

CLONING AND LOCALIZATION OF A PUTATIVE SEROTONIN RECEPTOR IN
THE PRIMARY OLFACTORY PATHWAY OF THE MOTH *MANDUCA SEXTA*

By

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Introduction

Serotonin is an important molecule in the nervous system. In humans, it is involved in sleep, depression, anxiety, schizophrenia, satiety, and other physiological and mental functions and disorders (Purves et al., 2008). Neuromodulation is one of the main ways through which serotonin acts. Neuromodulation is an important process underlying the functions of the nervous system, modifying the properties of neurons to adapt to the changing needs of neural networks and of the animal.

The serotonergic system in humans and mammals are highly complex. We seek to improve our understanding of serotonin and neuromodulation by studying them in a simpler system—the olfactory system of the moth, *Manduca sexta*. The moth primary olfactory pathway consists of its antenna, responsible for sensing odors, and antennal lobe (AL), which processes signals from the antenna. The AL is a structure homologous to the human olfactory bulb. It integrates and processes odorant signals from olfactory receptor neurons (ORNs) on the antenna, and sends the information to higher-order regions of the brain. In the AL (Fig. 1), the ORN axons sort into glomeruli, the functional units of the AL. At a glomerulus, ORN inputs converge, and the information is outputted by a small number of projection neurons (PNs). Another type of neurons, the local interneurons (LNs), tunes the signals from ORNs to PNs and connects multiple glomeruli. AL neurons are modulated by 5HT. Interestingly, the 5HT level in the AL fluctuates throughout the day, peaking when the animal is most active (Kloppenburg et al., 1999). This raises the question: how is neuromodulation by 5HT involved in the processing of olfactory information, and furthermore in the behavior of the animal?

Since the identification of the serotonin-immunoreactive neuron in the AL (Kent et al., 1987), serotonin has been studied extensively in this system. There is one serotonin-

immunoreactive neuron in each of the two ALs; it innervates all the glomeruli of the contralateral AL where it makes largely output synapses (Sun et al., 1993). Serotonin has modulatory effects on a subset of AL neurons. In cultured AL neurons (Mercer et al., 1995) and in some PNs (Kloppenburg et al., 1999), 5HT has been shown to reduce two potassium conductances, increasing cell input resistance. As a consequence, in certain AL neurons, 5HT has been found to increase cell excitability and broaden action potentials (Kloppenburg and Hildebrand, 1995), and to increase the response to pheromone stimulation (Kloppenburg et al., 1999). Examining the problem at the level of neural ensembles, 5HT was found to increase the sensitivity and resolution of olfactory processing in the AL (Dacks et al., 2008).

These studies approached the problem through anatomy and electrophysiology and taught us much, but we still only have a limited understanding because the molecular basis of 5HT's effects in the AL is not known. A molecular approach to the problem will elucidate the cellular mechanisms of 5HT's action, and give us tools to further investigate the role of 5HT in information processing at the AL. On the molecular level, the effects of 5HT are mediated by a variety of receptors, and studying these receptors would be the first step in this molecular approach.

In mammals, there are seven families of 5HT receptors. One advantage of simple insect models is that they possess a smaller number of receptors: orthologs to only three of the mammalian families of receptors have been identified in invertebrates (Tierney, 2001). They are the 5HT1, 5HT2, and 5HT7 families, all of them G-protein-coupled receptors. Two putative 5HT receptors, Ms5HT1A and Ms5HT1B, have been cloned from *Manduca* (Dacks et al., 2006). Here we describe the cloning of a third 5HT receptor, Ms5HT7, and progress in examining its localization in the primary olfactory pathway.

Materials and Methods

Animals

Manduca sexta were reared in the rearing facility of the Department of Neuroscience at the University of Arizona as described by Christensen and Hildebrand (1987). All moths used were 1-5-day-old adults.

Cloning of Ms5HT7 cDNA

Total RNA of adult brains was extracted using Aurum Total RNA Mini Kit (Bio-Rad). cDNA was then synthesized using Omniscript RT Kit (Qiagen). Amino acid sequences of 5HT7 receptors from *Aedes aegypti* (Aedes 5-HT7, accession number AAG49292), *Drosophila melanogaster* (5HT-dro, accession number AAA28305), and *Apis mellifera* (Am5-HT₇, accession number CAJ28210) were aligned, and degenerate primers were designed from conserved sequences. PCR on brain cDNA using one primer pair (sense: CCGTGTGCTGGCTGCCNTTYTTYRT, antisense: GCAGCATCGGAAGTACAGGATYTCNYKRAA) amplified a 196bp fragment. The fragment was cloned into pSTBlue-1 vector using pSTBlue-1 Perfectly Blunt Cloning Kit (Novagen) and sequenced. From this fragment, gene-specific primers were designed for RACE (rapid amplification of cDNA ends). RACE using SMART RACE cDNA Amplification Kit (Clontech) produced the 3' and 5' ends of the cDNA, which were cloned into pSTBlue-1 vector or into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen) and sequenced. All sequencing was performed by the Genomic Analysis and Technology Core at the University of Arizona.

Sequence and phylogenetic analysis

The sequences of the cDNA fragments were processed and assembled using Sequencher 4.7 (Gene Codes). Sequence similarity search was performed using blastx on the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignment was performed using ClustalW at the EMBL-EBI server (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Phylogenetic tree was created based on maximum-likelihood analysis.

Detection of Ms5HT7 mRNA in olfactory pathway using RT-PCR

Total RNA was extracted from adult antennae and from adult ALs using TRIzol LS (Invitrogen). cDNA was then synthesized using Omniscript RT Kit; to control for genomic DNA contamination, parallel reactions were run in which reverse transcriptase was omitted. PCR was performed on antenna cDNA and AL cDNA as well as the corresponding negative control samples using the primer pair GACGCCGTGAGTGCGCTGTTTCTTT (sense) and TGCTGACCTCGGGCATTGACAATCAT (antisense).

Riboprobe synthesis

Four pairs of digoxigenin-labeled riboprobes, ranging from 300 to 500 nt, for use in *in situ* hybridization were each synthesized as follows. A fragment of the Ms5HT7 cDNA was amplified using PCR from brain cDNA, cloned into pSTBlue-1 vector, and then subcloned into LITMUS 38i vector. The vector was linearized and transcribed in opposite directions (in two different reactions) to produce antisense probes and sense control probes. Transcription was performed using AmpliScribe T7 High Yield Transcription Kit (Epicentre) while substituting DIG RNA Labeling Mix (Roche) for regular ribonucleotides. The integrity of the probes was checked by denaturing gel electrophoresis.

In situ hybridization

Protocol for *in situ* hybridization was modified from Nighorn et al. (1998). Briefly, brains of adult male moth were dissected and fixed in 4% paraformaldehyde, either for 3 hours at room temperature or overnight at 4°C. Brains were cryo-protected with sucrose and sectioned using cryostat at 12-20 µm thickness. The sections are mounted on Superfrost slides and baked at 42°C overnight. The slides were then washed with PBS for 5 min, PBST for 15 min, 2X SSPE for 10 min, incubated in Proteinase K (2 µg/mL in TE) for 30 min, washed in PBS briefly, incubated in 4% paraformaldehyde for 5 min, and washed for 5 min twice with PBS. The slides were then incubated in prehybridization solution (10% dextran sulfate, 50% formamide, 1X Denhardt's Solution, 4X SSPE, 250 µg/mL salmon sperm DNA) for 2 hr at 50°C. Then, the slides were incubated in prehybridization solution containing 0.25 µg/mL of each of the four antisense riboprobes, at 50°C overnight. Parallel experiments were run with sense control probes. After hybridization, slides were washed twice for 15 min in 2X SSPE at 42°C, incubated for 30 min in RNase (20 µg/mL in NTE) at 37°C, and washed twice for 15 min in 0.1X SSPE at 42°C. The slides were blocked (1X DIG Buffer 1, 2% gelatin, 2% sheep serum, 0.1% Triton X-100) for 1 hr and incubated in alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) at 1:500 dilution overnight at 4°C. The slides were then washed four times for 15 min in 1X DIG 1 Buffer then for 20 min in DIG 3 Buffer. The slides were then stained in BCIP-NBT solution in dark for 2-6 hr. Finally, the slides were washed twice for 15 min in TE, for 5 min in distilled water, and mounted using Crystal/Mount.

Results

Cloning of Ms5HT7 cDNA

We cloned the full-length cDNA of a putative *M. sexta* serotonin receptor of the 5HT7 subtype, Ms5HT7. The cDNA consists of 3244 bp, and the deduced amino acid sequence consists of 563 amino acids.

Sequence and phylogenetic analysis

We performed BLAST searches, multiple sequence alignments, and phylogenetic analysis to examine the likely identity of the protein encoded by the Ms5HT7 cDNA. Search through blastx using the full-length Ms5HT7 cDNA sequence retrieved insect 5HT7 receptors with low E values. Among the sequences retrieved with the highest similarity are a honeybee 5HT7 receptor (accession number NP_001071289, E value: $3e^{-136}$), a fly 5HT7 receptor (accession number NP_524599, E value: $7e^{-117}$), and a mosquito 5HT7 receptor (accession number AAG49292, E value: $1e^{-69}$), along with uncharacterized putative serotonin receptors. Multiple sequence alignment was performed on the amino acid sequences of Ms5HT7 and 5HT7 receptors from *Aedes aegypti* (Aedes 5-HT7, accession number AAG49292), *Drosophila melanogaster* (5HT-dro, accession number AAA28305), and *Apis mellifera* (Am5-HT₇, accession number CAJ28210) (Fig. 2). The alignment shows high sequence similarity. To examine the evolutionary relationships between Ms5HT7 and other 5HT receptors, we generated a phylogenetic tree containing insect 5HT receptors (Fig. 3), in which Ms5HT7 clusters with 5HT7 receptors from other species. These sequence and phylogenetic analyses show that Ms5HT7 is most likely a serotonin receptor of the 5HT7 subtype.

Detection of Ms5HT7 mRNA in olfactory pathway using RT-PCR

To broadly examine the expression of Ms5HT7 in the moth olfactory pathway, we used RT-PCR to localize the mRNA of Ms5HT7 in antenna and AL tissues. RT-PCR amplified a

fragment of the Ms5HT7 cDNA from both adult antennal and AL cDNA (Fig. 4), showing that Ms5HT7 mRNA is expressed by adult moths in both elements of the primary olfactory pathway.

In situ hybridization

To identify specific AL cells or cell types where Ms5HT7 may mediate the effects of 5HT, we are investigating the Ms5HT7 mRNA localization pattern using *in situ* hybridization. For this purpose, we have generated four riboprobes as well as four control probes complementary to them (Fig. 5). We are currently in the process of performing the *in situ* hybridization experiments.

Discussion

The cloning of Ms5HT7 is an early step in the molecular approach to studying 5HT modulation in the AL, and it fills in gaps in our knowledge obtained from anatomical and neurophysiological studies. The existence of a putative 5HT7 receptor suggests a potential signaling mechanism for some of the known physiological effects of 5HT in AL cells. 5HT7 receptors are positively coupled to adenylate cyclase and increase the intracellular concentration of cAMP when activated by 5HT. Protein kinase A, activated by cAMP, can phosphorylate ion channels and change their conductance. For example, cAMP-dependent protein phosphorylation has been shown to mediate the decrease of K⁺ current in the sea slug *Aplysia californica* (Castellucci et al., 1982). Thus, the activation of Ms5HT7 by 5HT may lead to intracellular pathways that result in the decrease of two K⁺ currents and other effects observed in AL neurons.

To continue the study of Ms5HT7 and its role in serotonin modulation in the AL, several directions can be taken. The localization of the Ms5HT7 mRNA, through *in situ* hybridization, and the localization of the Ms5HT7 protein, through immunocytochemistry, will identify cells

where Ms5HT7 may play a role in mediating 5HT modulation. Pharmacological characterization of the receptor will reveal its affinity to 5HT, which is important in understanding the different effects of fluctuating levels of 5HT. Also, if selective drugs can be found that affects Ms5HT7, such as a selective antagonist, they could be used in electrophysiological studies to isolate the specific contribution of Ms5HT7 to the known physiological effects of 5-HT. We hope in the future to add to our understanding of serotonin modulation in the AL by characterizing Ms5HT7.

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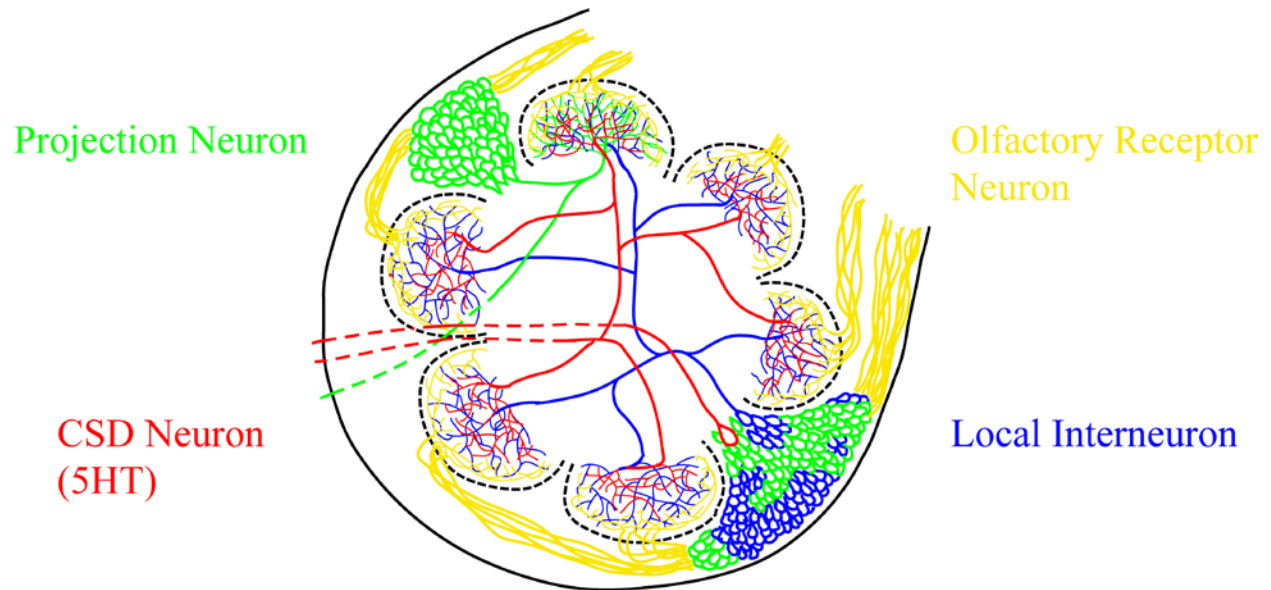


Fig. 1 Schematic diagram of the antennal lobe (AL). Olfactory receptor neurons (ORN, yellow) send their axons from the antenna into the AL, where they sort into glomeruli (delineated by dashed lines), where ORNs expressing the same odorant receptor project to the same glomerulus. Projection neurons (PN, green) receives input from a single glomerulus and outputs to higher-order regions of the brain. Local interneurons (LN, blue), with processes in multiple glomeruli, are involved in the processing of olfactory information. 5HT is released by the CSD neuron (red) into all glomeruli, modulating diverse AL cells.

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Ms5HT7 1 - - - - -MATQNHKSHSLP--SFVALPSCLEILVSLIILQPN--AVDSVDCVDSLLASEYN-YINPNSITNCTIFDTNSIHKHIK- - - - -
Aa5HT7 1 - - - - -MDPTVFPLLSTLQQSSAQVLPIGDGPSTSVASGVAEVAIINAIATINILEYLLIGNSS- - - - -
Am5HT7 1 - - - - -MEGKDAITTEDILLN- - - - -LITQDVDG- - - - -FLQGFPGKNSP- - - - -
Dm5HT7 1 MALS GDWRHRHQRHRHRHRTQGNHQLISTATLTLFVLFSSWIAAYAGKATVPAPLVEGETESATSQDFNSSALGAIASASSTGS GSGSGSGSGSGS
Ms5HT7 74 - - - - -HSKYSTPVTIFLVLFELIVCTIVGNILVCVAVRLVRRKLRPSNYLIIVSL
Aa5HT7 60 -SASVSATAIATSLPALVDRLTPTSS--TSLLDELGGTS--ESSPAEPVNVLTIQTVISLVLLAVLIGIIVGNVLCVAVCLVRRKLRPCNYLLVSL
Am5HT7 36 - - - - -TVTQAALLALVLSIIVGTIVGNILVCVAVFLVRRKLRPCNYLLVSL
Dm5HT7 103 GSGSYGLASMNSPIAIVSYQGITSSNLGDSNTTLVPLSDTPLLLEFAAGFEVLPPLTSLVSVLVLLIVLGLVVGIVLGIACMRRKLRPCNYLLVSL
Ms5HT7 125 AVSDLCVAIIVMPFATVNDIMGTWPGPVIICFFNANS DVLSQAASILNLCMSVDRYVAITKPLEYGVKRTPKRMLLCILIVMSAAFISLPPVILGNEK-
Aa5HT7 154 AISDLCVAIVLMPALLLYEIVLEEKFGTIFCDIWSFDVLSCTASILNLCMSVDRYVAITKPLEYGVKRTPRRMIAQIVLVWVAACISLPPVILGNEH-
Am5HT7 84 AVSDLCVALLVMPALLLYEISGNNSFGTIMCDLWSFDVLSCTASILNLCMSVDRYVAITKPIKYGKRTPRRMIVYVSLVWVAACISLPPVILGNEHT-
Dm5HT7 205 ALSDLCVALLVMPALLLYEIVLEKINFGPILLCDIWSFDVLCCTASILNLCMSVDRYVAITKPLEYGVKRTPRRMIVGLVWVAACISLPPVILGNEH-
Ms5HT7 226 - - - - -TETSNSDNQVYQIYATLGSFYIPLTVMVYVYKIFRAARKIKDEKRAQSHLETHCYLEINVKNGGAEEKLLGNQPAQPPPRGSTASTNTTCSV
Aa5HT7 255 MTN-GQBSCSVCQNFYQIYATLCAFYIPLAVMLFVYEQIFRAARRIYNEEKRAQKHLETAIN--GSATTEKK- - - - -
Am5HT7 186 YSETGPSHIVCQNFYQIYATLGSFYIPLFVMIQYVYKIFCAARRIVLEFRRAQSHLEAHYFDEFTVQHQPVTVNRQLNSDVQPGHSGPPVKQHRSSS
Dm5HT7 306 EDEEGQIITVCQNFAYQIYATLGSFYIPLSVMFVYVYQIFRAARRIILEKRAQTHIQQALNGTGSAPQAPPPLGHTELASSGNQQRHSSVGNSTLYST
Ms5HT7 324 DKAESGLGRCSGQRKSNESQCPMLQPKPTPKIHTINRSPPTLTPNNKPIVKDRRRPSETS NKMTTNRIRSSLSNFAQKSHIAKDLFHSQSHQKLRFP
Aa5HT7 326 - - - - -LSAGG- - - - -TVLVATPQHRRLRFO
Am5HT7 288 ASTTCSGHTVRCFTGGPRKSHESQCPMLQKLEKPVLSSTTTTSPMTSKSTI- - - - -VRNHLNSTCSVTNSPHQKLRPH
Dm5HT7 408 CGGLSSGGALAGHGS- - - - -GGGVSGSTGLLGSPPHKKLRFP
Ms5HT7 426 LAKERKASTTLGIMSATLVCWLPFFVLAIRPFVEE-DALPDAYSALFLWLGYNLSLNPVIYATLNRDERKPFQELIFERCNSLNMHMRREEFYHSQYQDP
Aa5HT7 346 LAKERKASTTLGIMSATVWLPFFVLAIRPFVEE-EDH-HLSSILFLWLGYNLSLNPVIYATLNRDERKPFQELIFERCNSLNMHMRREEFYHSQYQDP
Am5HT7 364 LAKERKASTTLGIMSATVWLPFFVLAIRPFVEE-DAIPAFSSLFLWLGYNLSLNPVIYATLNRDERKPFQELIFERCNSLNMHMRREEFYHSQYQDP
Dm5HT7 446 LAKERKASTTLGIMSATVWLPFFVLAIRPFVEE--MHVPASSLFLWLGYNLSLNPVIYATLNRDERKPFQELIFERCNSLNMHMRREEFYHSQYQDP
Ms5HT7 527 DQHYCGNNTKLHNYDEG--VEIVSAIDRETRASFSFL
Aa5HT7 445 G--SQRVMAAN--DGGGARESF
Am5HT7 466 I--NNCEIKAGEIDAERLNNQIESIDIAANAPNESFL
Dm5HT7 546 P--SQRVMLGD--ERHGARESF

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Fig. 2 Multiple sequence alignment of Ms5HT7 and characterized 5HT7 receptors from *Aedes aegypti* (Aa5HT7), *Apis mellifera* (Am5HT7), and *Drosophila melanogaster* (Dm5HT7). The seven putative transmembrane domains are indicated by black bars atop the sequences.

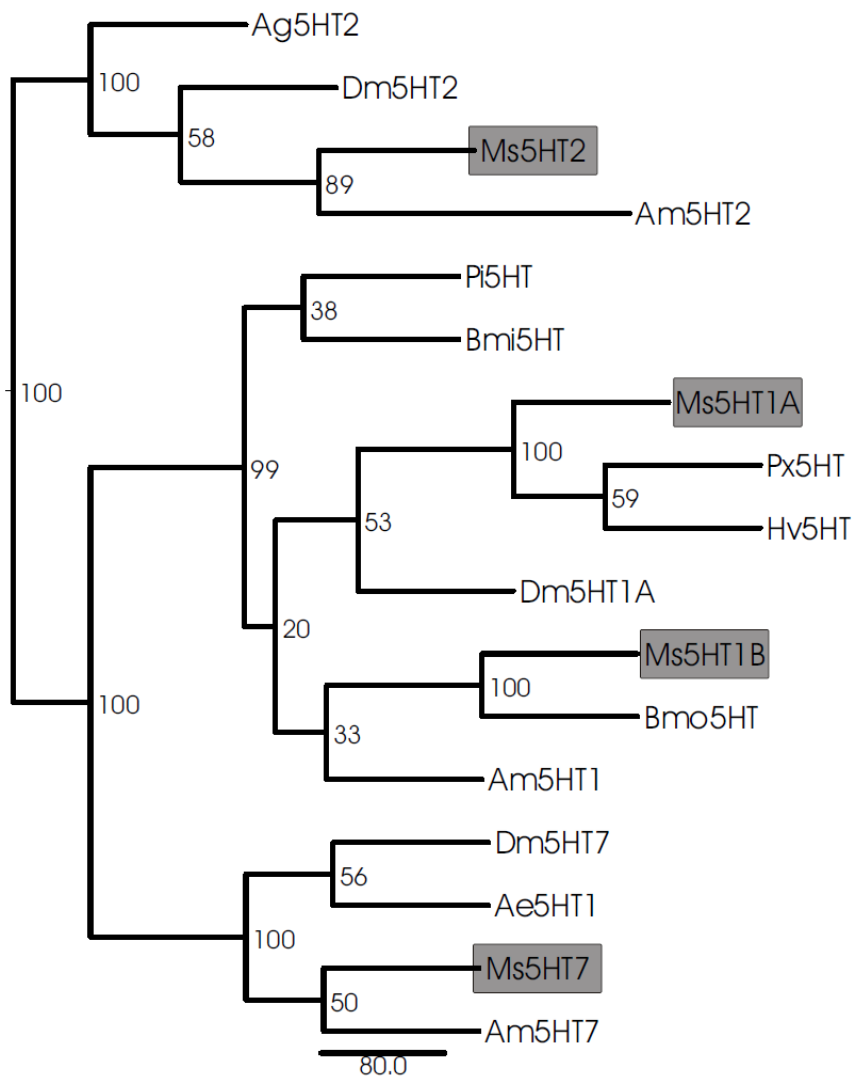


Fig. 3 Phylogenetic tree of insect 5HT receptors. Ms5HT7 clusters with 5HT7 receptors from other insects. Also highlighted are other putative *M. sexta* 5HT receptors.

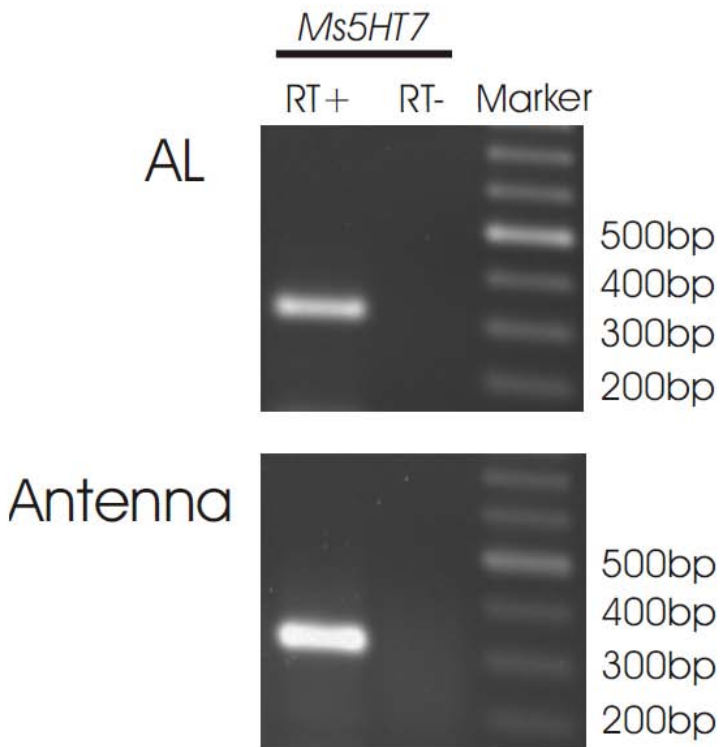


Fig. 4 RT-PCR amplifying a fragment of the *Ms5HT7* cDNA shows that its mRNA is expressed in the AL (upper panel) and antenna (lower panel). RT+ and RT- denote respectively the inclusion and omission of reverse transcriptase in the cDNA synthesis reaction.

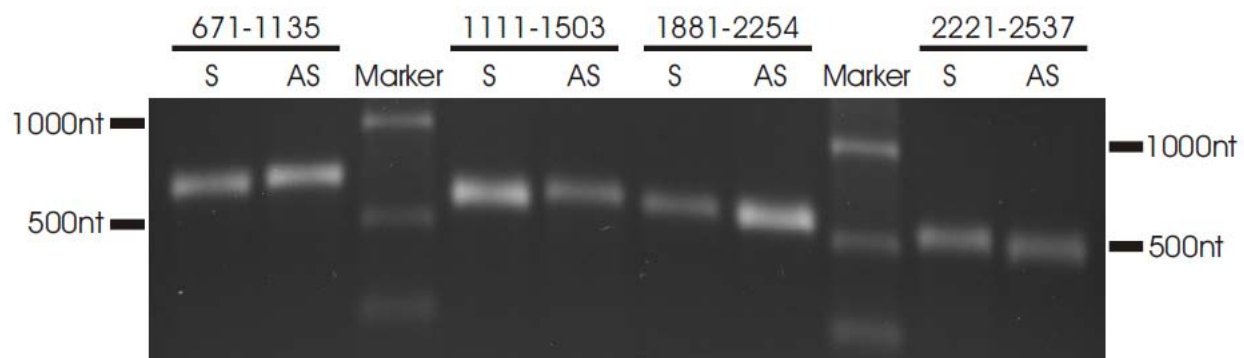


Fig. 5 Denaturing gel of Ms5HT7 riboprobes for *in situ* hybridization. Numbers above the lanes denote fragments of the Ms5HT7 cDNA that served as transcription templates for probes in the corresponding lanes. S and AS denote sense and antisense probe, respectively.