectioned and subsequently stained with hematoxylin–eosin to reveal its morphology. Control fragments showed tissue remodeling processes characteristic to WBR, including epithelial detachments from tunic embedment, epithelial divisions and the formation of regeneration compartments throughout the vasculature (Fig. 5D). In the SBTI-treated fragments, remodeling did not occur and regeneration compartments were not formed. Instead, blood vessel fields developed into large single lacuna (Fig. 5C) and the entire extracellular space of the blood vessel was packed with cellular and matrix material, creating a dense scaffold. Inside the scaffold, some buds started development (Fig. 5E), but at advanced regeneration stages II and III (Rinkevich et al., 2007), they developed into disorganized structures with irregular morphologies (Fig. 5F), which failed to complete their regeneration ($n=8/9$).

Discussion

In this study, we have taken an EST-based genome-wide subtractive expression approach to uncover the signaling machinery underlying *B. leachi* WBR. The remarkable complexity of morphological processes and molecular cascades in this developmental system (Rinkevich et al., 1995, 1996, 2007) make the botryllid WBR assay a highly suitable model for explaining basic and common themes for regeneration, in general, and various aspects of ascidian's developmental biology, in particular. The cDNA subtraction technology allowed us to screen the expressed ESTs with a high degree of relevance to *Botrylloides* WBR. In the functionality analysis performed here, we focused on currently known immune defense-related gene homologues, although homologues of other categories in our EST library could be equally important for the WBR process (Y. Rinkevich, unpublished). Results revealed that 67 different transcripts were orthologous to immune-related genes with characteristic domains of innate immune family members, elucidating harnessing of immune-related genes within a tunicate developmental context. A gene ontology (GO) search performed on the immune gene category revealed that these genes could perform two major processes during *Botrylloides* early regeneration stages: peptidase and peptidase inhibitory activities. This renders a new level of understanding of the biological processes essential for WBR, which at the single gene level of examination, might be concealed because of redundancy. Studying a representative peptidase (Bl-TrSP, a serine protease), we demonstrated that this molecule is specifically confined, during regeneration first, to the subpopulation of macrophage-like cells and then, to organogenesis throughout bud development. Bl-TrSP is also expressed cyclically in developing buds during colonial blastogenesis. These outcomes indicate possible intimate roles for innate immune-related genes in developmental processes, resembling the activation of innate immune signaling genes during larval metamorphosis in a solitary ascidian (Davidson and Swalla, 2002).

Many of the 67 immune-related transcripts found in the EST library match genes known to participate in complement cascades. The complement system consists of three activation pathways, the classical, the alternative and the lectin pathway that convolutes more than 30 serum and cell surface molecules (Fujita, 2002, Matsushita and Fujita 1996). In the lectin pathway, mannose-binding lectin (MBL) and/or ficolins (Holmskov et al., 2003; Matsushita and Fujita, 2001, 2002) operate as pattern recognition receptors that trigger the pathway through association of MASPs (Matsushita et al., 1998; Schwaeble et al., 2002). These proteolytic enzymes cleave the complement components C4, C2 and C3 (Fujita et al., 2004) and activate the lectin pathway. Although there is no evidence for adaptive immunity in urochordate ascidians (Azumi et al., 2003), sequence analysis of the draft genome of the solitary ascidian *C. intestinalis* suggests the presence of many innate immune system components. Ascidians also possess two different MASPs (Ji et al., 1997), for which two corresponding cDNA clones with high homology were identified in the EST library (asMASPa and asMASPb; seq. 37 and 38). Another MBL molecule, galactose-binding lectin (GBL) that, in

Table 4
"Protease inhibitor activity" sub ontology group of genes

	Gene Id	Description
1	EL784712	Inter-alpha-trypsin inhibitor heavy chain H3 precursor
2	EL784713	Inter-alpha-trypsin inhibitor heavy chain H3 precursor
3	EL784732	Inter-alpha trypsin inhibitor heavy chain precursor 5 isoform 2
4	EL784748	Isoform 2 of STE20-like serine/threonine-protein kinase
5	EL784694	Selectin E
6	EL784749	20 kDa protein
7	EL784707	Thrombospondin-1 precursor
8	EL784733	C3 and PZP-like alpha-2-macroglobulin domain containing 8
9	EL784721	53 kDa protein

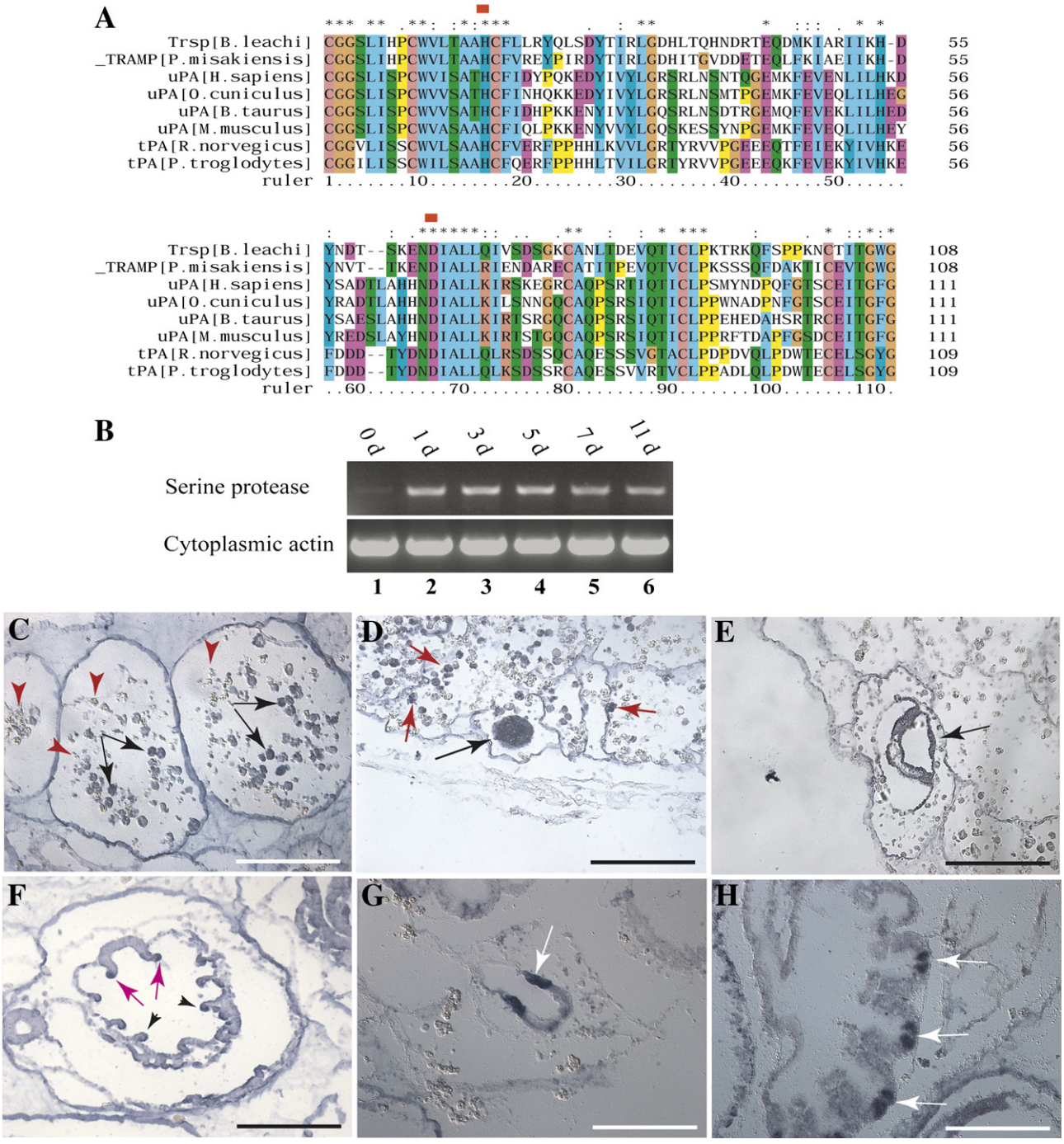

Fig. 4. Expression pattern of Bl-TrSP, a serine protease, during WBR and colony astogeny. (A) A conserved 108 amino acid fragment corresponding to a part of the characteristic domain of trypsin-like serine proteases from diverse animals. Amino acids corresponding to part of the active site are marked by red rectangles. (B) RT–PCR analysis performed on naïve and regenerating blood vessels at different developmental stages revealed a weak transcript expression in naïve ampullae (lane 1) while transcript levels increased during regeneration and persist throughout the entire process up to 11 days (lane 2–6). Cytoplasmic actin served as a positive control to normalize Bl-TrSP levels. (C) Whole-mount RNA *in situ* hybridization on paraffin sections of regenerating ampullae and *Botrylloides* colonies at representative blastogenic stage C revealed in naïve ampullae a specific staining in macrophage cells (arrows) compared to other blood cells (arrowheads) that showed no staining. (D) During early stages of regeneration, Bl-TrSP-positive macrophage cells conglomerate in sites of regeneration niches (red arrows) where bud primordium was formed and expressed Bl-TrSP transcripts (black arrow). (E) Later, Bl-TrSP localized to developing buds at early stages of organogenesis. (F) During colony astogeny, Bl-TrSP-stained primary buds at the tips of the endostylar groove (pink arrows) and at the tips of the prospective gill slits (black arrowheads). (G) In adult zooids, staining was confined to the upper mid section of the endostylar groove (arrow) and in localized areas within the stomach folds (H, arrows). Scale bar represents 100 μm.

ascidians, binds specifically to glucose (Sekine et al., 2001) and is associated with MASPs, thereby activating C3, was also confined to the EST library.

Ten putative lectin family representatives are found in the WBR library, including two intelectins, hepatic lectin, a C-type lectin, two selectin P, two cortical granule lectins, a single

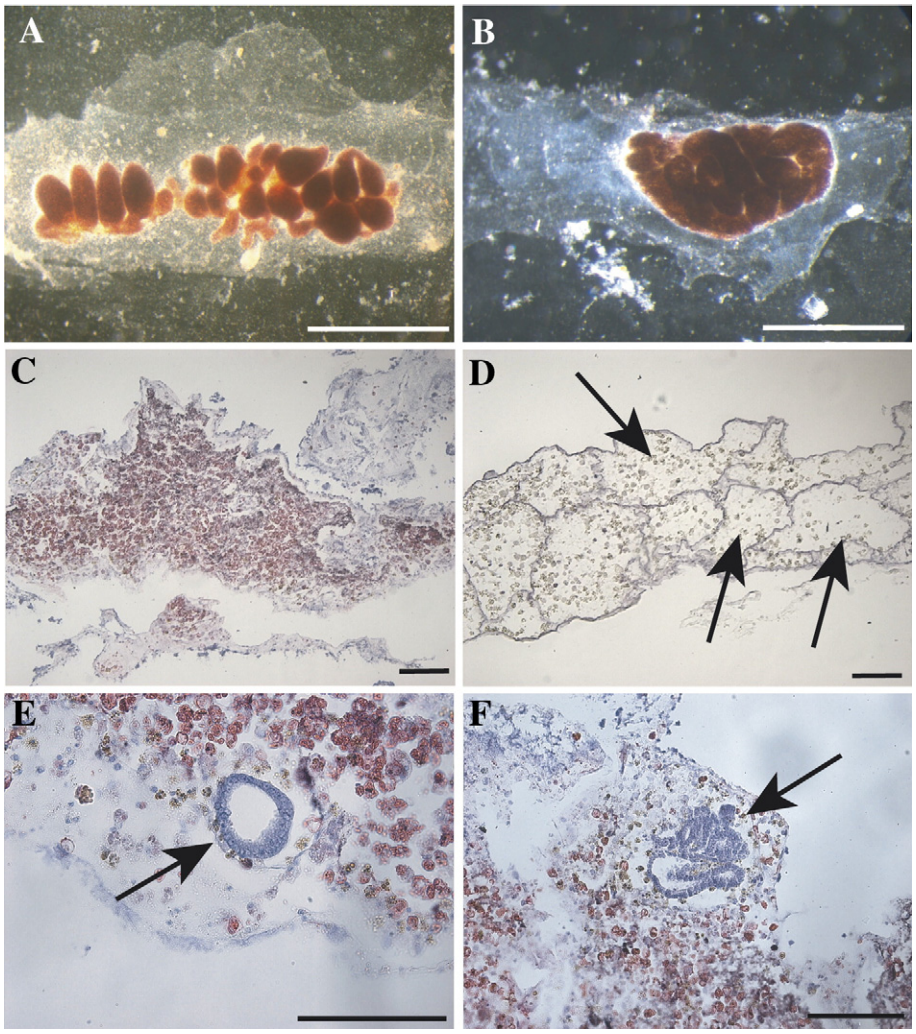

Fig. 5. Serine protease inhibition disrupts vessel microenvironment and functional regeneration. (A) Morphology of a regenerating vasculature fragment after 19 days following treatment with SBTI. Blood vessels stopped in an intermediate stage of regeneration with no formation of vessel lacuna. (B) Control regenerating fragments show characteristic vessel aggregations and lacuna formations after 6 days. (C) Hematoxylin–eosin-stained section of a representative SBTI-treated experiment in which blood vessels failed to compartmentalize, developing into a large single space. In addition, the entire vessel lumen was saturated with cellular and matrix material, creating a dense scaffold. (D) Hematoxylin–eosin-stained section of a control experiment showing characteristic microenvironment: regeneration niches (arrows) with normal vessel contents. (E) In SBTI-treated fragments, buds at early stages (arrow) are regenerating within the saturated scaffold. (F) At later stages, these buds develop into disorganized structures with irregular morphologies (arrow) and fail to regenerate successfully. Scale bar in panels A and B, 1 mm and in panels C–F, 100 μm.

galactose-binding lectin (GBL) and techylectin a lectin that recognize acetyl groups, altogether revealing a significant presentation of lectin pathway components in the regeneration phenomenon. The library also presents four lectin family members called ficolins, all matching ficolins from the solitary ascidian *H. roretzi* (Kenjo et al., 2001). Ficolins are a group of proteins containing both collagen-like and fibrinogen-like domains with structure, function and activity similar to lectins. In humans, serum ficolins act in aggregation and opsonization of pathogens as well as associate and activate downstream serine proteases thereby activating the complement through "the lectin pathway" (Matsushita and Fujita, 2001; Fujita, 2002; Endo et al., 2007). Thus, both lectins and ficolins are instrumental for activating the lectin pathway. The lectin-complement pathway acts in jawless vertebrates and in many invertebrates (including the tunicates) as a highly organized effector arm for innate immunity (Endo et al., 2006). In addition, it activates in vertebrates' diverse biological processes, including disposal of apoptotic cells (Walport, 2001), activation and chemotaxis of leukocytes (Fujita, 2002) and production of cytokines and chemokines (McGreal et al., 2004). In mammals, lectin pathway components help in recruiting leukocytes to inflamed tissues by promoting leukocytes' adherence to blood vessel walls (Vestweber and Blanks, 1999; Ley, 2001). In addition, several proteins which possess a fibrinogen-like domain and are structurally similar to ficolins (tenascin, microfibril-associated glycoprotein 4) are actively involved in cell adhesion (Chiquet-Ehrismann et al., 1986; Zhao et al., 1995). It is therefore possible that members of the lectin-complement pathway act as cell adhesion proteins during the 'inflammation-like process' in early stages of regeneration, enabling recruitment of blood cells to regeneration niches

(Rinkevich et al., 2007) and their conglomeration. This tenet is supported by the presence of two selectins (seq. 5 and 6; Table 1), molecules that share roles in leukocytes recruitment into inflamed mammalian tissues (Ley, 2001; Vestweber and Blanks, 1999). Although we have identified major lectin pathway components, our EST library failed to show matches for the classical and alternative complement pathways, such as C1q-binding protein and Factor B, as in other tunicates (Endo et al., 2006).

To elucidate further the roles of immune-related genes in *Botrylloides* WBR, we analyzed the expression of a representative candidate gene from the "peptidase activity" subgroup, the Bl-TrSP. Results demonstrated expressions at elevated levels throughout the WBR process. *In situ* hybridization localized Bl-TrSP to macrophage-like cells, which conglomerate after vasculature fragmentation, from early stages of WBR. The macrophage-like cells are usually large (10–15 μm in diameter) and round possessing one to a few vacuoles with ingested material of heterogeneous appearance, occupying most of the cell volume during the takeover process of blastogenesis (Lauzon et al., 1993). They hold hydrolytic enzymes and are positive for specific lectins such as WGA and ConA (Cima et al., 2001). Recent studies have revealed that botryllid macrophages, while coordinating between death and clearance signals in blastogenesis (Voskoboynik et al., 2004), express aldehyde dehydrogenase transcript, the source for retinoic acid synthesis, a molecule that plays a central role in WBR (Rinkevich et al., 2007). These outcomes implicate macrophage cells as important cell constituents coordinating between immune responses and developmental traits in botryllid ascidians. Bl-TrSP is not exclusive to the macrophage-like cells. The transcript is present in advanced WBR stages, in regeneration niches throughout organogenesis of buds, in blastogenic buds throughout colony astogeny and in areas within zooidal organs (similarly to the expression of retinoic acid; Y. Rinkevich, unpublished observations). Similarly, a serine protease expressed in blood cells in a closely related ascidian *P. misakiensis*, was found to modulate proliferation of a cell line derived from the atrial epithelium – the cellular source for budding processes (Ohashi et al., 1999). More importantly, this serine protease (termed TRAMP) was upregulated following retinoic acid induction. The high sequence similarity between TRAMP and Bl-TrSP, and their concurrence expression patterns during regeneration and colony budding, suggests a role to Bl-TrSP as exerting a developmental process downstream of retinoic acid and implicates serine protease activity as an important biological feature during ascidians' developmental traits.

The list of immune-related genes from our EST dataset is significantly enriched for GO terms, where two major entries, "peptidase activity" (to whom Bl-TrSP belongs) and "peptidase inhibitor activity", were found to be highly significant, in total revealing 74% of the whole 'immunity section'. Peptidase activity had been described from blood cells of solitary as well as colonial ascidians. In solitary ascidians, proteolytic activity was documented in blood cells of *Ciona intestinalis*, *Halocynthia roretzi*, *Phallusia mammillata* and *Microcosmus sulcatus* (Scippa et al., 1996), with specific serine–protease activities (Smith and Peddie, 1992; Shishikura et al., 1997; Guerrieri et al., 2000) documented during immunological functions. One such example in invertebrates is the pro-phenoloxidase (proPO) system which is activated by humoral serine proteases that proteolyse proPO to active PO (Soderhall and Cerenius, 1998; Ballarin et al., 1998), thereby activating immune-related reactions such as blood cell aggregation at tunic wound (Taylor, 1992; Takahashi et al., 1994). More recent evidence points to the importance of protease activities in remodeling developmental processes in both solitary and colonial ascidians. In the solitary ascidian *B. villosa*, a subtractive library has revealed protease transcripts upregulation during metamorphosis (Davidson and Swalla, 2002), suggesting that these genes may function in ascidian metamorphosis. Interestingly, BvMASP, a serine protease from *B. villosa*, is expressed in anterior larval tissues specifically in a population of migratory cells resembling motile immune cells or macrophages (Roberts et al., 2007). Furthermore, larvae treated with the serine protease inhibitor SBTI failed to complete remodeling of the body plan and subsequent metamorphosis (Roberts et al., 2007), again demonstrating a functional role for serine protease activity during life history traits of ascidians.

In the colonial ascidian *P. misakiensis*, both the serine protease P-trefoil and the serine protease inhibitor P-serpin have overlapping expressions in coelomic cells, during bud primordium and throughout bud growth (Kawamura et al., 2006). Similarly, in *P. misakiensis*, these proteins are upregulated following epidermal injury, collaborating to promote cell growth, motility and cell differentiation of a multipotent *P. misakiensis* cell line (Kawamura et al., 2006). In *Botrylloides* WBR, functional inhibition of serine protease activity using the pharmacological inhibitor SBTI disrupts early remodeling processes of the vasculature microenvironment and inhibits WBR. Bl-TrSP is closely similar to the mammalian serine protease urokinase-type plasminogen activator (uPA) which regulates matrix remodeling in various mammalian tissues. In uPA-deficient mice, tissue repair and remodeling in response to injury is impaired. uPA-deficient mice show regeneration defects in skeletal muscle concomitant with abnormal deposition of ECM and decreased recruitment of blood cells to sites of injury (Lluis et al., 2001). Similarly, uPA-deficient mice also show regeneration defects in liver concomitant with impaired clearance and accumulation of ECM components (Bezerra et al., 2001; Shanmukhappa et al., 2006). The morphological and cellular outcomes of serine protease inhibition in WBR highlights serine protease activity as a potential biological process for remodeling and clearance of the extracellular matrix during *Botrylloides* WBR, of which pharmacological inhibition abrogates zooidal regeneration.

This whole genome view of immune-related transcripts could disclose common and basic routes for innate immunity and developmental systems as revealed for immunity and neuroendocrine systems (Ottaviani and Franceschi, 1997). Activation of innate immune signaling cascades during developmental processes was found in metamorphosis of the solitary ascidian *B. villosa* (Davidson and Swalla, 2002) and recently in *C. intestinalis* (Chambon et al., 2007). Whole

Table 5
Comparative expression of representative immune-related genes from 5 different biological phenomena in urochordate ascidians

Gene ID		Allorecognition (*B. schlosseri*)	Metamorphosis (*B. villosa*)	Metamorphosis (*C. intestinalis*)	WBR (*B. leachi*)	Budding (*P. misakiensis*)	Blastogenesis (*B. schlosseri*)
1	Serine protease	+	+[a]	+	+[a]	+	+
2	Von Willebrand factor	+	+	+	+	+	+
3	META	+	+	+	+	−	+
4	Trypsin-like serine protease	−	+	−	+	+[a]	+
5	Selectin	+	+	+	+	−	−
6	MASPb	+	+	−	+	−	−
7	Coagulation factor	−	+	−	+	+	−
8	Ficolin	+	−	+	+	−	+
9	Serpin (serine protease inhibitor)	+	−	−	+	+[a]	−
10	C-type lectin	+	−	−	+	+[a]	−
11	Cathepsin	+	−	−	+	−	+
12	Trypsin inhibitor	−	−	+	+	+	−
13	Thrombospondin	−	+	+	+	−	−
14	Toll receptor	−	+	−	+	−	−
15	Scavenger receptor	+	−	−	+	−	−
16	Cell adhesion molecule (sushi)	−	−	+	+	−	−
17	ADAM precursor	−	−	−	+	−	+
18	Cysteine proteinase	−	−	+	−	−	+

Relevant library information includes the following ascidian species: *Botryllus schlosseri* (Oren et al., 2007; J. Douek, unpublished), *Botrylloides leachi* (this paper), *Boltenia villosa* (Davidson and Swalla, 2002; Roberts et al., 2007), *Ciona intestinalis* (Nakayama et al., 2002; Chambon et al., 2007), *Polyandrocarpa misakiensis* (Kawamura et al., 1998, 2006).
(+) Transcript represented in the above ascidian library; (−) Not represented.
[a] Functional roles were found for these transcripts.

genome comparisons for transcript expressions in five different biological processes in ascidians (allorecognition, metamorphosis, budding, blastogenesis and WBR; Table 5) were developed to reveal shared transcripts. A list of 18 immune-relevant genes from various family members (Table 5) depicts common expressions in both immune and developmental processes. For example, both von Willebrand factor and serine protease are expressed in all five different developmental processes, metamorphosis, WBR, blastogenesis and budding events recorded in solitary and colonial ascidians, and during allorecognition. Three immune genes, i.e. META, trypsin-like serine protease and ficolin, are found in four processes. Seven additional immune genes, i.e. C-type lectin, selectin, cathepsin, serpin, MASP, trypsin inhibitor and coagulation factor, are represented in three different biological processes, showing a broad participation of immune genes in development and imply possible novel functions of these gene families during developmental processes. More importantly, most of the conserved list of immune genes outline two major biological processes of cell adhesion (selectin, coagulation factor, ficolin, C-type lectin and cell adhesion molecule) and protease activity (cysteine proteinase, ADAM precursor, thrombospondin, trypsin inhibitor, cathepsin, serpin, MASPb, trypsin-like serine protease and serine protease), both processes which have been previously described as significant in developmental traits of ascidians (Matsumoto et al., 2001; Kawamura and Sugino, 1999, Kawamura et al., 2006; Roberts et al., 2007). We therefore, propose that in ascidians, immune cascades through two main biological processes of cell adhesion and protease activity, are co-opted to function in developmental processes, imposing innate–immune signaling as common players and dynamic participants in ascidians' developmental and regeneration circuits. This, in turn, may have enabled the diversity of life history traits exemplified in urochordate ascidians.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.09.005.

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