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Animal and Plant Members of a Gene Family with Similarity to Alkaloid-Synthesizing Enzymes

Marco Fabbri,* Gabriele Delp,† Otto Schmidt,* and Ulrich Theopold*,[1]
**Department of Applied and Molecular Ecology and †Department of Soil and Water, University of Adelaide, Glen Osmond, South Australia 5064, Australia*

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Here we describe novel members of a gene family which have similarity to strictosidine synthase (SS), one of the key enzymes in the production of monoterpene indole alkaloids. In addition to the first animal member of the family described previously (*Drosophila* hemomucin), a second *Drosophila* member has been identified, which appears to differ in subcellular distribution from hemomucin. In *Arabidopsis*, SS-like genes form a multigene family, compatible with a possible function as antifeedants and antibacterial compounds. In *Caenorhabditis*, two members have been identified and one member each in mouse and human. Interestingly, the human SS-like gene is strongly expressed in the brain, the very organ many of the indole alkaloids act upon.

Key Words: **alkaloids; dopamine; insect immunity; neurotransmission; biogenic amines; strictosidine synthase.**

Amongst plant alkaloids, some monoterpenoid indole alkaloids have attracted particular interest due to their ability to act as toxins (strichnin), antimalarial (quinine), antineoplastic (vincristine and vinblastine, 1) or antipsychotic drugs (reserpine, 2). This led to the identification of a number of enzymes that are part of their biosynthetic pathway including tryptophane decarboxylase and strictosidine synthase (SS), two key enzymes in the production of monoterpenoid indole alkaloids (3). SS has been isolated from two plant species which are known for their pharmaceutical benefits, namely *Catharanthus roseus* (madagascar periwinkle, 4) and *Rauvolfia serpentina* (sarpagandha plant, 3).

We have previously described a cell-surface molecule (hemomucin) isolated from a hemocyte-like cell line from *Drosophila melanogaster* (5). Hemomucin is composed of two domains, one with mucin-type repeats and the other with sequence similarity to SS. We also found evidence for the existence of hemomucin in two other insect species, the hymenopteran *Venturia canescens* (6) and the lepidopteran *Galleria mellonella* (7). Here we report on a novel gene family with similarity to SS and hemomucin using expression data and information obtained from genomic sequencing and EST projects.

MATERIALS AND METHODS

Flies. D. melanogaster w118 flies were kept on cornmeal/yeast food at 25°C with a 10/14 h light/dark cycle.

Preparation of antisera. For the production of an antiserum against recombinant hemomucin, a PCR amplified fragment covering amino acids 178–299 (5), position 216–341 in Fig. 1, was expressed in the expression vector pQE32 (Quiagen). The resulting fusion protein was purified according to the instructions of the manufacturer and excised from a preparative polyacrylamide gel. Rabbits were immunized according to Harlow and Lane (8) with approximately 10 μg protein/immunization.

Electrophoretic techniques. SDS–polyacrylamide gel electrophoresis on a Mini-Protean II electrophoresis unit (Bio-Rad) was performed according to Laemmli (9). Molecular weights were determined using prestained SeeBlue molecular weight markers (Novex). The proteins were blotted onto a nitrocellulose membrane (Amersham) as described before (7). The amount of protein loaded was as indicated in the figure legends. The blotting efficiency was determined by staining the blot with Ponceau S.

Sequence similarity searches were performed on the NCBI server or the BDGP server using the blastp or tblastn program (10). Sequences with a significant similarity to the original sequence were downloaded into the Lasergene program package (DNASTAR Inc., Madison, WI) and further aligned using Megalign. Genomic regions were analysed using Genfinder. Chromosomal localisations for P1 clones and BACs were available from the BDGP/HHMI EST Project (11, 12).

Northern blots. RNA blots were performed as described (7). The multiple tissue Northern blot was purchased from Invitrogen.

RESULTS

In an attempt to identify genes that are related to hemomucin, we used the hemomucin amino acid se-

[1] To whom correspondence should be addressed at Department of Applied and Molecular Ecology, University of Adelaide, Waite Campus, Glen Osmond, SA 5064, Australia. Fax: +61-8-8379 4095. E-mail: utheopol@waite.adelaide.edu.au.

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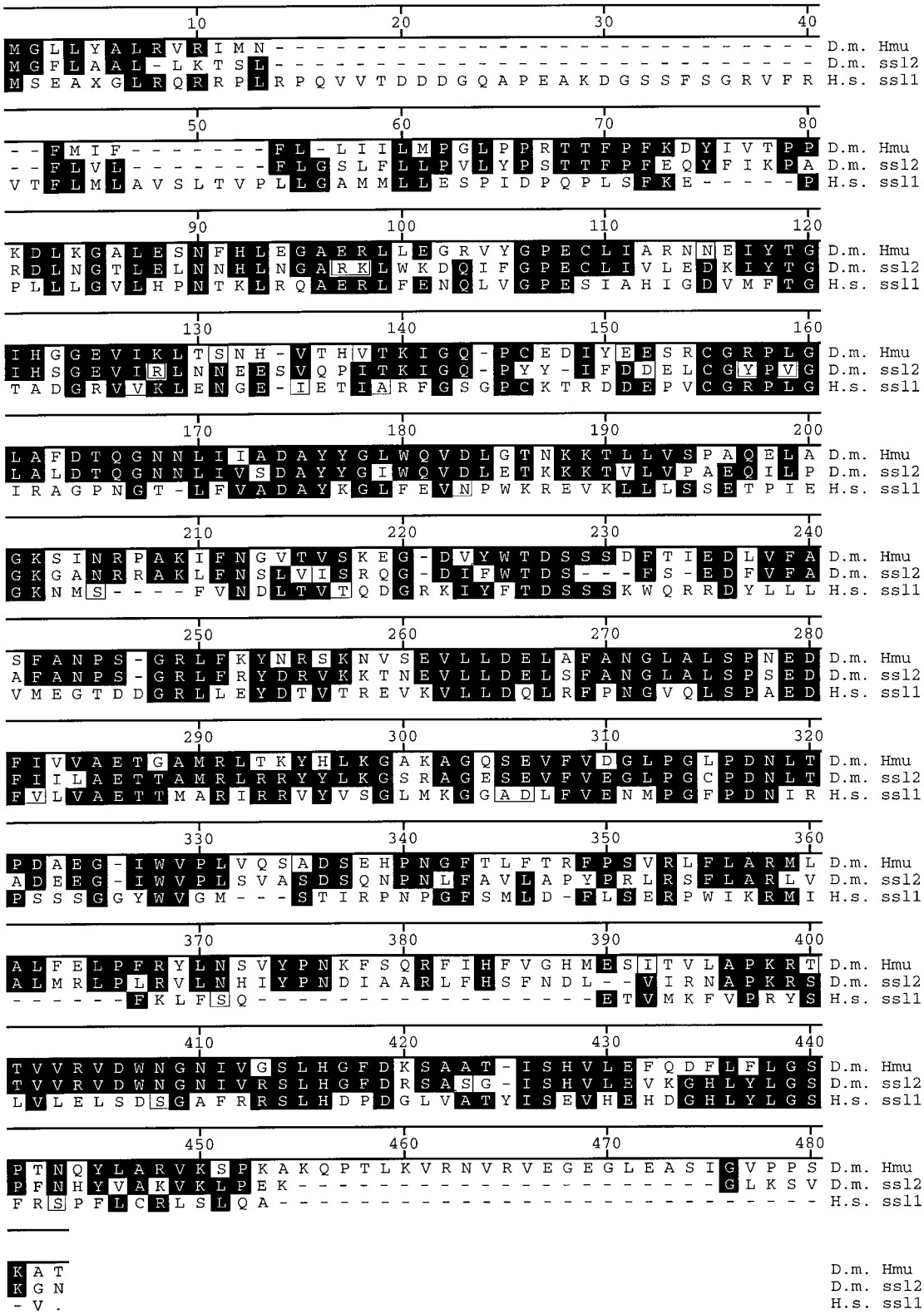

Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.

Decoration 'Decoration #2': Box residues that match the Consensus within 1 distance units.

FIG. 1. Sequence alignment of 3 members of the SSl family. *Drosophila* (Dm) and human (H.s.), members of the SS family were aligned using the Megalign program.

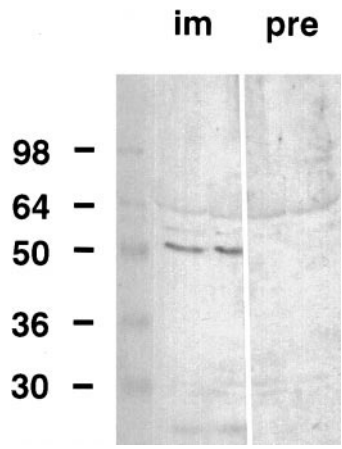

FIG. 2. (A) A candidate for DmSSl2 in *Drosophila* hemolymph. Two different concentrations of *Drosophila* hemolymph (equivalent to the hemolymph of 40 and 20 third instar larvae) were analysed with an antiserum specific for hemomucin and the preimmune serum as a control. A band of the size expected for DmSSl2 was only detected with the immune serum.

quence to run comparisons with sequences obtained from sequencing projects of the major model organisms, including *Drosophila melanogaster, Arabidopsis thaliana, Caenorhabditis elegans, Homo sapiens* and *Mus musculus.* In order to reduce background due to the mucin domain, which shows sequence similarity with a large number of glycoproteins, only the domain with similarity to SS was used for comparison. Interestingly, one additional ORF with similarity to SS could be identified in *Drosophila* (Fig. 1). Further inspection of the genomic region comprising this sequence revealed no ORFs with mucin-type character. Using Genefinder, a complete ORF could be assembled from the genomic sequence, which also did not contain any mucin-like sequences. The novel gene localises to the same chromosomal position as hemomucin (98F). Because of the sequence similarity to SS and the absence of a mucin domain, we decided to call the gene coding for the second member of this family *Drosophila melanogaster* strictosidine synthase-like 2 (DmSSl2). The ORF predicts a product of approximately 46 kDa molecular mass. Since the sequence conservation between hemomucin and DmSSl2 is strong enough to expect antibody cross-reactions, we performed Western-blot analyses using an antiserum produced against recombinant hemomucin. Moreover structural prediction programs predicted DmSSl2 to be secreted (13, 14). Therefore, we analysed hemolymph samples for hemomucin-like proteins. We were indeed able to detect a labeled band of the predicted molecular mass (and different from the 100 kDa of hemomucin), which was completely absent in a blot developed with the pre-immune serum (Fig. 2). We therefore conclude that DmSSl2 is antigenically related to hemomucin and present in *Drosophila* hemolymph. It does not contain the mucin domain and differs in subcellular localisation from hemomucin, which is a cell surface protein. DmSSl2 also lacks two of the three N-linked glycosylation sites present in hemomucin and is expected to be less glycosylated or not glycosylated at all.

In order to identify members of the SS family from other organisms, we performed sequence searches on several organisms with genomic and/or EST sequencing projects. In *A. thaliana,* 13 members could be identified, two with the highest similarity to SS from *Catharanthus roseus* (AtSSl11 and AtSSl12) and other less related members (Figs. 3A and 3B). Amongst these, four sequences seem more closely related to hemomucin than to SS (AtSSl4-7). Three sequences we derived from soybean (*Glycine max*) ESTs that were long enough to allow an alignment were included in the analysis. Additional members were identified from *C. elegans* and both mouse and human. The human sequence looks complete whereas the mouse ORF was incomplete at the time of preparation of this manuscript. None of the newly identified members contain a mucin domain as with hemomucin, which seems to be unique to insects. Since SS has been grouped together with paraoxonases and gluconolactonases into a superfamily based on sequence similarity, we also included human paraoxonase in our comparison (15). In a phylogenetic analysis, all SS-like sequences are more related to each other than to paraoxonase (Fig. 3B). The majority of the plant sequences group together and are separate from the animal sequences. Sequences AtSSl4-7 from *A. thaliana,* and one of the soybean sequences (GmSSl3) are an exception as they show higher similarity to the animal members than to other plant members of the family even from *A. thaliana.*

Since the EST sequencing projects are performed on tissue-specific libraries, a preliminary indication of the specificity of expression can be obtained from the number of ESTs obtained from one particular tissue. Judged from this, high expression for the human SS-like EST is expected in the brain (a majority of the ESTs in the clot with hemomucin similarity are from the brain, see: http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG = Hs&CID = 22391). In order to confirm high expression in the brain, a human multiple tissue Northern blot was probed with one of the ESTs (clone image 258726), confirming that the strongest expression amongst the tissues chosen is in fact in the brain (Fig. 4). The chromosomal localisation of the human SSl-sequence is to chromosome 20 between regions 11:21 and 11:23.

DISCUSSION

Here we present evidence for the existence of a novel gene family with similarity to the plant enzyme strictosidine synthase, one of the key enzymes in plant alkaloid biosynthesis. Since secondary metabolites like

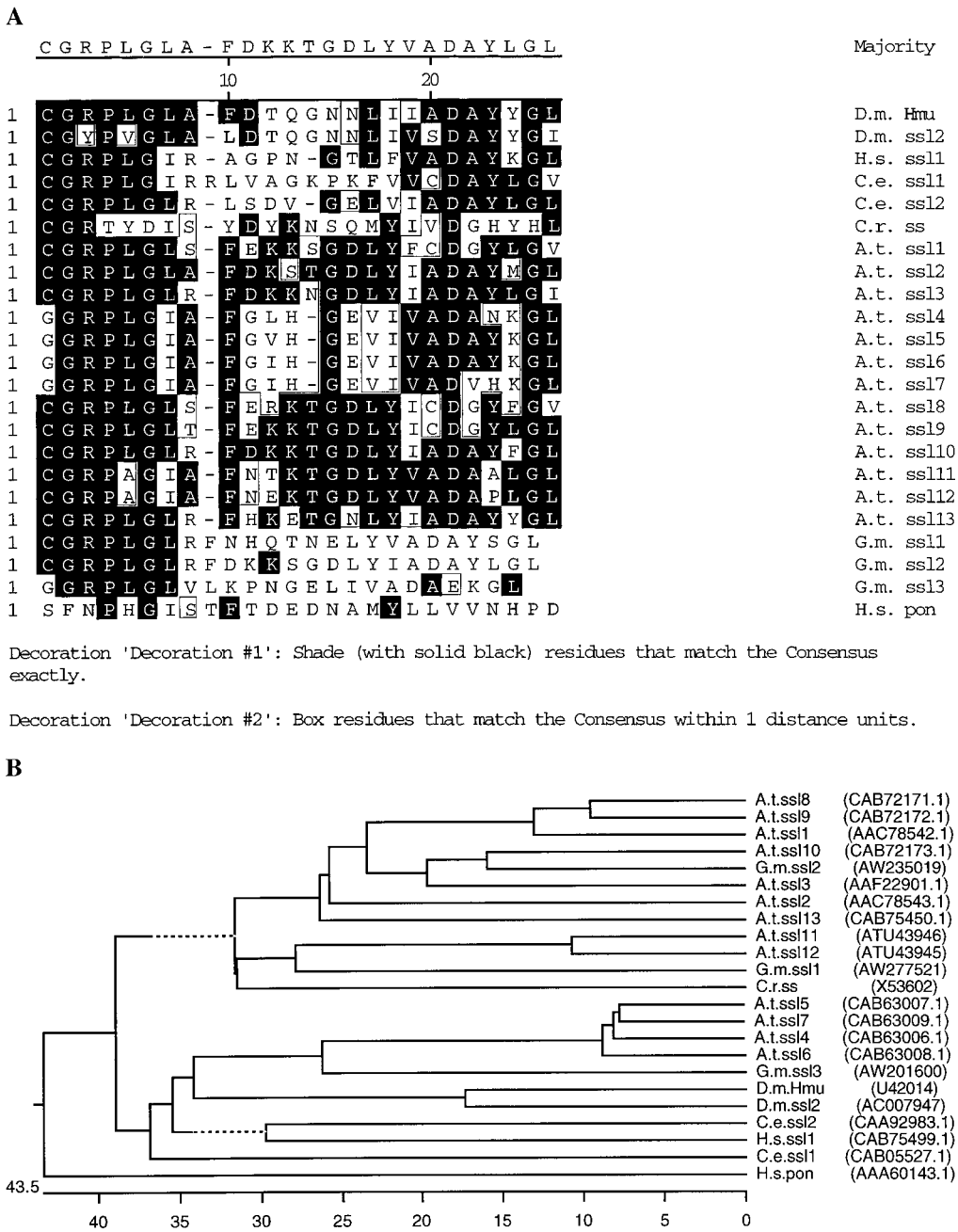

FIG. 3. Relationship between members of the SSl family: The same sequences as in Fig. 1 and sequences from *C. elegans* (C.s.) and *C. roseus* (C.r.) strictosidine synthase as well as *Arabidopsis* sequences were analysed for their possible phylogenetic relationship (unbalanced display, where branch distances correspond to sequence divergence) using the CLUSTAL program as part of Megalign. One of the conserved parts (positions 155–179 in Fig. 1) is shown in A. Some of the sequences are identical in this part but differ otherwise. The phylogenetic tree in B is based on the sequence between positions 116 and 281 in Fig. 1.

indole alkaloids are widely distributed in plants but less often found in animals, the existence of members of this family in a number of animal species came as a surprise. We speculate that the animal members of the family have a different function than SS. The fact that the *Arabidopsis* members fall in different groups, with one group being more similar to hemomucin than other plant genes, indicates that also in plants, SSl proteins may perform more than one function and be involved in more than one biochemical pathway. The existence of at least 13 members of the SSl family in *Arabidopsis* is in agreement with the identification of multiple isoforms of the enzyme in *C. roseus* (16) and with the observation that this plant is known to contain over 100 different monoterpenoid indole alkaloids (1). Each of these SSl family members may differ in their enzy-

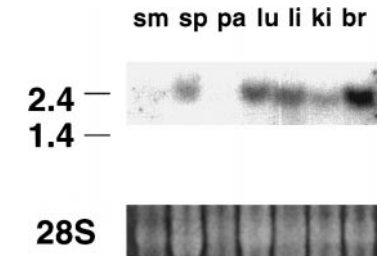

FIG. 4. Expression pattern of the human SSl member. Total RNA from brain (br), kidney (ki), liver (li), lung (lu), pancreas (pa), spleen (sp), and skeletal muscle (sm) was analysed using the EST coding for the human SSl protein as a probe. The 28S RNA is shown as an internal control for the amount loaded.

matic activities and/or substrate specificities for the different products of secondary metabolism. In addition, they might differ in their expression, which was shown to be induced by fungal elicitors and jasmonate in the case of SS from *C. roseus*. The selective pressure for the production of a variety of phytoalexins may have lead to the duplication of an ancestral SSl gene in *Arabidopsis* and other plants (like soybean). In *Arabidopsis,* multiple gene duplication events have happened leading to the 13 observed members of the family (for example, AtSSl8 and 9 and AtSSl4-7 are closely linked and have arisen by gene duplication).

The gene coding for the next enzyme in the biosynthetic pathway leading to indole alkaloids, strictosidine glucosidase (17) also seems to be part of a multigene family in *Arabidopsis* (unpublished observations). The most intensely studied members of this family are myrosinases, which are believed to be part of the plant's defense against insects and possibly pathogens (18). In contrast to that and similar to the situation with SS, we could only identify one gene with significant similarity to strictosidine glucosidase in *Drosophila* (unpublished results). Strictosidine itself and the deglucosylation product of strictosidine could be shown to have antibacterial activity but seem to lack the antifeedant activity of further downstream products in the biosynthetic pathway for indole alkaloids (19). As for insects, hemomucin, the previously identified member from *Drosophila* seems to be exceptional in that it contains a mucin-like domain attached to the SS-like domain. Therefore in *Drosophila* and possibly in other insects, there seem to exist two members of the family, one on the cell-surface and a second one which is present in hemolymph. At this stage, it can only be speculated what the substrates for SSl members in insects might be, but it is interesting to note that a number of components of the phenoloxidase cascade (like dopamine) show structural similarity with indole ring-containing substances. The enzyme upstream of SS in the biosynthetic pathway for monoterpene indole alkaloids (tryptophane decarboxylase) shows high sequence similarity with the enzymes dopa decarboxylase and the product of the *l(2)amd* gene, which are known to be involved in both the generation of neurotransmitter substances (like dopamine and serotonin) and central components of the phenoloxidase cascade (20, 21). The *amd* product is also expressed in the lymph glands suggesting a possible role in immunity (21). In agreement with a possible function of hemomucin in defense reactions which may include antimicrobial activity and/or protein crosslinking, we have previously gained evidence that it is involved in hemolymph coagulation (7).

An interesting finding is the fact that the human member of the SSl family shows the strongest expression in the brain, the very organ some indole alkaloids like reserpine act upon by interfering with the synaptic transmission mediated by dopamine (2). Learning about the function of the human SSl protein(s) might therefore help to understand the effect of these alkaloids in the brain. It will also be interesting to analyse a possible function of the insect genes in the nervous system and to look into a possible correlation between neurological defects in the genomic and mutations in the corresponding gene. With the availability of the sequence information presented here it is now possible to address these questions.

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