# Molecular Physiological Studies on Cellular and Tissue Osmoregulation in the Silkworm, *Bombyx mori*

**Physiology of V-ATPase and Aquaporin –water channel– in Insects**

（昆虫の水分調節におけるアクアポリン水チャネルの細胞生理機能に関する研究）

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# はじめに

## 1．昆虫のデザイン

昆虫は今から３億５千万年前に出現したとされ，最初に陸上生活を始めた動物界のパイオニア的存在である。種の数，個体の数が他の動物と比べて桁外れに多く，生活型（ライフスタイル）の多様性に極めて富んでいる。このような昆虫の繁栄を可能にした大きな要因は，小型化・軽量化であるといわれている。「虫けら」という言葉に象徴されるように体は小さい。小型であるが外形の複雑さから，体の大きさに比べてその体表総面積は陸生脊椎動物よりはるかに大きい。また，開放血管系であるので皮膚（integument，いわゆる外骨格）からの蒸散は，体液（haemolymph：血リンパ）の損失に直結する。したがって，陸上を主たる棲息環境としている多くの昆虫にとって体からの水分蒸散は日常的に直面する課題であり，体内水分のホメオスタシス（恒常性維持）は昆虫が陸地で生き延びるために最重要課題の一つであるにちがいない。

乾燥や渇きという陸上生物の宿命を克服するために，昆虫は多層構造の表皮（cuticle: クチクラ）を持ち，昆虫種によっては鎧のように強固な皮膚を構成するものもいる。一方，気象変化による冠水・水没による酸素欠乏という事態も昆虫たちは避けてゆかねばならない。そのために昆虫皮膚は防水性や撥水性も有している。外骨格動物である昆虫は水代謝からみるとすべてを閉鎖したような系であるが，昆虫の表皮には呼吸（ガス交換）のための空気の通路（気管: trachea）が体節ごとに網目状に分布している。肺が存在しないので，酸素は表皮上に開口している気門から拡散によって気管へ流入し，気管は毛細気管（tracheole）となって体内のすみずみに分枝し，各組織細胞にまで直接酸素を到達させている（酸素直達方式）。気管系は全身に分布しているので，ガス交換と同時に換気・体内湿度管理の役割を果たしていると考えられる。

昆虫の一生は変化の連続である。 小さな体に存在する僅かな水分を最大限に利用して個体のホメオスタシス，成長・変態，生殖まで完結している。形態を変え，生活様態を劇的に変化させるために，変態機構や休眠機構の発達が昆虫の生活史に多様性を与え，外界の環境に影響を受けやすい変温動物であることをむしろ逆手に取って有効に活用してきたといえる。ヒトよりもはるかに短い一生の中で，自らの存在のスクラップ＆ビルドを完遂している（山下，2001）。脊椎動物のような閉鎖血管系を持たない昆虫では，それぞれの組織・細胞は体液に浸った状態にあり，細胞は細胞外（体液環境）との交流を原形質膜に在る輸送システムを介して直接行い，組織＝組織間の相互作用よりも直接的である。能動輸送機構に

よって創出された組織内外の pH・イオン組成の勾配や偏りは，体内の各組織の働きへダイレクトに作用し，個体としての浸透圧維持や耐乾燥能力に反映されていると考えられる。

## 2．昆虫の能動輸送・浸透圧調節機構の特質

昆虫は熱帯から極地まで，地球上のあらゆる地理・気候帯にその生存圏を有することが正にその繁栄を示しているが，海洋からは排除されている（Maddrell, 1998）。系統的に近縁の甲殻類は海を利用しているが，海中を生活の場としている昆虫はいない。哺乳類の多くも確かに陸上生活であるが，ミネラルとしての $Na^+$ が生命維持に欠かせない。それはヒトをはじめ脊椎動物では$Na^+$が常に要求され，能動輸送機構として$Na^+$ポンプ（$Na^+$, $K^+$-ATPase）が生命維持に必要不可欠なエネルギー生産装置として働いているからである。ナトリウムポンプが機能していない生物，すなわち $Na^+$ の駆動力（"sodium-motive force"）を得ることができない細胞・組織ではいかにして物質輸送の駆動力を獲得するのであろうか。昆虫では鱗翅目昆虫（蛾・チョウの仲間）（Fig. 1A, B and C）や淡水にいる水生昆虫（例：ボウフラなど）のようにプロトン（$H^+$）の能動輸送機構（プロトンポンプ，$H^+$輸送性液胞型ATPase，V-ATPase）を具備することで，$Na^+$不在下での生存を可能にしている。さらにプロトンポンプによるプロトンの移動が原形質膜を介して起こるので，pH 変化が細胞内の酵素作用やタンパク質の性質に直接的に影響を与えることになる。1990年代以降，精力的に推進された研究から，V-ATPase が細胞内外での電気化学的電位差を形成し，二次的なイオン・溶質輸送（solute transport）に必要な駆動力（"proton-motive force"）を創出していることが次々と明らかになってきた（Harvey and Wieczorek, 1997；Wieczorek et al., 1999）。現在では生物界に広く存在する普遍的なプロトンポンプとして個々の細胞生理における重要性が認識され，定着している（Beyenbach and Wieczorek, 2006；森山，2000；孫ら，2002）。

原形質膜に存在する V-ATPase（plasma membrane V-ATPase）は細胞外に $H^+$ を放出し，原形質膜を介して pH 勾配を形成し，この場が多くの生理機能と関係している。元来，V-ATPase は酵母細胞の液胞で発見されたのが最初で（1981年），その後 lysosome 膜など細胞内膜系のプロトンポンプ（endomembrane V-ATPase）と考えられていたが（Harvey and Nelson, 1992），脊椎動物の破骨細胞・腎尿細管上皮細胞・膀胱上皮や，淡水の両生類ヒキガエル（Bufo marinus）の皮膚上皮細胞など，昆虫細胞に限らず様々な動物組織で原形質膜での重要性が報告されている（Brown and Breton, 1996；Wieczorek et al., 1999）。つまり，F-ATPase (ATP synthase)，P-ATPase（$Na^+$,$K^+$-ATPase など）に次

ぐ第３の ATPase としての V-ATPase の地位が確立していった（Harvey and Nelson, 1992；東，1995）。プロトンポンプ（V-ATPase）の系で共通していることは，前段で述べたように "sodium-motive force" の供給がない環境下にあるということである。特に鱗翅目幼虫の系（中腸やマルピーギ管）（Fig. 1D）は，血液自体にナトリウムイオンがなく，虫体内の全身の組織が V-ATPase による "proton-motive force" に依存して，二次的な輸送系（対向輸送，共輸送やイオンチャネル）の働きを調節していることが大きな特徴であり，非ナトリウム輸送系細胞の典型であるといえる（Wieczorek, 1992；Lepier et al., 1994；東，1995；Wieczorek et al., 2000）。このように昆虫細胞においては，原形質膜 V-ATPase が普遍的に分布しており，陸上生活する昆虫の多様なライフスタイルを保証するための pH 調節や水分（浸透圧）調節に対する V-ATPase の関与の高さを伺い知ることができる（Harvey et al., 1998）。

生体内水溶液系でのイオン濃度の動的平衡状態は，第一義的に能動輸送機構（イオン輸送性 ATPase）によって支配されている。細胞の内外で様々な溶質の濃度勾配が創出されると，それらの総体として浸透圧が生じ，水分子自体の流れを生み出すことになる。つまり，溶質濃度の薄い場所から濃い場所へ水のフローが発生する。原形質膜が水専用の通過路を装備し，その通路が存在すると，水分子が10～100倍の効率で輸送されることが1992年に発見され，2003年のノーベル化学賞（Peter Agre 教授）に輝いた。これが Water Channel -アクアポリン（aquaporin: AQP）- である（Agre, 2006）。水は生命現象にとって必須の物質であり，ヒトの体ではその６～７割が水である。体内ではみかけ以上にダイナミックな水輸送が行われ生命が維持されている（佐々木編，2005）。本研究では，大型鱗翅目昆虫であるカイコ幼虫の生命維持基盤になると考えられる V-ATPase と AQP を取り上げる。

### ３．絹糸昆虫のデザイン

カイコの特徴を一つ挙げるとすると，“糸を吐いて繭（シルク）を造る昆虫である”といえる（Fig. 1）。絹糸腺は繭を造るために絹タンパク質を合成，分泌することに特化した組織で，そこでは莫大なタンパク質生産を行っている。絹糸腺は上皮細胞が縫合するように一層に並んで構成された管状の腺組織で，他の昆虫種にもみられる口部に開口する下唇腺（labial gland）である（Julien et al., 2005）。その腺組織の内部（腺腔）に絹タンパク質が分泌される。絹糸腺の研究といえば，絹タンパク質であるフィブロインや，フィブロインを被う糊としての役割を持つタンパク質，セリシンに関する研究が中心で，カイコ絹糸腺は

これまでに絹タンパク質の遺伝子発現の分子生物学あるいは絹タンパク質の高分子化学や高分子繊維としての物性研究としての研究対象であった（Fedic et al., 2002）。

カイコの吐糸生理に関する研究は，腺腔に貯留されている液状絹の物性や物理化学的性質に関する調査に基づいて，かなりの推測を交えた上でこれまでに議論されてきている。しかし，細胞生理学・分子生理学的な視点から絹糸腺を眺めたとき，カイコはどのようなメカニズムで盛食期に，腺腔内に絹タンパク質を高濃度で一旦貯留するのであろうか。絹糸腺が極大まで肥大した時点で，確かに腺腔内の絹タンパク質溶液（液状絹）はゲル化している。そのゲル様態の液状絹が，吐糸期においてはいかにしてゾル化し，円滑に吐き出され，カイコは繭を造るのであろうか。幼虫期のクライマックスにおけるダイナミックな営繭行動の下支えをしている，絹糸腺の pH 調節や水分（浸透圧）調節の実体解明へのアプローチはこれまで充分になされてきたとはいえなかった。

絹糸腺細胞で合成され，腺腔内へ分泌されたフィブロインやセリシンの溶液は，カイコが変態期をむかえて吐糸を開始するまでは，変性することなく20～30％にも達する高濃度タンパク質溶液（液状絹：liquid silk）として，とりわけ中部絹糸腺の腺腔内において莫大な量が貯留される（Jin and Kaplan, 2003）。液状絹の物性研究から，液状絹は吐糸開始まで一時的に貯留されている腺腔内において脱水が進行するといわれている（馬越・馬越, 1996）。しかし，どのような生理生化学的過程が進行するのかについては明らかではない。また，フィブロイン溶液を用いた in vitro での実験から，フィブロインのゲル—ゾル転移は，pH 依存的に起こることが証明されている（Ayub et al., 1993）。フィブロインやセリシンのタンパク質としての性質を考えると，腺腔内の液状絹の pH や水分を適正なレベルに維持する機構が存在することによって，そのような高濃度タンパク質溶液の生体内での安定した分泌や貯蔵が可能になり，そこでの液状絹のゲル—ゾル転移も可逆的に進行するものと考えられる。

そこで，そのような高濃度タンパク質溶液の物理化学的性質の維持には水分管理・浸透圧調節が必要であり，腺腔内の液状絹の pH 調節も厳密に維持されているに違いないとの作業仮説のもと，絹糸腺の能動輸送系による pH 調節の研究から着手した（**Chapter 1**）。絹糸腺細胞にはプロトンを能動輸送する V-ATPase が腺腔側原形質膜に見い出されている（Azuma and Ohta, 1998; Azuma et al., 2001）。幼虫が活発にエサを食べて成長し，絹糸腺は肥大成長を続け（Fig. 1E），その腺腔内に絹タンパク質を吐糸開始するまで蓄える。高濃度ゲル状態の絹タンパク質の pH の実態と V-ATPase の分布については，別の絹糸昆虫（野蚕）であるエリサンの絹糸腺についても調査した（**Chapter 1**）。さらに，絹糸腺細胞

の水分調節の細胞生理機能については，カイコ AQP をクローン化（Chapter 2）した後に，Chapter 3 において追究した。

## 4．昆虫の水分・浸透圧調節に関わるアクアポリン研究はヨコバイから始まった

昆虫は種類によって植食性や吸血性など食べるものが異なるので，食下物（植物汁液・植物葉・動物血液など）が引き金となった体内へ流入する各種イオンや浸透圧の調節は，消化・吸収・排泄における水代謝の問題として1950年代から研究されてきた（Maddrell, 2004参照）。脊椎動物と比較するとシンプルなデザインである昆虫においても，乾燥を防ぎ，恒常性維持のために上皮系組織を介した溶質輸送（fluid secretion）が活発である。水専用の通過路である AQP の機能について詳細な研究が昆虫においてもなされるようになったのは最近10年くらいのことである（東，2005）。本研究で絹糸腺細胞の水分調節機能を究明するにあたって，AQP 遺伝子の探索と同定は避けて通れない課題である。カイコ AQP の遺伝子クローニングと幼虫体内での組織特異的な遺伝子発現を Chapter 2 で取り上げる。

昆虫で最初に AQP が遺伝子としてクローンが得られ，それが水輸送機能を有することを証明したのは，フランス・レンヌ大学の Hubert らによるオオヨコバイ（Cicadella viridis）の腸管を用いた1996年のリポートであった（Le Caherec et al., 1996）。このヨコバイ（Fig. 2A）は半翅目（Hemiptera）という昆虫の大きなグループのうち，セミやウンカと同じ仲間で同翅亜目（Homoptera）に属する。オオヨコバイは自身の体液と比べてはるかに低張で栄養価の低い植物の木部道管液（僅かに糖・アミノ酸やミネラルを含む）をもっぱら吸汁し，水だけをバイパスさせて速やかに腸管の末端へ濾し取る装置（ろ過室：Filter Chamber）を有するのが特徴である（Fig. 2B）。食道直下の腸管前端部に位置する Filter Chamber 内部で腸管前端と腸管末端が密着した構造になっており，大部分が水である吸汁液は腸管末端（こちらの方が高張液となっている）へ向かって，浸透圧にしたがった水の流れが生じる。その結果，濃縮された液体として腸管内容物が腸管本体（midgut：中腸）へ移行し，糖やアミノ酸を効率よく吸収することが可能になる。

オオヨコバイの Filter Chamber には，MIP (Major Intrinsic Protein) ファミリーに属する疎水性の高い膜タンパク質 (25 kDa hydrophobic polypeptide) が大量に存在することが分かっていた（Fig. 2C）。そこで MIP ファミリーに保存された NPA モチーフ（Asn-Pro-Ala）のアミノ酸配列情報を利用して，Filter Chamber の mRNA から MIP ファミリーに相同性のある cDNA がクローン化された。このクローンはまさに25 kDa hydrophobic

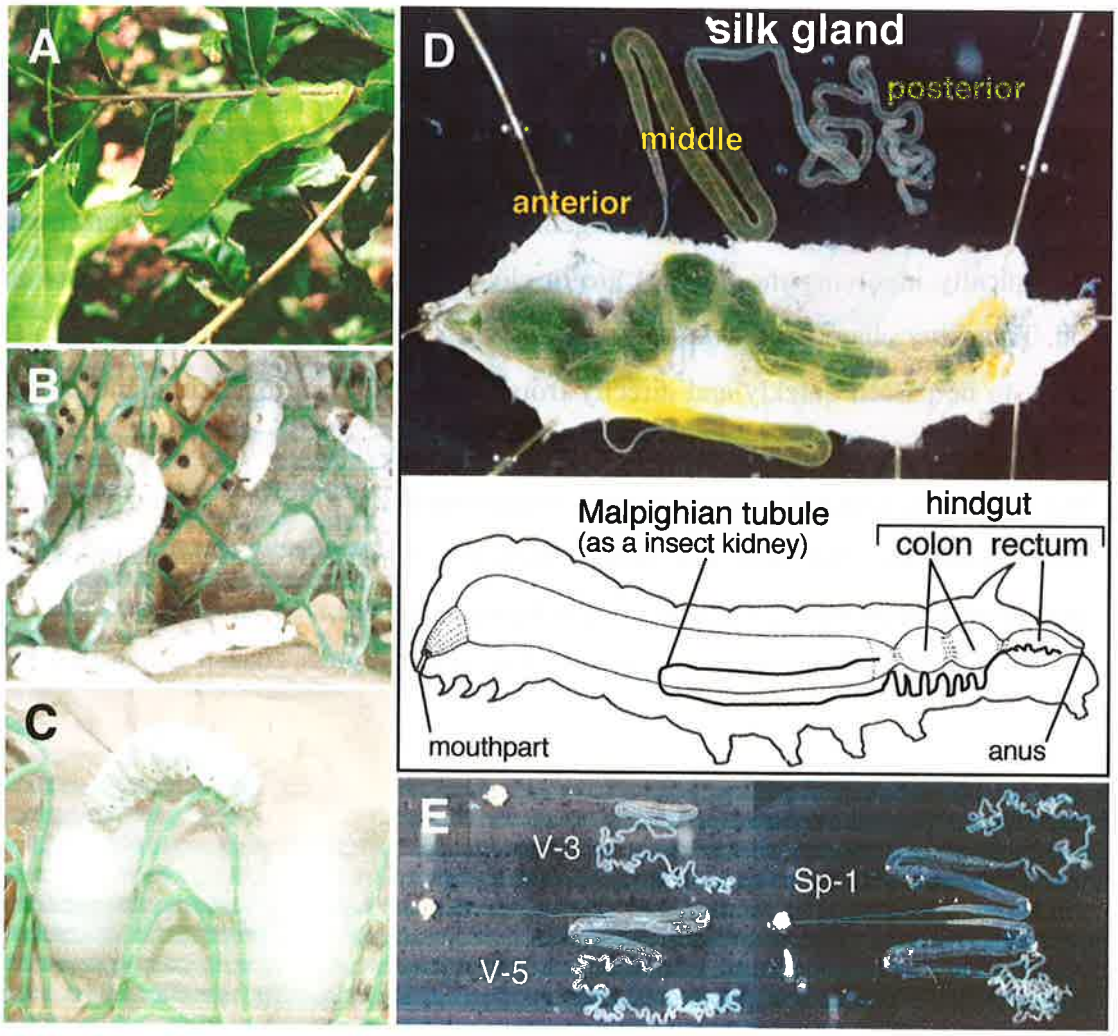
polypeptideに相当し，ヒトの AQP 1 や AQP 4 （Fig. 19A）とアミノ酸配列で43％のホモロジーを有するタンパク質をコードしていた。体長 1 cm程度のオオヨコバイの体内に，その数倍の長さの腸管（大部分は中腸）がコンパクトに収まっているが，その腸管の Filter Chamber の領域でのみ，AQP の mRNA 発現が確認された。

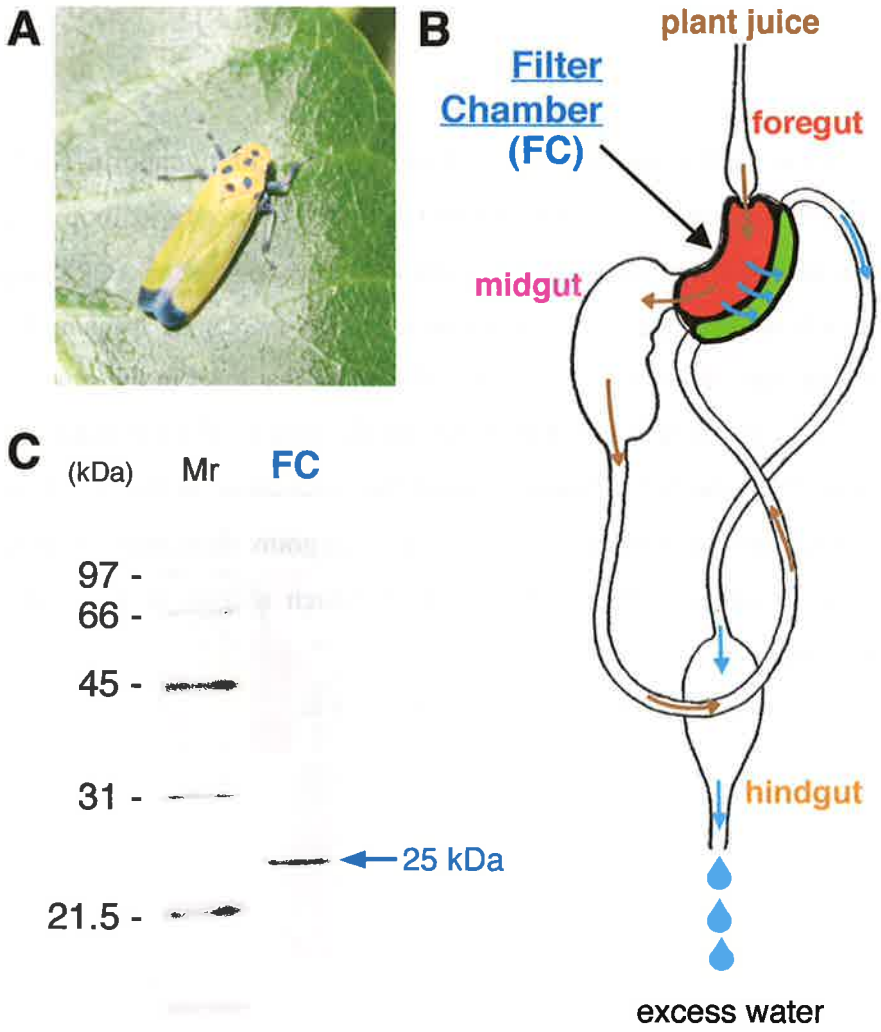
アクアポリンの分子構造はヒト赤血球から大量に得られる AQP 1 で電子線結晶構造解析により初めて明らかにされ，全てに共通の立体構造であると考えられている。それは，6本の膜貫通ヘリックス以外に，保存された Loop B と Loop E に位置する NPA モチーフを有する細胞外ループ（２番目と５番目）が膜の両側から入り込み，それらの凸と凹が真ん中で最狭部を形成している（Fig. 3A）。AQP は細胞膜を挟んで内と外から対称であり，中心部分が狭く，ちょうど砂時計の形に似ていることから，アクアポリンフォールドと呼ばれる構造をとる（Fig. 3B）（藤吉，2005）。この最狭部の孔の径（3.0 Å）が水分子の径（2.8 Å）に近いことが，水に対する高い選択性を規定している。さらに NPA モチーフの２つのアラニンが水通過路に沿って並び，水のみの通過，そして $H^+$ の非通過を決定している（Fig. 3C）。**Chapter 2** においては，この普遍的構造に基づいたホモロジークローニングによって，カイコ幼虫より AQP ホモログを２種類（AQP-Bom1 および AQP-Bom2）をクローン化し，さらにこれらのカイコ AQP（Bommo AQP）について組織特異的な発現を調査した。AQP-Bom1 が絹糸腺で発現していることを確認することができたので，**Chapter 3** では，再度，絹糸腺組織で，AQP の発現領域を組織・細胞レベルで特定し，カイコの糸作りにおける AQP の生理的役割について考察した。

Fig. 1. Lepidopteran larvae and its general anatomy. (A) *Antheraea yamamai*. (B) *Bombyx mori*. (C) *Samia cynthia ricini*. (D) Upper panel; dissection of *B. mori*. Digestive tract and a pair of silk glands occupied the haemocoel. Lower panel; Schematic diagram of arrangement of alimentary canal (midgut) and excretory system (hindgut and Malpighian tubule) of the left side of *B. mori*. (E) Growth of the silk gland during the final (fifth) instar stadium. V-3: Day 3 larvae, V-5: Day 5 larvae, Sp-1: Day 8 larvae at the spinning phase.

Fig. 2. Digestive tract of hemiptera. (A) Leafhopper, *Bothrogonia japonica*, related species of *Cicadella viridis,* in Japan. (B) An arrangement of the gut. Anterior and posterior parts of the gut (typically involving the midgut) are in close contact to allow concentrate of the liquid food. This filter chamber allows excess water and relatively small molecules such as simple sugars, to be passed quickly and directly from the anterior gut to the hindgut, thereby short-circuiting the main absorptive portion of the midgut. (C) Western blotting of filter chamber. Filter chambers from *B. japonica* were fractionated by SDS-PAGE and transferred onto PVDF membrane. The blot was treated with anti-*C. viridis* aquaporin antibody. Positive signal corresponding to a 25 kDa was reproducibly obtained.

(BはKlowden, M. J., Physiological Systems in Insects, Academic press, 2002, p. 243の図をもとに作成)


A
B
C
D
silk gland
posterior
middle
anterior
Malpighian tubule
(as a insect kidney)
hindgut
colon rectum
mouthpart
anus
E
V-3
Sp-1
V-5


A
B
plant juice
Filter
Chamber
(FC)
foregut
midgut
hindgut
excess water
C
(kDa)
Mr
FC
97 -
66 -
45 -
31 -
25 kDa
21.5 -

# Chapter 1

# Acidification of the silk gland lumen in *Bombyx mori* and *Samia cynthia ricini* and localization of $H^+$-translocating vacuolar-type ATPase

（絹糸昆虫の成長発達過程における液状絹の酸性化
—液状絹pHの推定と絹糸腺細胞のV-ATPaseの分布—）

## Summary

The silk gland of *Bombyx mori* and *Samia cynthia ricini* produces vast amounts of silk proteins and stores them in the glandular lumen as a liquid silk during the larval growth and development. We have explored the system regulating pH in the silk gland, because the gelation of fibroin is pH-dependent. By injecting the pH-sensitive dye (phenol red) into silkworm larvae, we have estimated the pH in the glandular lumen. Although the entry of dye was unsuccessful in the anterior silk gland (ASG) of *Bombyx*, the lumen of the middle silk gland (MSG: major reservoir for fibroin) and that of the posterior silk gland (PSG: the fibroin factory) were colored with phenol red. The coloration by phenol red indicated that the MSG was acidic (pH 5~6) in the vigorously feeding larvae leading to gelation of silk proteins at the MSG and that the PSG was neutral (pH 7~8). When the larvae started spinning, the lumen in the MSG became neutral. A similar pattern in the luminal pH shift was obtained in the silk gland of *Samia cynthia ricini* (Eri-silkworm) with a dye-injection experiment. In *Samia*, the $H^+$-translocating vacuolar-type ATPase (V-ATPase) locates at the apical surface of PSG, where the fibroin is produced, secreted and temporarily stored. The V-ATPase was also distributed at the apical surface of the anterior MSG as well as that in ASG. These V-ATPases became undetectable after the onset of spinning. The V-ATPase at the plasma membrane of silk gland cells regulates the physico-chemical state of liquid silk in the glandular lumen, in particular at the MSG of *Bombyx* and at the PSG of *Samia,* respectively.

**Key words:** liquid silk, acidification, $H^+$-translocating vacuolar-type ATPase, silk gland, *Bombyx mori, Samia cynthia ricini*

# INTRODUCTION

The caterpillars are characterized by the gluttonous feeding at the final stadium of their larval life, increasing their body weight and mass (Reynolds *et al.*, 1985). At that growing phase, the explosive development of the silk gland occurs in the cocoon-producing lepidopteran insects such as a silkworm, *Bombyx mori*. The gland is a protein factory specialized in fibroin production at the posterior silk gland (PSG), where the fibroin is synthesized, secreted and temporarily stored in the glandular lumen as a liquid silk (Prudhomme *et al.*, 1985; Fedic *et al.*, 2002; Julien *et al.*, 2005). In particular, the middle silk gland (MSG) in *Bombyx mori* greatly increases its tissue mass at the vigorously feeding phase of the fifth instar larvae and functions as a huge reservoir for the liquid silk in addition to the production of sericins. The anterior silk gland (ASG) is a conduit for the liquid silk from the MSG to the spinneret at the mouthpart (Akai, 1998). It is believed that the liquid silk with unusually high content of proteins (> 20%) has to be maintained within the glandular lumen without any irreversible coagulation and/or denaturation before spinning and that it does not flow easily out of the spinneret in vigorously feeding larvae due to the physico-chemical nature of fibroin *in vivo*.

The pH and water homeostasis in the silk gland is crucial, since the sol-gel transition point of fibroin solution lies at pH 5.0-5.5 in an *in vitro* study (Ayub *et al.*, 1993). The pH in the haemolymph of *Bombyx* larvae is usually in between 6.5 and 7.0, suggesting that there exists the active acidification mechanism in the silk gland as that in the lepidopteran midgut alkalinization (Dow, 1992; Azuma *et al.*, 1995). A vacuolar-type ATPase (V-ATPase) is an ubiquitous $H^+$-translocating ATPase typically residing in the plasma membrane of many insect epithelia (Harvey *et al.*, 1998; Wieczorek *et al.*, 2000; Beyenbach and Wieczorek, 2006). The diversity in cellular functions of the V-ATPase is attributed to its properties of generating both electrochemical and pH gradients across the plasma membrane. It has been suggested that the

plasma membrane V-ATPase plays an important role in the luminal acidification of the silk gland of *Bombyx* (Azuma and Ohta, 1998; Azuma *et al.*, 2001). The pH gradients established by the V-ATPase across the apical plasma membrane would contribute to the gelation of fibroin *in vivo*, increasing the viscosity of the liquid silk and plugging it in the narrow canal structure of ASG. Since this V-ATPase at ASG disappears in spinning larvae, we have speculated that the pH shift is neutral in the lumen of ASG, causing the liquid silk to become mobile and resulting in an easy flowage at the spinneret for making a cocoon (Azuma and Ohta, 1998). In the MSG, the distribution of V-ATPase was rather limited along the length of the silk gland, clearly lacking at the anterior division of MSG and mainly existing at the posterior division of MSG. This V-ATPase at MSG seems to be important for the deposition of liquid silk in the lumen (Azuma *et al.*, 2001).

The present report aims to demonstrate the above speculation on the physiology of V-ATPase in the silk gland more directly using the pH-sensitive dye, phenol red. Using not only *Bombyx* larvae but also another silkworm, *Samia cynthia ricini* (Eri-silkworm), we have observed the occurrence of acidification and neutralization in the silk gland as a whole during its development. Additionally, we have explored the silk gland V-ATPase in *Samia*, which distributed along the length of the gland at the feeding stage and disappeared at the spinning phase.

# MATERIALS AND METHODS

## Insects

Hybrid races (Shunrei x Shogetsu and Kinshu x Showa) of the silkworm, *Bombyx mori*, were reared on fresh mulberry leaves or an artificial diet (Aseptic Sericulture System Lab., Kyoto, Japan) at 24-26 °C. The final (fifth) larval instar stadium of *Bombyx* continues 7 days for feeding, followed by 3 days of spinning for cocoon production. Eri-silkworms (*Samia cynthia ricini*) were reared on an artificial diet (Silkmate L4M, Nosan Corporation, Japan) at 26-28 °C. The final (fifth) larval instar stadium continues 6 days for feeding, followed by 2 days of spinning. For the immunocytochemistry of both species, larvae were studied at the active feeding phase (*Bombyx*: 5-day-old fifth instar larvae, *Samia*: 3- or 4-day-old fifth instar larvae) and at the spinning stage (1 day after the onset of spinning). At this developmental point, in *Bombyx*, just after the gut purge, we could observe the spinning behavior through a thin cocoon layer, while the spinning larva of *Samia* could not be seen inside a cocoon layer.

## Injection of pH-sensitive dye into the living larvae

Silkworms of both sexes at the several stages of development in fifth instar larvae were anesthetized on ice for 10 min, then 5 mM phenol red in 5 mM Mops (3-morpholinopropane sulfonic acid)-Tris buffer (pH 7.0) was injected into the haemocoel at various stages during the larval development. The volumes of injection (50-200 $\mu$l) depends on the body size of larvae. The fully matured larvae of *Samia* accepted with 300 $\mu$l injection. Maximun staining in the gland by the dye was normally obtained when the larvae were sacrificed at 2-3 h after the dye-injection. Checking a normal feeding or spinning behavior of each larva, a pair of silk glands was dissected from the dye-injected larvae carefully, rinsed quickly with the phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4), and photographed as soon as possible. The accumulated dye in the lumen of the silk gland was diffused and disappeared in the PBS in most cases within 3 h.

Comparing with the pH standards prepared with 0.1 M Mops-Tris (pH 6.0-8.5) and 0.1 M Mes (2-morpholinoethane sulfonic acid, monohydrate)-Tris (pH 4.0-6.0), we obtained an approximation of the pH inside the silk gland. Under these buffers, phenol red was yellow below pH 6, upon neutralization, gradually changed to a variable yellow/orange color in between and then red above pH ~ 7.5 (Fig. 4B). For example, when we measured by the pH meter, the pH value of haemolymph from dye-injected larvae corresponded well with the titration shown in Figure 4B (pH ~ 6.7). The dye was also accumulated in the midgut lumen

(pH > 8.2, out of range for phenol red), indicating the higher alkalinity in the midgut (Dow, 1992; also see Fig. 5B in the **RESULT**). Thus, we considered that the visual transition interval of phenol red was not influenced in living tissues and that an approximation of the pH was reliable. Since the liquid silk was quite sticky on the surface of the pH electrode, the precise handling of the pH meter was impossible.

Injection of the dye did not cause any artificial effects in both silkworms. The dye-injected larvae produced the yellow cocoons with a normal shape (data not shown), suggesting that phenol red is permeable into the lumen of the silk gland of both species.

### Immunocytochemistry of V-ATPase

The silk gland used for immunocytochemistry were dissected and fixed for 5-6 h in Bouin's fixative as described previously (Azuma and Ohta, 1998; Azuma *et al.*, 2001). The specimens studied were referred to as depicted in the respective figures of the text. The tissue was then dehydrated through a graded ethanol series, cleared and embedded in paraffin (HISTOSEC®, Histologie, MERCK, Darmstadt, Germany). Sections (~5 $\mu$m) were placed on clean glass slides coated with 3-aminopropyltriethoxysilane and allowed to dry at 40 °C overnight. Sections were then deparaffinized in xylene and rehydrated through an ethanol series into PBS. Sections were incubated at room temperature in 10% normal goat serum in PBS for 2-3 h and then with a primary antibody at 4 °C overnight. Rabbit antiserum against the V-ATPase holoenzyme from *Manduca sexta* midgut (Wieczorek *et al.*, 1991) was used for all immunological experiments (usually 1:10,000 dilution). After four rinses (10 min each) in PBS, sections were incubated with a goat anti-rabbit biotin-conjugated antibody (Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 2-3 h, rinsed four times in PBS and incubated with an avidin-biotinylated peroxidase complex (ABC) reagent (Vector Laboratories, Inc.). Sections were rinsed four times in PBS and incubated with 3,3'-diaminobenzidine (DAB) /$H_2O_2$ until sufficient color development was attained (usually 5-10 min at room temperature). Control incubations were also done on adjacent tissue sections and included substituting a non-immune rabbit serum (1:2,000 dilution) for the primary antibody.

In *Samia*, using the membrane fraction from PSG extracts as well as the MSG extracts on the western blotting test as an antigen, the specificity of V-ATPase antiserum failed to check due to the presence of liquid silk. The membranes from ASG showed the similar pattern to *Bombyx* ASG (data not shown).

# RESULTS

## Direct observation of the luminal acidification in the silk gland of *Bombyx mori* with phenol red

Among the several pH-sensitive dyes tested, the significant and reliable intensity of staining in a pair of the silk glands was obtained by injecting phenol red (visual transition interval : pH 6-8) to the 5th instar feeding larvae of *Bombyx*. The hybrid strain used in this experiment has an usual whitish color in the gland. The epithelia of the silk gland did not stained but the liquid silk itself as a gel-like state was colored with this dye, suggesting that the dye penetrated into the glandular lumen. The silk gland from active feeding larvae (5-day-old fifth instar) exhibited yellow staining at the middle and posterior division of MSG (Fig. 4A). The most posterior region of MSG showed a gradient of increasing orange staining, then became rose in the PSG. As judged from the pH standards of phenol red (Fig. 4B), the lumen in the MSG was acidic (pH 5-6). The glandular lumen rose above the pH ~ 7 transition point of this dye at the junction between MSG and PSG. Then, it was shifted to rather neutral (pH > 7) in the lumen of PSG. The injection of bromphenol blue (visual transition interval : pH 3.5-4.5) made the MSG and PSG deep blue color indicating a pH > 4.5 (data not shown).

At the onset of spinning, the middle division of MSG became slightly orange indicating the luminal neutralization to pH > 6 (Fig. 4C). Thereafter, the posterior division of MSG gradually increased the reddish zone proximally towards the middle MSG (Fig. 4D, 4E), indicating that the lumen rose above the pH ~ 7 during the most active spinning period. When the larvae almost finished spinning (3 days after the onset of spinning), the middle MSG exhibited crimson staining in the lumen. The PSG did not accept the dye at this stage (Fig. 4F). There were no considerable changes in staining at the PSG until the spinning was completed. Also, phenol red did not provide any visible coloration in the ASG and the anterior division of MSG.

## Direct observation of the luminal acidification in the silk gland of *Samia cynthia ricini* with phenol red

We applied the dye-injection experiment to another silkworm, *Samia cynthia ricini*. The general anatomy of the silk gland in *Samia* is characterized by zigzag-shaped MSG and PSG with a long fine duct of ASG (Fig. 5A). Not only MSG but PSG of *Samia* became yellow when the phenol red was injected to a feeding larva (3-day-old fifth instar) indicating the lumen of both parts of the gland at pH 5-6 (Fig. 5B). The most anterior part of the midgut was stained by the dye, showing pH > 8 (out of range of phenol red) due to the alkalinity of gut lumen (Dow, 1992). In a spinning larva (7-day-old fifth instar), the MSG and PSG became red or crimson with the dye-injection. The color intensity was more prominent than that in *Bombyx*, indicating pH ~ 8 at the *Samia* PSG (Fig. 5C). When we injected phenol red at the first day of fifth instar larva, the lumen of *Samia* ASG showed yellowish color, indicating the lumen at pH 5-6 (Fig. 5D). This was exceptionally common at this stage of *Samia*. The fifth instar larvae of older stages did not show a considerable staining at the lumen of ASG (data not shown). The observation in MSG and PSG of *Samia* exhibited the similar staining pattern to that in *Bombyx* (Fig. 4) during the gland development. The acidification seems to have occurred at the glandular lumen where the liquid silk is stored during the feeding phase, although the majority of liquid silk accumulates at the different region of the gland in each silkworm (Akai, 1998), that is, at the MSG in *Bombyx* and at the PSG in *Samia*, respectively.

## Immunocytochemistry of V-ATPase along the length of the silk gland of *Samia cynthia ricini*

The dye-injection studies described above strongly suggests the presence of the plasma membrane V-ATPase which pumps the proton to the glandular lumen for acidification as suggested in *Bombyx* silk gland (Azuma and Ohta, 1998; Azuma *et al.*, 2001). By using the

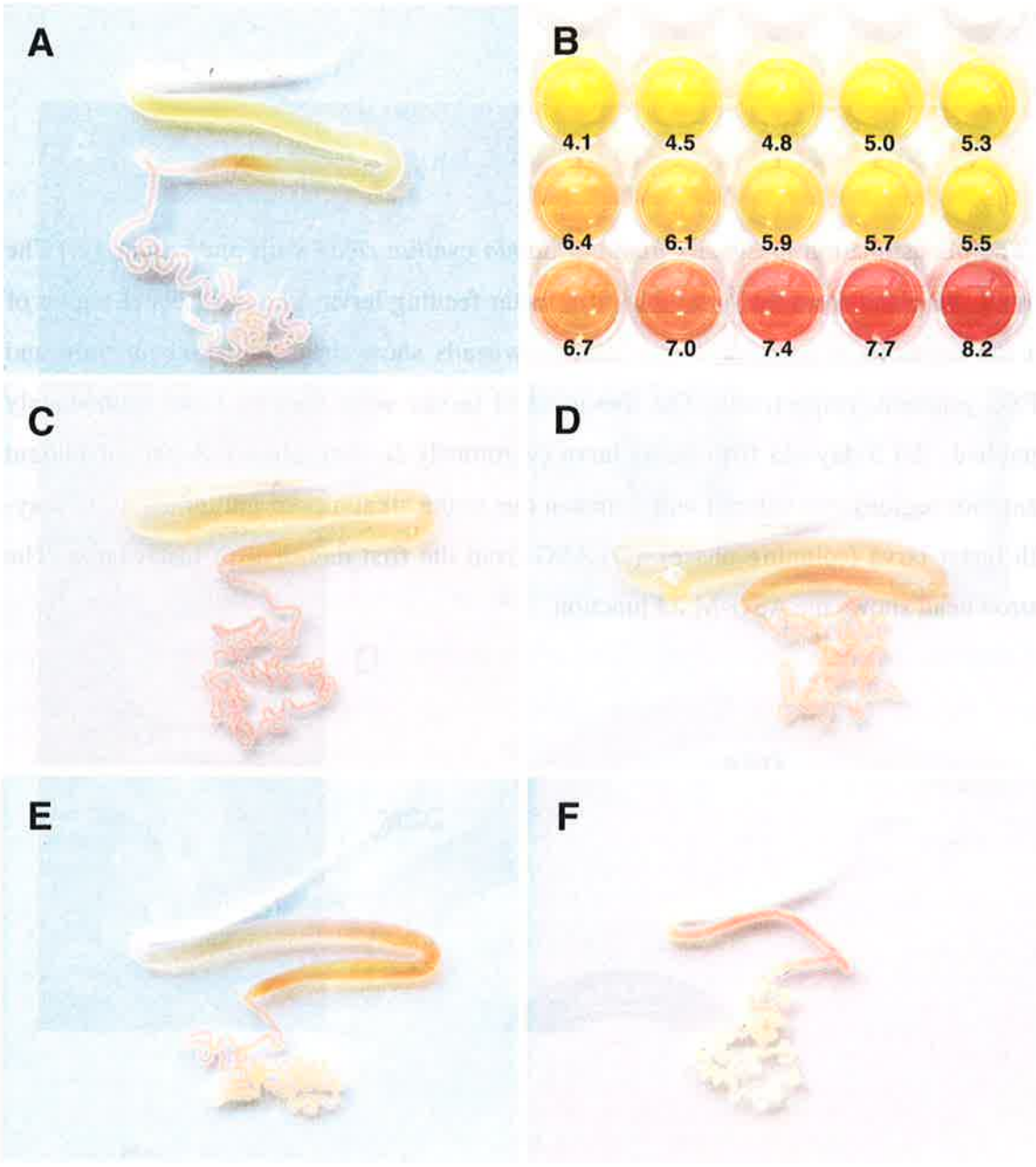
*Manduca sexta* V-ATPase antibody, we surveyed the distribution of V-ATPase in the *Samia* silk gland. In *Samia*, observing histologically, the liquid silk (almost fibroin) is temporarily stored in the lumen of PSG (Akai, 1998), where the fibroin is produced. The significant staining of V-ATPase was detected at the apical surface of the PSG cell (Fig. 6A). The control showed no significant staining at the apical surface (Fig. 6B). This was different from the *Bombyx* PSG, which showed no V-ATPase immunostaining (Azuma *et al.*, 2001). Further, the apical immunolabelling disappeared from the PSG cell of the spinning larvae (Fig. 6C). This explains that the PSG cell ceased to acidify the lumen after the onset of spinning. The apical distribution of V-ATPase in a feeding larva became undetectable at PSG-MSG junction (Fig. 6D). The weak but significant immunoreaction in the cytoplasmic space in PSG (Fig. 6A, 6C) may be due to the presence of the endomembrane type of V-ATPase such as secretory vesicles or central vacuolar systems (Futai *et al.*, 1998; 2000).

We checked the V-ATPase distribution in other parts of *Samia* silk gland. The posterior region of MSG showed no considerable staining (Fig. 7A), confirming the immunocytochemistry shown in Figure 6D. Although the tissue mass of MSG is homogeneous throughout the MSG (see Fig. 5A), the specific immunolabelling reappeared at the apical surface of the anterior region of MSG (Fig. 7B, 7C). This is a great difference from the anterior division of *Bombyx* MSG where the V-ATPase is absent (Azuma and Ohta, 1998; Azuma *et al.*, 2001). Since the *Samia* MSG is not the main reservoir site for fibroin deposition, a part of MSG near ASG may have a similar function to the *Bombyx* ASG. The *Samia* ASG also showed the apical immunolabelling for V-ATPase (Fig. 7D). At the spinning stage of the gland, the positive immunolabelling at the apical surface disappeared from the anterior MSG and ASG (Fig. 7E, 7F). The cytoplasmic region of ASG was stained significantly (Fig. 7F). This may be due to the occurrence of the endomembrane-type V-ATPase such as autophagic vacuoles. Metamorphic changes in V-ATPase at the ASG and the

anterior MSG of *Samia* showed a similar pattern to those in *Bombyx* ASG (Azuma and Ohta, 1998).

We failed to detect the clear disappearance of V-ATPase in the posterior division of *Bombyx* MSG. The apical immunoreaction actually became weak but the precise detection was technically difficult, because the MSG cell in thickness became thinner, sections were always cracked at the apical regions, and the cytoplasmic area increased the immunostaining of the endomembrane-type V-ATPase. It was microscopically difficult to discriminate the positive signal at the apical surface from that at the cytoplasm (data not shown).

Fig. 4. The pH changes in the silk gland of *Bombyx mori* with phenol red during metamorphosis. The silk gland of the fifth instar larvae is characterized by the thick S-shaped MSG and the convoluted PSG. Phenol red as a pH-sensitive dye was injected into the fifth instar larvae at various physiological stages. (A) 5-day-old fifth instar larvae (vigorously feeding phase). The most posterior division of MSG shows a variable yellow/red color. (B) pH standards obtained with 5 mM phenol red. The number indicates the pH value of each solution prepared by 0.1 M Mops-Tris or 0.1 M Mes-Tris buffer system. (C) The silk gland at the onset of spinning. The neutralization (pH > 6) starts in the MSG. (D) 1 day after the onset of spinning (after the gut purge). (E) 2 days after the onset of spinning. (F) 3 days after the onset of spinning. Note that the dye did not penetrate into the PSG at this stage. The ASG and the anterior division of MSG showed no coloration with the dye in all developmental stages examined.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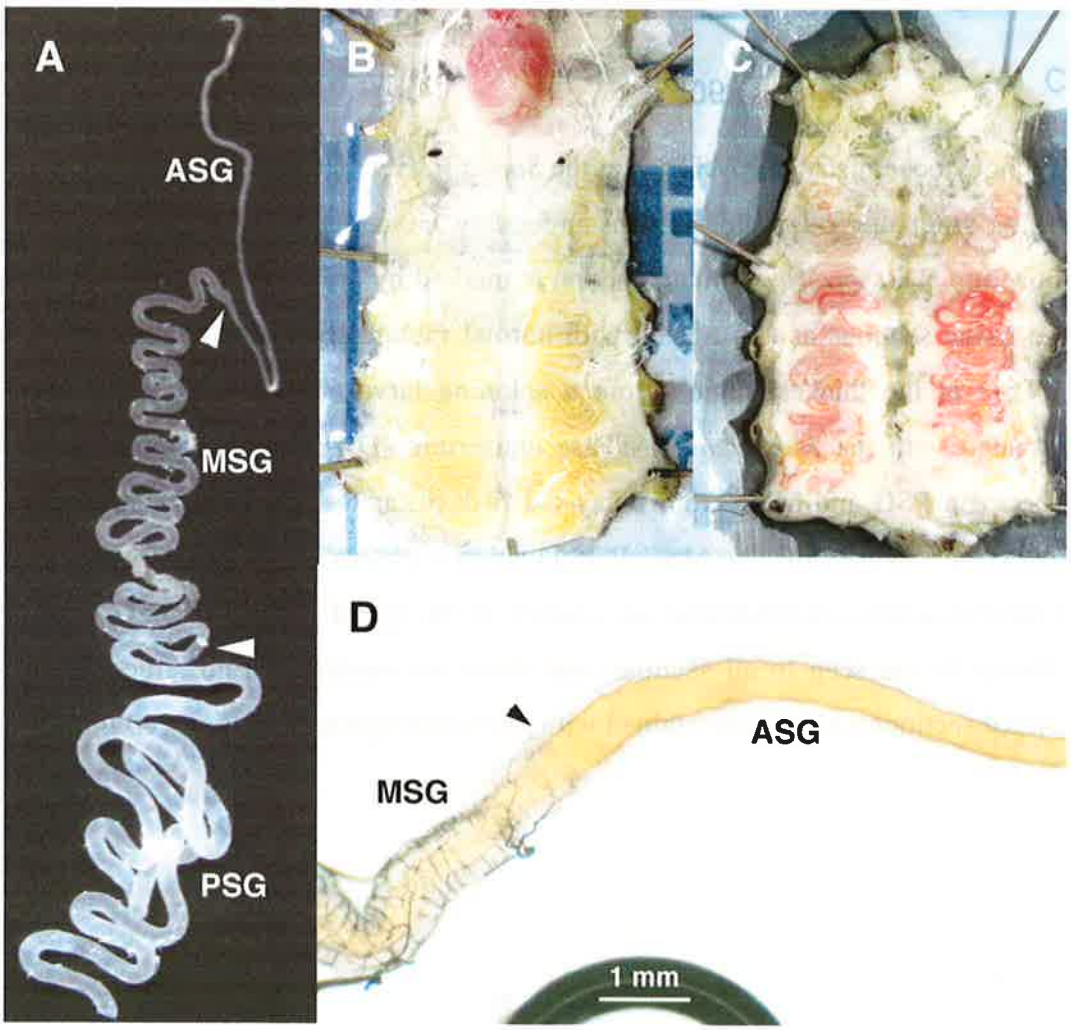
6.1
5.9
5.7
5.5
6.7
7.0
7.4
7.7
8.2
C
D
E
F

Fig. 5. The pH estimation in the silk gland of *Samia cynthia ricini* with phenol red. (A) The anatomy of the gland from the 4-day-old fifth instar feeding larva. The most distal region of PSG at the terminus is omitted. The white arrowheads show the ASG-MSG junction and MSG-PSG junction, respectively. The dye-injected larvae were dissected and immediately photographed. (B) 3-day-old fifth instar larva (vigorously feeding phase). A part of midgut (most anterior region) was colored with crimson due to the alkalinity of gut lumen. (C) 7-day-old fifth instar larva (spinning phase). (D) ASG from the first day of fifth instar larva. The black arrowhead shows the ASG-MSG junction.


A
ASG
MSG
PSG
B
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D
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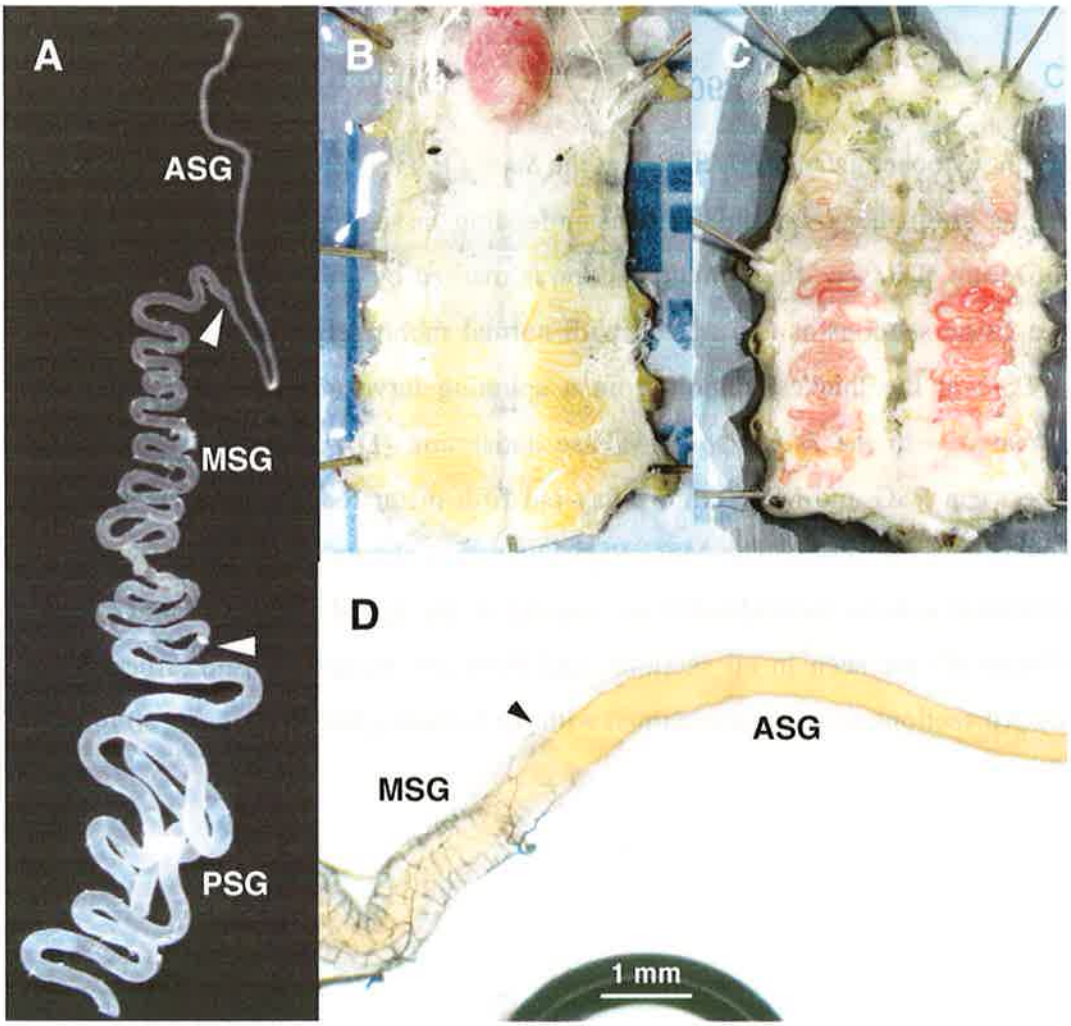
MSG
1 mm


A
ASG
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MSG
1 mm

Fig. 6. Immunocytochemistry of V-ATPase at the *Samia* PSG. (A) Cross section of PSG at the thickest region from the 4-day-old fifth instar feeding larva treated with the *Manduca* V-ATPase antiserum. The specific immunoreaction is marked by arrowheads. (B) Cross section of the same tissue sections as (A) treated with normal rabbit serum as a control. (C) Cross section of PSG at the thickest region from a spinning larva (one day after the onset of spinning) treated with the *Manduca* V-ATPase antiserum. (D) Longitudinal section at the boundary between PSG and MSG from 4-day-old fifth instar feeding larva treated with the *Manduca* V-ATPase antiserum. The MSG-PSG junction is shown by the arrow. Note that the specific immunoreactions (arrowheads) are limited at the apical surface of PSG cells. The secreted fibroin (f) are seen in all sections, and there are numerous void structures in the lumen (Lu). All sections were counterstained with the hematoxylin. Scale bar, 100 $\mu$m.

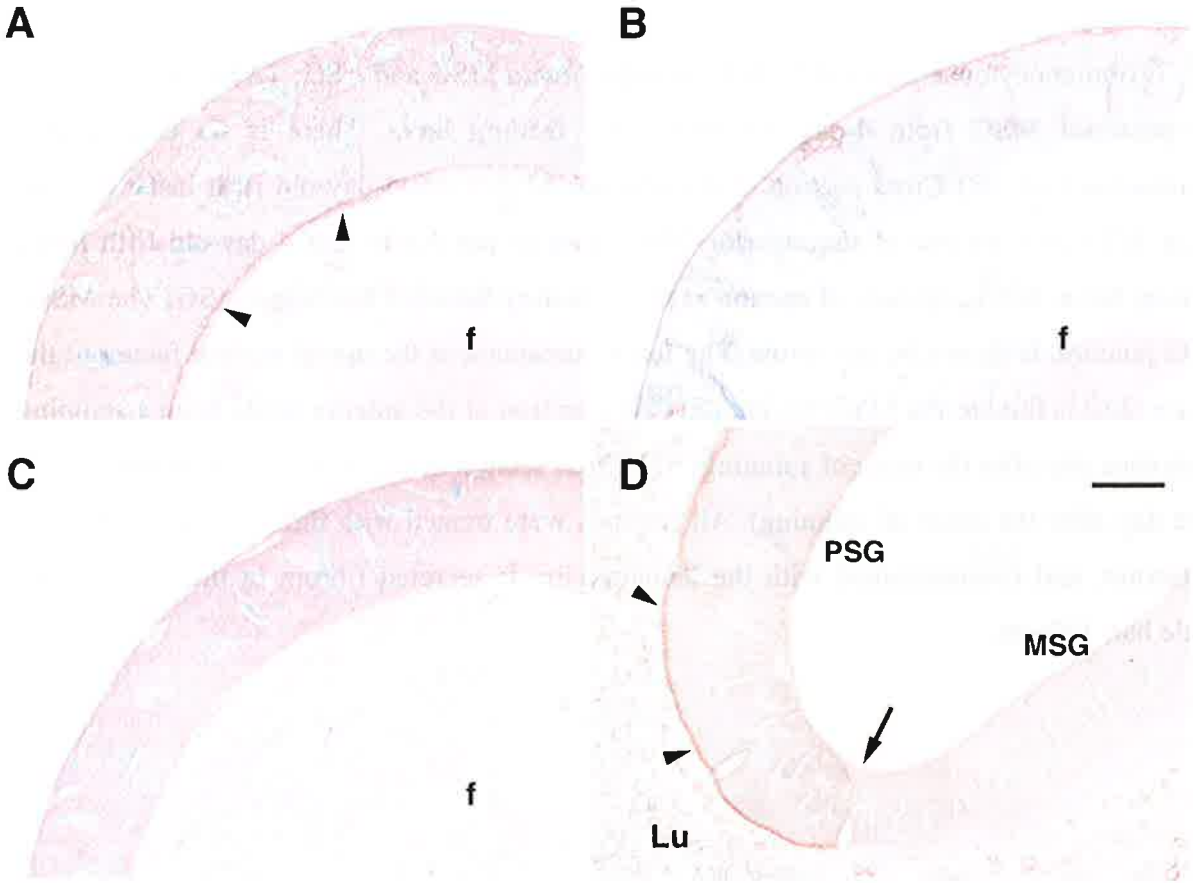
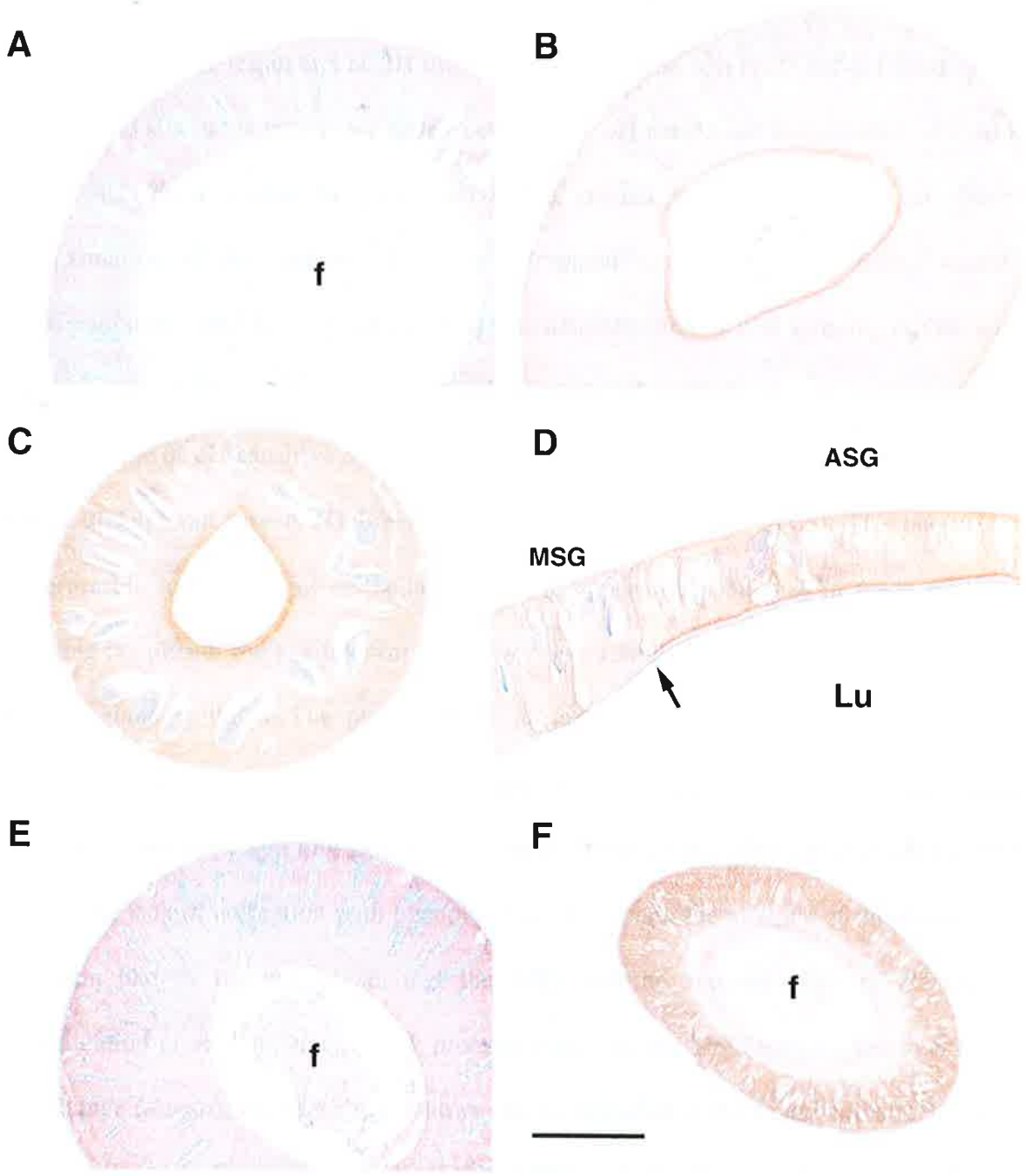

A
f
B
f
C
f
D
PSG
MSG
Lu

Fig. 7. Immunocytochemistry of V-ATPase at the *Samia* MSG and ASG. (A) Cross section of the posterior MSG from 4-day-old fifth instar feeding larva. There is no considerable immunoreaction. (B) Cross section of the anterior MSG from 4-day-old fifth instar feeding larva. (C) Cross section of the anterior MSG close to the ASG from 4-day-old fifth instar feeding larva. (D) Longitudinal section at the boundary between MSG and ASG. The MSG-ASG junction is shown by the arrow. The immunoreaction at the apical surface facing to the lumen (Lu) is faint at the MSG region. (E) Cross section of the anterior MSG from a spinning larva (one day after the onset of spinning). (F) Cross section of the ASG from a spinning larva (one day after the onset of spinning). All sections were treated with the *Manduca* V-ATPase antiserum, and counterstained with the hematoxylin. f: secreted fibroin in the lumen (Lu). Scale bar, 100 $\mu$m.


A
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## DISCUSSION

Fibroin is the major constituent of the liquid silk (Akai, 1984; 1998, Fedic *et al.*, 2002). During the period of fibroin production in *Bombyx* and *Samia*, the feeding larvae do not spin a cocoon but accumulate silk proteins in the glandular lumen as a liquid silk until the onset of spinning. Since the regulation of pH must be critical for the reversible sol-gel transformation of the liquid silk in the lumen, we have examined the pH nature and the presence of V-ATPase in the silk gland. Using the pH-sensitive dye, phenol red, we were able to obtain an approximation of the luminal pH of the silk gland *in vivo*. We have demonstrated the acidification of liquid silk in feeding larvae, the neutralization of it in spinning larvae, and the existence of V-ATPase for contributing to pH control in the silk gland.

The use of pH-sensitive dyes has made it possible to assess the pH profile in the midgut lumen of Dipteran insects (Dubreuil *et al.*, 1998; Corena *et al.*, 2005). The phenol red is impermeable to the midgut epithelia by feeding experiments of dyes in these studies. By injecting the phenol red to silkworm larvae, we have found that this dye is permeable through the silk gland epithelia. The phenol red is an anionic dye of sulfonates, which might be transported via a sulfonate transporter (Quinlan and O'Donnell, 1998) or nowadays called as an organic anion transporting polypeptide (oatp, Torrie *et al.*, 2004) if it exists in the silk gland. The lack of coloration with phenol red might explain the absence of this transporter in a certain part of the silk gland, e.g. the ASG and the anterior MSG of *Bombyx*. The accumulation of vast amounts of silk proteins occurs in the MSG lumen (*Bombyx*) or in the PSG lumen (*Samia*), where we have shown the acidification of the liquid silk at pH 5-6 (Figs. 1 and 2). The significant staining of MSG and PSG in both silkworms by phenol red must be due to the mechanism on such an organic anion transporter at the basal and/or apical plasma membrane of silk gland cells, though the precise mechanism of dye entry remains unknown.

The extent of acidification by V-ATPase is normally at pH value around 4 ~ 7 depending on the tissue physiology of living organisms (Harvey and Wieczorek, 1997; Harvey *et al.*, 1998; Wieczorek *et al.*, 1999). The plasma membrane V-ATPase in the posterior MSG of *Bombyx* (Azuma *et al.*, 2001) explains the weak acidic pH nature in the MSG (Fig. 1). These data obtained from *Bombyx* prompted us to explore the V-ATPase in another silkworm, *Samia*. The anatomy of *Samia* silk gland is characterized by the zigzag-shaped MSG and PSG, and the PSG of this group (wild silkmoths) increases its tissue mass for the deposition of fibroin (Akai, 1998), which is functionally similar to the *Bombyx* MSG. Such a histological feature in between *Samia* and *Bombyx* may reflect upon some differences of the V-ATPase distribution (Figs. 3 and 4). The V-ATPase immunoreaction at the PSG-MSG junction (Fig. 3D) was totally opposite to that at PSG-MSG junction of *Bombyx* (Fig. 2F in Azuma *et al.*, 2001). The anterior MSG of *Samia* showed the immunoreaction of V-ATPase, but that of *Bombyx* was absent (Azuma and Ohta, 1998). Regarding the apical V-ATPase at ASG, both silkworms showed the similar patterns. Taken together, the luminal acidification by the plasma membrane V-ATPase seems to be necessary for the accumulation of the liquid silk during the feeding period of silkworm larvae. At the spinning phase, the disappearance of the plasma membrane V-ATPase (Figs. 3 and 4) corresponded well with the pH inside the silk gland (Fig. 2). Since the spinning was completed within 2 days in *Samia*, rather shorter than *Bombyx*, the pH milieu would have to be shifted more quickly by switching off the V-ATPase function.

The acidification and alkalinization in a certain compartment of living organisms is a key factor for regulating the protein function in the tissue specific physiology (Wieczorek *et al.*, 1999). The V-ATPase is conceived to be the sole energizer in the silk gland like the midgut because the lepidopteran insect is a typical living system of the "non-sodium world" where the cell has to require the $H^+$-motive force instead of $Na^+$-motive force for the plasma

membrane energization (Harvey and Wieczorek, 1997). Consequently, it is concluded that the silk gland V-ATPase contributes to pH homeostasis in maintaining the liquid silk under the weak acid milieu at the lumen, leading to the reversible sol-gel transformation of the liquid silk, because the gelation of fibroin (Ayub *et al.*, 1993) and sericin (Zhu *et al.*, 1995) solution *in vitro* are pH-dependent. Not only the pH shift in this study, but also the water withdrawal and other modifications must be indispensable for the conversion of liquid silk into solid fiber. Water as well as pH regulation in the silk gland awaits further investigation.

# Chapter 2

# Molecular cloning, tissue and cellular characterization of two isoforms of aquaporin in silkworms, differently expressed in the hindgut and midgut of *Bombyx mori*

（カイコアクアポリンのcDNAクローニングとその遺伝子発現
―カイコ幼虫個体の水コントロールにおけるアクアポリンの組織特異的発現―

## Summary

Two different cDNAs encoding a homologue of aquaporin (AQP) were identified by a reverse transcription-polymerase chain reaction from the silkworm larva, *Bombyx mori.* The deduced amino acid sequence shares 40-60% homology with other insect AQP homologues. The first cDNA (AQP-Bom1) cloned from the anterior silk gland encodes a 25 900 Da protein homologous to insect AQPs isolated from the liquid-feeding insect of *Aedes aegypti, Anophelles gambiae, Cicadella viridis* and *Haematobia irritans exigua* and from *Drosophila melanogaster* (DRIP). The second cDNA (AQP-Bom2) cloned from the posterior midgut encodes a 27 963 Da protein similar to putative AQPs identified in genome sequences of some insects such as a *Drosophila melanogaster* (GH16993 or Aqp17664). Northern blot analysis has revealed that the AQP-Bom1 mRNA (2.3 kb) is expressed predominantly in the hindgut (colon and rectum); moderate or lower expression in the silk gland, midgut and Malpighian tubules, while the AQP-Bom2 mRNA (1.3 kb) is mainly expressed in the posterior midgut and Malpighian tubules; lower expression in the hindgut. *In situ* hybridization studies confirmed the AQP-Bom1 mRNA expression, but no detectable AQP-Bom2 mRNA expression at the cryptonephric Malpighian tubules as well as the colonic and rectal epithelia. Immunocytochemistry using an antibody raised against a partial peptide of AQP-Bom1 protein could detect the positive reaction at the apical surface of the colonic and rectal epithelial cells. These results indicate that the AQP-Bom1 mRNA encodes an aquaporin working actively for the water-recycling machinery in the hindgut of lepidopteran larvae (solid/ plant feeder), excreting even dried faeces during a gluttonous feeding phase.

**Key words:** *aquaporin, cryptonephric complex, silk gland, water retrieval, Bombyx mori*

# INTRODUCTION

Water conservation in insects is critical because of their large surface area relative to volume and the generally arid environment where they survive. Insects employ a variety of structural and physiological adaptations to keep their water loss minimum. For phytophagous caterpillars, which are often gluttonous habitants in an agricultural field, the gain and loss of body fluids and the water balance has to be strictly controlled throughout their growth and development (Reynolds *et al.*, 1985; Reynolds and Bellward, 1989). The economy of internal water in tissues is directly mediated by the quality of food conditions via the alimentary canal as well as the circulating haemolymph, where tissues were dipped directly (Klowden, 2002).

The molecular aspects of water regulation in living organisms has been greatly progressed in recent years (Agre *et al.*, 1998; Borgnia *et al.*, 1999; Agre, 2006). Aquaporins (AQPs) function as water channels that allow rapid osmotic water flow across the cell membranes. Currently thirteen AQP isoforms have been identified in mammals, and are expressed in many fluid-transporting epithelia. Despite extensive physiological evidence on insect osmoregulation (Harvey *et al.*, 1998), studies on water transport itself in insect epithelia has been paid little attention for long years. Molecular mechanisms on water permeability in insects has been investigated from a plant sap sucking insect (Le Caherec *et al.*, 1996), followed by the studies in blood sucking insects (Elvin *et al.*, 1999; Pietrantonio *et al.*, 2000; Echevarria *et al.*, 2001). AQPs in these liquid feeder are responsible for osmoregulation in diuresis and excretion after the intermittent or periodical feeding. For the phytophagous caterpillar (solid/plant feeder), it seems to be important how the gluttonous larvae conserve water from plant, producing very dry faeces. Little is known about the molecular and cellular mechanisms of the possible AQP(s) in fluid-transpoting epithelia of lepidopteran insects, although Reynolds *et al.* (1985) speculated that water is resorbed in the hindgut and recycled via the haemolymph to the contents of the midgut.

Regarding the economy of body water in the silkworm, *Bombyx mori*, we cannot ignore the existence of the silk gland, because the tissue mass of the gland enormously increases at the vigorous feeding phase of the final (fifth) instar stadium, reaching to approximately 40% of the body weight (usually 5-6 g in a fully matured larva) at the onset of spinning (Kajiura and Yamashita, 1989). The gland stores vast amount of silk proteins in the glandular lumen as 'liquid silk', a solution containing up to 30% (wt/vol.) protein solution (Akai, 1998; Jin and Kaplan, 2003). The water balance and osmoregulation in this gland must be critical to maintain the liquid silk *in vivo* without fear of irreversible coagulation or denaturation until the completion of spinning. We have identified a proton-translocating vacuolar-type ATPase ($H^+$ V-ATPase or V-ATPase) in the silk gland (Azuma and Ohta, 1998; Azuma *et al.*, 2001; also see **Chapter 1**). This V-ATPase is responsible for the luminal acidification during the accumulation of silk proteins in this gland.

The electrochemical and pH gradients established by V-ATPase usually evoke several secondary active processes such transporters and channels (Harvey and Wieczorek, 1997; Beyenbach and Wieczorek, 2006). Silkworm larvae at the gluttonous feeding phase must carry out the active osmoregulatory work in such a high energy-requiring tissue as the silk gland and midgut. Therefore, it would be worthwhile to investigate some functional AQPs which are abundantly expressed and involved in the epithelial physiology of silkworms. In the present study, first we identify two putative AQPs of *Bombyx mori* (*Bommo* AQPs) from the silk gland and midgut by PCR-based cloning. Here, we name two *Bommo* AQPs as AQP-Bom1 and AQP-Bom2, which are closely related to the mammalian AQP4. Second, we demonstrate the different tissue distribution of mRNA expression, and the cellular expression *in situ* of them. One major isoform (AQP-Bom1), which is higher homology to other insect AQPs, is predominantly expressed in the hindgut not so highly at the silk gland. The other

isoform (AQP-Bom2), which has lower homology to AQP-Bom1 as well as other insect AQPs, is found in the midgut and Malpighian tubules.

## MATERIALS AND METHODS

### Insects and tissues

Hybrid races (Shunrei × Shougetu and Kinshu × Showa) of the silkworm, *Bombyx mori,* Linnaeus were reared on fresh mulberry leaves or an artificial diet at 24-26 °C. The final (fifth) larval instar stadium lasts 10 days: 7 days for feeding, followed by 3 days for spinning to build a cocoon. The larvae were studied at 4- or 5-day-old fifth-instar larvae (gluttonous feeding phase) in all experiments.

Larval tissues (silk gland, midgut, hindgut, Malpighian tubules, trachea) were collected and rinsed with diethylpyrocarbonate (DEPC)-treated phosphate buffered saline (Dulbecco's PBS without $CaCl_2$ and $MgCl_2$). The midgut was divided into three parts (anterior, middle and posterior) at the position of abdominal spiracles (Azuma *et al.*, 1991). The hindgut was divided into colon and rectum. The ileum in *B. mori*, which differs from that of *M. sexta*, is a junction between midgut and hindgut with one-way valve not to flow the gut contents backwardly. Collected tissues (0.1-0.2g) were weighed, immediately frozen with liquid nitrogen and then were stored at -80 °C until utilized for RNA extraction.

### Design of primers and PCR Cloning of the full-length cDNA

Based on the highly conserved amino acid sequence around the first NPA motif and the sixth transmembrane region after the second NPA motif in cloned insect aquaporins, we designed a pair of degenerate primers (sense: 5'-TG(C/T) CAC AT(C/G/T) AA(C/T) CC(C/T) GC(C/T) GT-3', and antisense: 5'-AC (A/G)AT (A/G)GG (A/G/T)CC (A/G)AC CCA G(A/T) A (A/G)A(C/T) CCA-3'), which was optimized for the codon preference in *B. mori* proteins (Frohlich and Wells, 1994).

Poly(A) RNA was prepared from several tissues of *B. mori* larvae using the QuickPrep Micro® mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). cDNA (~2 $\mu$g) was reverse transcribed from the mRNA with the oligo(dT) $_{12\text{-}18}$ primer (AMV Reverse Transcriptase First-strand cDNA Synthesis Kit, LIFE SCIENCES, Inc., Florida, USA). Using the degenerate primers, PCR amplification was performed by a hot-start technique with an initial 10 min incubation at 95 °C, followed by 40 cycles of 94 °C for 30 sec, 55 °C for 30 sec

and 72 °C for 30 sec, with a final incubation of 72 °C for 10 min. The resulting 440 bp product was purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and was ligated into pGEM®-T Easy Vector (Promega, Madison, WI, USA) as described by the manufacturer. The plasmid DNA was purified, and both strands of the DNA were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

According to the partial-length cDNA information on a possible candidate of *Bombyx mori* aquaporin (*Bommo* AQP), the 5'- and 3'-RACE (Rapid Amplification of cDNA Ends) DNA fragments were produced from the anterior silk gland mRNA, the rectum mRNA and the posterior midgut mRNA using the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Mountain View, CA, USA). Longest open reading frames corresponding to each candidate were obtained by joining the three overlapping PCR-derived fragments. To obtain the full sequence, appropriate primers were synthesized to allow determination of sequences from both strands. Finally, the nucleotide sequence of the open reading frame was further confirmed by checking the nucleotide sequence of a PCR product amplified with first strand cDNA from the anterior silk gland for AQP-Bom1 and that from the posterior midgut for AQP-Bom2 using the gene-specific primers, respectively (see Fig. 2).

Kyte-Doolittle hydropathy profile of the deduced amino acid sequence was analyzed using GENETYX-MAC version 13.0 (Genetyx Corporation, Tokyo, Japan) at a 10-residue window. The amino acid identities between *Bommo* AQP and other AQPs were analyzed by the BLAST search.

### mRNA expression studies

All reactions were prepared with ribonuclease (RNase) free-reagents and all procedures described below were carried out under the RNase-free conditions at room temperature as long as not specified in the text.

We carried out the hybridization with the single-stranded RNA (ssRNA) probe in order to increase the sensitivity and specificity as much as possible. The open reading frame of each *Bommo* AQP was subcloned into pGEM®-T Easy Vector (Promega, Madison, USA), which was linearized by an appropriate restriction enzyme. A digoxigenin (DIG)-labeled ssRNA probe was synthesized by *in vitro* transcription using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany), T7 or SP6 RNA polymerase and the linearized plasmid as a template DNA. For *in situ* hybridization, the positive signal probe (antisense strand) and the

negative control probe (sense strand) were synthesized by T7 and SP6 RNA polymerases, thus the probe length of each strand was in a range of 850-950 bp including the multiple cloning site of pGEM®-T easy. The DIG-labeled probes were cleaned up with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). A dot-blot was performed according to the manufacture's instructions to quantify the concentration of the probes.

For northern blotting and analysis, total RNA from several tissues was first extracted by the guanidium-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) followed by the further purification of poly(A) RNA with the QuickPrep Micro® mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Aliquots of 1 $\mu$g were loaded onto a 1.2 % agarose/formaldehyde gel and blotted onto a Nylon Membrane (positively charged, Roche Diagnostics) according to the manufacturer's instructions. After UV crosslinking, the poly(A) RNA was hybridized with a DIG-labeled ssRNA probe (25-50 ng/ml) overnight at 68 °C in 50% formamide, 5x SSC buffer, 0.02% SDS, 0.1% *N*-lauroylsarcosine and 2% (w/v) blocking reagent (Roche Diagnostics). Stringency washing was performed at 68 °C in low salt buffer (0.1x SSC, 0.1% SDS), which ruled out the probe cross-hybridization between the AQP-Bom1 and Bom2 when we compared the intensity of gene expression in the same tissue analyzed. The detection of hybridized probe was performed according to the Roche DIG protocol using alkaline phosphatase-conjugated anti-DIG antibody with the NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) colorimetrically. The specimens were kept in a dark at room temperature for 6-12 h depending on the color development.

*In situ* hybridization was performed following established procedures (Xu and Wilkinson, 1998) and the manufacturer's recommendations in order to optimize the conditions for a better signal with good histological resolutions. The colons and rectums from 4-day-old fifth-instar larvae were fixed by 4% paraformaldehyde (PFA) dissolved in DEPC-treated PBS (4% PFA/PBS, pH 7.4) for 5-6 hours at 4 °C. After fixation, tissue pieces were dehydrated through a graded series of methanols and embedded in a Paraplast embedding medium (Paraplast-Regular, Sigma, St. Louis, Missouri, CA, USA). Serial sections of approximately 5 $\mu$m thick were mounted on the fresh slides and dried overnight at 40 °C similar way for the immunocytochemistry (Azuma and Ohta, 1998). Prior to hybridization, the sections were dewaxed, rehydrated, equilibrated with PBS-DEPC, and then treated with Proteinase K (16 $\mu$g/ml, Roche Diagnostics) for 10 or 20 min at 37 °C. Longer treatment tends to decrease the positive signals. After fixing the preoteinase K-treated sections with 4% PFA/PBS for 30 min

at room temperature, the sections were dehydrated through a graded series of methanols. The hybridization mixture (50% formamide, 5x SSC, 1% SDS, 200 $\mu$g/ml tRNA, 50 $\mu$g/ml heparin) including the DIG-labeled RNA probe (100~200 ng/ml) was placed directly on the dehydrated sections and overlaid with a fresh coverslip. Hybridization was continued in a humidified box overnight (usually 14~18 h) at 55 °C. To remove the excessive probes, the specimens were successively washed with 2x SSC containing 25% formamide, 2x SSC and 0.2x SSC at 55 °C, then finally with 100 mM maleic acid buffer containing 150 mM NaCl and 0.1% Tween 20 at room temperature. The detection of hybridized probe was performed according to the Roche DIG protocol using alkaline phosphatase-conjugated anti-DIG antibody with the NBT/BCIP colorimetrically as the similar way to northern hybridization. The hindgut sections normally showed the clear specific signals up to 6 h. The color development in the midgut sections needed overnight (~12 h) in a dark. Stained sections were rinsed in PBS, fixed for 60 min in 4% PFA/PBS, and then mounted in Entellan® neu (Merck, Darmstadt, Germany). The sections were observed under a microscope (Olympus BX51) equipped with a differential interference contrast device (Olympus Corporation., Tokyo, Japan).

### Immunocytochemistry

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of most hydrophilic loop D region, namely amino acid residues 165-179 (C165DPQRNDLKGSAPLA179) in the AQP-Bom1 (see Fig. 10A). The antigen peptide was conjugated via bovine serum albumin (BSA, Minimum 98%, Sigma, St. Louis, CA, USA) as a carrier, and was then injected into rabbits (Operon Biotechnology, Ltd., Tokyo, Japan). The obtained antiserum for the AQP-Bom1 was further purified by passing through the column of IgG Purification Kit-A (Dojindo Laboratories, Kumamoto, Japan). The obtained IgG fractions were diluted with 1: 1,000 by 1% BSA in PBS. Tissues used for immunocytochemistry were dissected and fixed for 5-6 h in Bouin's fixative. All procedures followed our previous studies (Azuma *et al.*, 1991; Azuma and Ohta, 1998; Azuma *et al.*, 2001). The section were stained using the avidin-biotinylated enzyme complex (ABC) method using commercial reagents (Vectastain® *elite* ABC kit or Vectastain® ABC-AP kit, Vector Laboratories, Burlingame, CA, USA). Control incubations were done on adjacent tissue sections and included substituting a non-immune rabbit serum (1: 2,000 dilution) for the primary antibody.

# Results

## cDNA cloning and sequence analysis of the putative *Bombyx mori* aquaporins

We designed a pair of degenerate oligonuleotides for the highly conserved regions of insect aquaporins encompassing two NPA motifs, and PCR was carried out with first-strand cDNA prepared from silk gland, midgut and Malpighian tubules. After optimization of PCR parameters, the PCR product of the expected size (approximately 440 bp) was prominently obtained from the anterior silk gland mRNA (Fig. 8A). The 440 bp PCR product was specifically amplified under the presence of both primers (Fig. 8B). The nucleotide sequence of this product was determined and the deduced amino acid sequence exhibited high degrees of similarity with insect AQPs published previously (data not shown).

A full-length cDNA consisted of 2,246 bp in length and contained an open reading frame of 252 amino acids (nucleotide positions 229-984) encoding a polypeptide with a deduced molecular mass of 25,900 Da (Fig. 9A). This was designated as the first putative *Bommo* AQP (AQP-Bom1, DDBJ/EMBL/GenBank accession no. AB178640). There are two consensus sequences for N-linked glycosylation site [NX(S/T)] at Asn-50 and Asn-213, one consensus sequences for phosphorylation by protein kinase C at Thr-6 and one tyrosine kinase phosphorylation site at Tyr-250.

We tried to confirm the identical clone from first-strand cDNAs of the posterior midgut, since the degenerate PCR study showed the same molecular size of PCR product from the midgut mRNA (Fig. 8A). We obtained another AQP-like cDNA, however, it was different from the first clone, which was 1346 bp in length and contained an open reading frame of 259 amino acids (nucleotide positions 179-955) encoding a polypeptide with a deduced molecular mass of 27,963 Da (Fig. 9B). This was designated as the second *Bommo* AQP (AQP-Bom2, DDBJ/EMBL/GenBank accession no. AB245966). No potential N-linked glycosylation site

was found in AQP-Bom2. There are two consensus sequences for phosphorylation by casein kinase II at Ser-10 and Ser-138.

According to the hydropathy analysis, two cloned *Bommo* AQPs contained six putative transmembrane domains, five connecting loops, and cytoplasmic N- and C-terminal domains, all of which are widely conserved among AQP molecules (Fig. 10A). The second (B) and fifth (E) loops contained consensus NPA motifs in the first clone (AQP-Bom1), while the AQP-Bom2 has an alanine-serine replacement at the first NPA motif in loop B. The potential N-linked glycosylation sites for AQP-Bom1 (Asn-50, -213) were located in loop A and loop E, respectively. The sequence identity between the AQP-Bom1 and Bom2 (41.6%) was comparatively low and both *Bommo* AQPs exhibited a lower sequence conservation with the mercury-insensitive water channel (MIWC), namely AQP4 among the mammalian AQPs (Fig. 10B).

BLAST search comparions of the coding sequence of AQP-Bom1 revealed that the highest amino acid identity (>50%) with several insect AQPs published previously (Fig. 11A), all of which has been isolated by the cDNA cloning from each insect species. A number of highly conserved motifs are distributed throughout the protein sequences, notably around the loop B, loop E and the sixth transmembrane domains. Furthermore, the C-terminal tail contains the 'SYDF' as a consensus sequence across the Insecta. The other isoform, AQP-Bom2, also showed a sequence similarity with several other insect AQPs, all of which were the predicted AQP from each insect species (Fig. 11B).

## Tissue distribution of mRNA expression in silkworm larvae

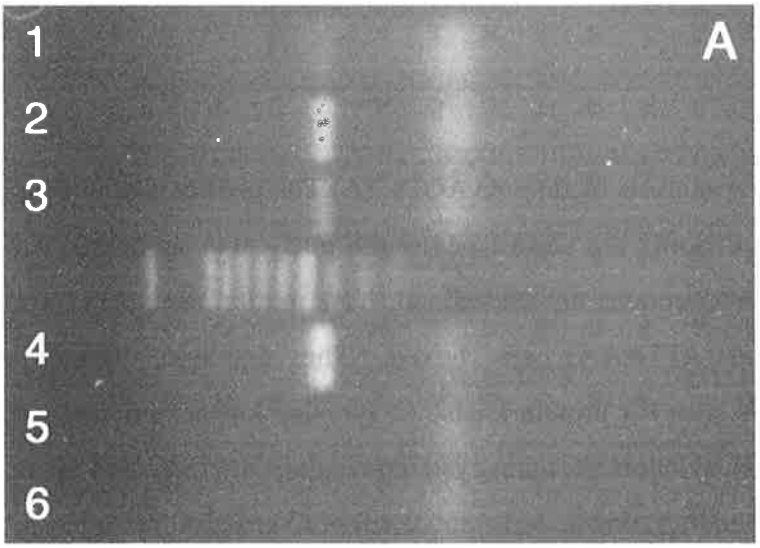
Aquaporins are found in tissues in which water movements are abundant and/or physiologically important. The presence of different AQP isoforms in silkworm larvae suggests that they serve different functions in active fluid-transporting epithelia. We prepared specific DIG-labelled anti-sense ssRNA probes for hybridization and detection of AQP-Bom1 and Bom2 mRNA, respectively, and tissue-specific expression was analyzed by northern hybridization (Fig. 12). Since we have hybridized the same amounts of mRNA preparation with two different ssRNA probes by northern blots, we could compare the relative abundance of each *Bommo* AQP transcripts at the respective tissue with the signal intensity. The hindgut (colon and rectum) as well as the anterior silk gland predominantly expressed the AQP-Bom1. The hindgut showed the highest expression of AQP-Bom1 as a single band of approximately 2.3 kb. Much lower levels of AQP-Bom1 expression were present in the midgut and Malpighian tubules. Instead, alternatively, the posterior midgut and Malpighian tubules dominated the AQP-Bom2 as a major 1.3 kb band with a faint 1.9 kb band. Expression of the AQP-Bom2 was notably absent in the anterior silk gland and the colon. The size of each transcript was the same as that of the full-length cDNA cloned by RACE-PCR (Fig. 9). The midgut showed significant regional differences on two *Bommo* AQPs. Search of a *Bombyx* EST database (Silkbase; http://papilio.ab.a.u-tokyo.ac.jp/silkbase/index.) (Mita *et al.*, 2003) with the AQP-Bom2 revealed that two different clone 'mg--0505' from midgut and 'maV30588' from Malpighian tubules were a part of the nucleotide sequence of AQP-Bom2. The strong mRNA expression of AQP-Bom1 from the colon as well as the rectum suggests the higher water permeability in the hindgut.

### *In situ* hybridization and immunocytochemistry of AQP in the hindgut

We have examined the hindgut region in more details by *in situ* hybridization, because the rectum of lepidopteran larvae consists of not only rectal epithelia but the cryptonephric Malpighian tubules, which has been called as a cryptonephric complex (Bradley, 1985; Liao *et al.*, 2000; Klowden, 2002; Schooley *et al.*, 2005). An intense signal of AQP-Bom1 was found in the cytoplasm of the rectal epithelial cells (Fig. 13A) and that of the colon (Fig. 13B), but very weakly in the columnar cells of posterior midgut (Fig. 13C). The cryptonephric Malpighian tubules also showed the significant staining (Fig. 13A), whereas the Malpighian tubules surrounding the colon were negative (Fig. 13B). The control experiments using the sense probe of AQP-Bom1 did not show any specific signal in these three tissues (Fig. 13D-F) except for nonspecific colorations at the cuticular lining, which was also seen in Figure 13A and 13B. The AQP-Bom2 was found to be expressed in neither the rectum nor the colon (Fig. 13G,H), but prominently in the columnar cells of posterior midgut (Fig. 13I). The controls using the sense probes for AQP-Bom2 showed no coloration (data not shown, similar image to Fig. 13D-F). The cellular expression patterns on AQP-Bom1 corresponded well with the data of northern hybridization (Fig. 12).

Finally, we try to confirm the localization of the AQP-Bom1 in the hindgut epithelia immunohistochemically, since AQP in most cases resides at the plasma membrane (Agre *et al.*, 1998; Borgnia *et al.*, 1999). The strong positive immunostaining of AQP-Bom1 clearly distributed at the apical surface of colonic epithelia (Fig. 14A) and at that of rectal epithelia (Fig. 14B). Very thick stainings in both epithelia imply that the apical plasma membrane has highly developed villous projections, extending the membrane surface just like the epithelial cells in the filter chamber of *C. viridis* (Le Caherec *et al.*, 1997). In addition, the cryptonephric Malpighian tubules in the rectum showed the positive immunostainings, but the resolution of AQP-Bom1 localization was not clear (Fig. 14B, *lower right corner*). This

would be due to the quite thin cell shape with the reduced cytoplasmic space, as we also observed that the AQP-Bom1 mRNA expression was very limited in the cytoplasm of the cryptonephric Malpighian tubules cells (Fig. 13A). Taken together, it is suggested that the water transport actively occurs at the hindgut epithelia, which seems to play a different role in fluid transport at the midgut epithelia.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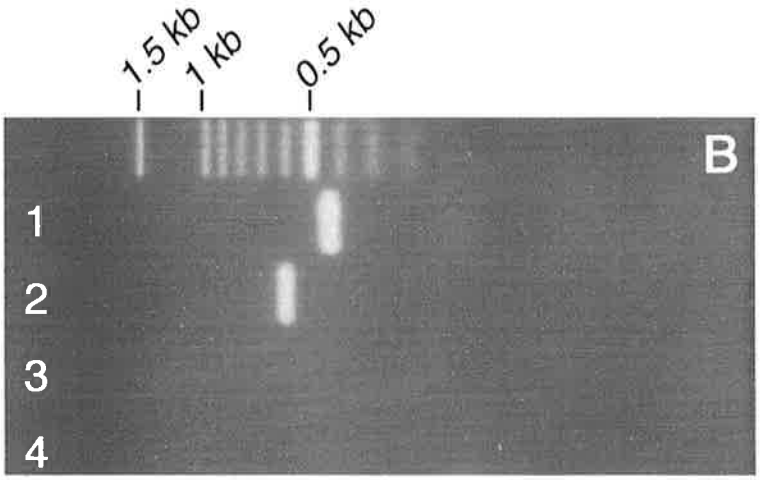
A


1.5 kb
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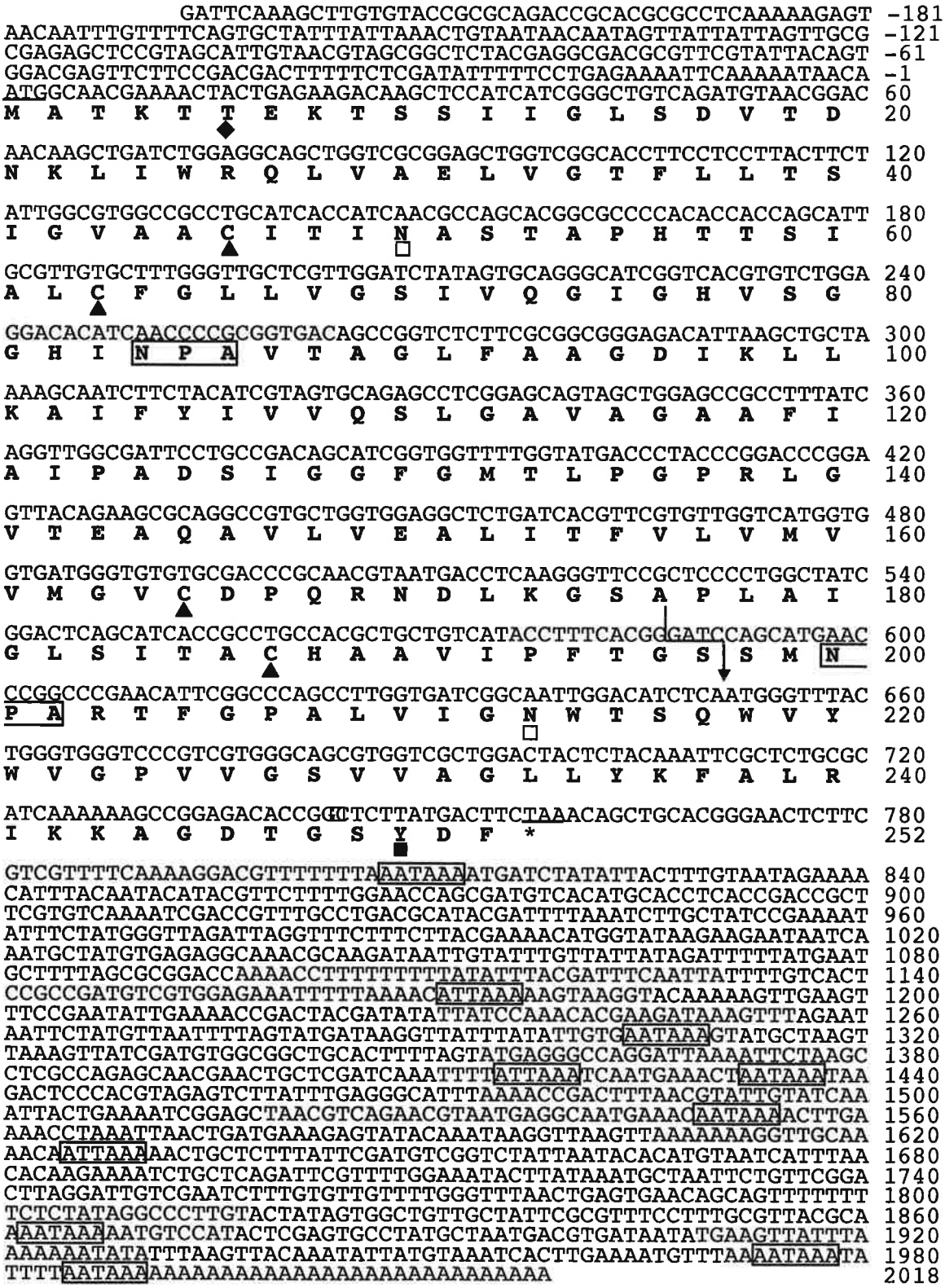
1
2
3
4

Fig. 9. Sequence analysis of *Bommo* AQPs. (A) The predicted amino acids of AQP-Bom1 and (B) that of AQP-Bom2 are shown below the nucleotide sequence. An initiation (ATG) and termination (TAA) codons are underlined. NPA motifs and putative polyadenylation signals (AATAAA and ATTAAA) are boxed. The diamond, square and circle indicate phosphorylation sites for protein kinase C, tyrosine kinase and casein kinase II, respectively. Potential N-glycosylation sites and cysteine residues are indicated open squares and triangles, respectively. Bending arrow indicate restriction enzyme recognition site (GGATCC and ACTAGT) of *Bam* HI and *Spe* I, respectively.

A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GGACGAGTTCTTCCGACGACTTTTTCTCGATATTTTTCCTGAGAAAATTCAAAAATAACA -1
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M A T K T T E K T S S I I G L S D V T D 20
AACAAGCTGATCTGGAGGCAGCTGGTCGCGGAGCTGGTCGGCACCTTCCTCCTTACTTCT 120
N K L I W R Q L V A E L V G T F L L T S 40
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I G V A A C I T I N A S T A P H T T S I 60
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A L C F G L L V G S I V Q G I G H V S G 80
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G H I N P A V T A G L F A A G D I K L L 100
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V T E A Q A V L V E A L I T F V L V M V 160
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V M G V C D P Q R N D L K G S A P L A I 180
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G L S I T A C H A A V I P F T G S S M N 200
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P A R T F G P A L V I G N W T S Q W V Y 220
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W V G P V V G S V V A G L L Y K F A L R 240
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I K K A G D T G S Y D F * 252
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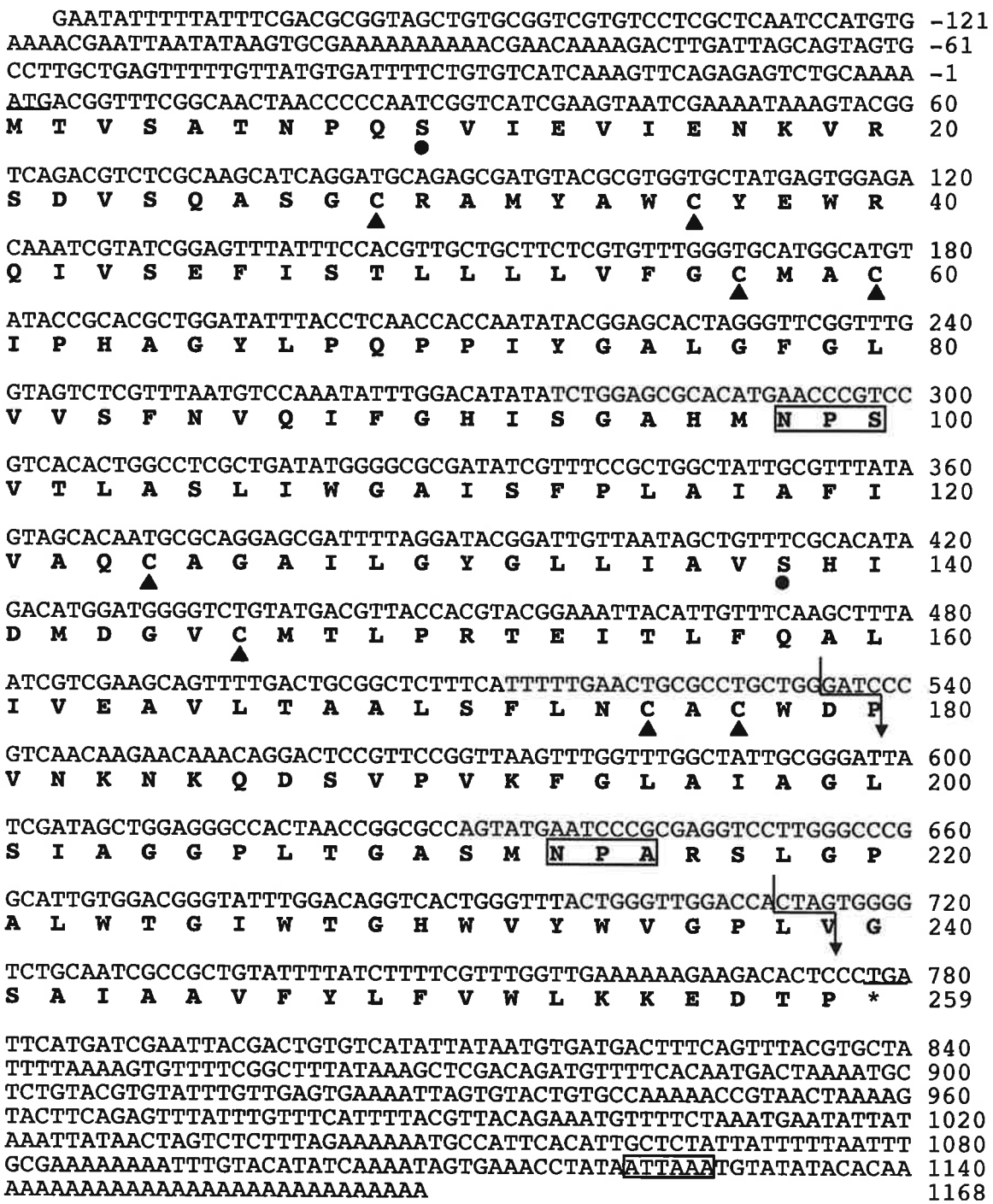
B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M T V S A T N P Q S V I E V I E N K V R 20
TCAGACGTCTCGCAAGCATCAGGATGCAGAGCGATGTACGCGTGGTGCTATGAGTGGAGA 120
S D V S Q A S G C R A M Y A W C Y E W R 40
CAAATCGTATCGGAGTTTATTTCCACGTTGCTGCTTCTCGTGTTTGGGTGCATGGCATGT 180
Q I V S E F I S T L L L L V F G C M A C 60
ATACCGCACGCTGGATATTTACCTCAACCACCAATATACGGAGCACTAGGGTTCGGTTTG 240
I P H A G Y L P Q P P I Y G A L G F G L 80
GTAGTCTCGTTTAATGTCCAAATATTTGGACATATATCTGGAGCGCACATGAACCCGTCC 300
V V S F N V Q I F G H I S G A H M N P S 100
GTCACACTGGCCTCGCTGATATGGGGCGCGATATCGTTTCCGCTGGCTATTGCGTTTATA 360
V T L A S L I W G A I S F P L A I A F I 120
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D M D G V C M T L P R T E I T L F Q A L 160
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I V E A V L T A A L S F L N C A C W D P 180
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A L W T G I W T G H W V Y W V G P L V G 240
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S A I A A V F Y L F V W L K K E D T P * 259
TTCATGATCGAATTACGACTGTGTCATATTATAATGTGATGACTTTCAGTTTACGTGCTA 840
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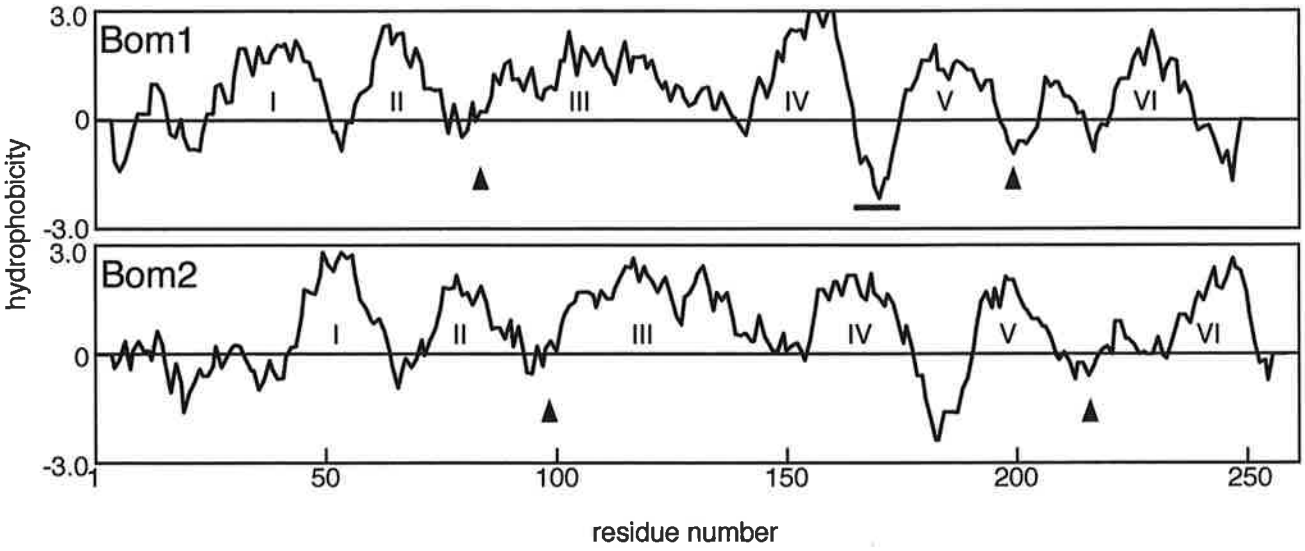
AAAAAAAAAAAAAAAAAAAAAAAAAAA 1168

Fig. 10. Hydropathy analysis of deduced amino acid sequences of *B. mori* homologues of mammalian AQP4, *Bommo* AQPs and comparison between *Bommo* AQPs and human AQP4 (AQP4). (A) Kyte-Doolittle hydropathy profile (window 10) of the deduced amino acid sequences of *Bommo* AQPs. Putative six transmembrane domains are numbered from I to VI. Position of two NPA motifs were exhibited by arrow heads. Solid bar in profile of Bom1 indicate location of the antigen peptides for antibody production corresponding to most hydrophilic loop D region (see Fig. 3A). (B) Alignment of *Bommo* AQPs and AQP4. *Bommo* AQPs have highly similarity to AQP4 (the mercury insensitive water channel, MIWC). Transmembrane domains deduced from hydropathy profile (see Fig. 10A) are underlined and are numbered from I to VI. Identical amino acids with AQP4 are highlighted in black. Dark and light tints indicate conserved amino acid residues between AQP4 and each *Bommo* AQPs, respectively. The two NPA motifs, a hallmark of many AQPs, are boxed. Percentages represent the amino acid homology of each sequence of *Bommo* AQPs to that of AQP4.

A

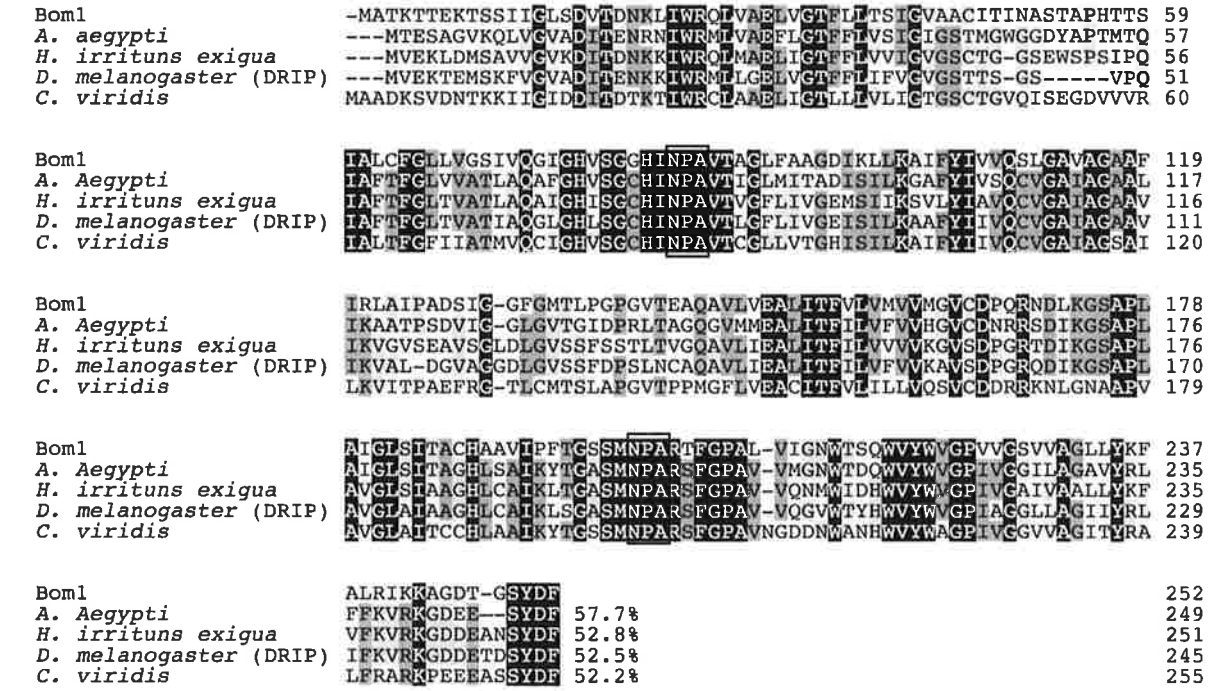



B

```
Bom1          ------------------------------MATKTTEKTSSIIGLSDVTDNKLIWRQLVAE  31
Bom2          ----------------MTVSATNPQSVIEVIENKVRSDVSQASGCRAMYAWCYEWRQIVSE  45
AQP4(MIWC)    MRKNHACFVETPNLAGEGMSDRPTARRWGKCGPLCTRENIMVAFKGVWTQAF-WKAVTAE  59

                                                                    I
LVGTFLLTSIGVAACITINASTAPHTTS---IALCFGLLVGSIVQGIGHVSGGHINPAVT  88
FISTLLLLVFGCMACIPHAGYLPQPPIY---GALGFGLVVSFNVQIFGHISGAHMNPSVT 102
FLAMLIFVLLSLGSTINWGGTEKPLPVDMVLISLCFGLSIATMVQCFGHISGGHINPAVT 119
          I                    II

AGLFAAGDIKLLKAIFYIVVQSLGAVAGAAFIRLAIPADSIGGFGMTLPGPGVTEAQAVL 148
LASLIWGAISFPLAIAFIVAQCAGAILGYGLL-IAVSHIDMDGVCMTLPRTEITLFQALI 161
VAMVCTRKISIAKSVFYIAAQCLGAIIGAGILYLVTPPSVVGGLGVTMVHGNLTAGHGLL 179
                  III                                  III

VEALITFVLVMVVMGVCDPQRNDLKGSAPLAIGLSITACHAAVIPFTGSSMNPARTFGPA 208
VEAVLTAALSFLNCACWDPVNKNKQDSVPVKFGLAIAGLSIAGGPLTGASMNPARSLGPA 221
VELIITFQLVFTIFASCDSKRTDVTGSIALAIGFSVAIGHLFAINYTGASMNPARSFGPA 239
          IV                   V

LVIGNWTSQWVYWVGPVVGSVVAGLLYKFALRIKKAGDTGSYDF---------------- 252
LWTGIWTGHWVYWVGPLVGSAIAAVFYLFLWLKKEDTP---------------------- 259
VIMGNWENHWIYWVGPIIGAVLAGALYEYVFCPDVEFKRRFKEAFSKAAQQTKGSYMEVE 299
                  VI

---------------------------------------- 38.7%              252
---------------------------------------- 35.9%              259
DNRSQAKTDDLILKLGVVHVIDVDRGEEKKGKDQSGEVLSSV                  341
```

Fig. 11. *Bommo* AQPs share similarity with insect aquaporins. (A) Multiple alignments of the deduced amino acid sequence of AQP-Bom1 (Bom1) with its homologous proteins; *A. aegypti* (AF218314), *H. irritans exigua* (U51638), DRIP (ABA81817) and *C. viridis* (X97159), and (B) that of the deduced amino acid sequence of AQP-Bom2 (Bom2) with its homologous proteins speculated from genomic sequence of several insects; *T. castaneum* (XP_970728), *A. gmbiae* (XP_554502), *D. melanogaster* (GH16993, AAL39296) and *A. mellifera* (XP_624194) . The two NPA motifs, a hallmark of many AQPs, are boxed. Identical amino acids are highlighted in black. Dark and light tints indicate partial identity. Percentages represent the amino acid homology of each sequence to that of AQP-Bom1 and AQP-Bom2.

A


Bom1 -MATKTTEKTSSIIGLSDVTDNKLIWRQLVAELVGTFLLTSIGVAACITINASTAPHTTS 59
A. aegypti ---MTESAGVKQLVGVADITENRNIWRMLVAEFLGTFFLVSIGIGSTMGWGGDYAPTMTQ 57
H. irrituns exigua ---MVEKLDMSAVVGVKDITDNKKIWRQLMAELIGTFFLVVIGVGSCTG-GSEWSPSIPQ 56
D. melanogaster (DRIP) ---MVEKTEMSKFVGVADITENKKIWRMLLGELVGTFFLIFVGVGSTTS-GS-----VPQ 51
C. viridis MAADKSVDNTKKIIGIDDITDTKTIWRCLAAELIGTLLLIVLIGTGSCTGVQISEGDVVVR 60
Bom1 IALCFGLLVGSIVQGIGHVSGGHINPAVTAGLFAAGDIKLLKAIFYIVVQSLGAVAGAAF 119
A. Aegypti IAFTFGLVVATLAQAFGHVSGCHINPAVTIGLMITADISILKGAFYIVSQCVGAIAGAAL 117
H. irrituns exigua IAFTFGLTVATLAQAIGHISGCHINPAVTVGFLIVGEMSIIKSVLYIAVQCVGAIAGAAV 116
D. melanogaster (DRIP) IAFTFGLTVATIAQGLGHLSGCHINPAVTLGFLIVGEISILKAAFYIIVQCVGAIAGAAV 111
C. viridis IALTFGFIIATMVQCIGHVSGCHINPAVTCGLLVTGHISILKAIFYIIVQCVGAIAGSAI 120
Bom1 IRLAIPADSIG-GFGMTLPGPGVTEAQAVLVEALITFVLVMVVMGVCDPQRNDLKGSAPL 178
A. Aegypti IKAATPSDVIG-GLGVTGIDPRLTAGQGVMMEALITFILVFVVHGVCDNRRSDIKGSAPL 176
H. irrituns exigua IKVGVSEAVSGLDLGVSSFSSTLTVGQAVLIEALITFILVVVVKGVSDPGRTDIKGSAPL 176
D. melanogaster (DRIP) IKVAL-DGVAGGDLGVSSFDPSLNCAQAVLIEALITFILVFVVKAVSDPGRQDIKGSAPL 170
C. viridis LKVITPAEFRG-TLCMTSLAPGVTPPMGFLVEACITFVLILLVQSVCDDRRKNLGNAAPV 179
Bom1 AIGLSITACHAAVIPFTGSSMNPARTFGPALL-VIGNWTSQWVYWVGPVVGSVVAGLLYKF 237
A. Aegypti AIGLSITAGHLSAIKYTGASMNPARSFGPAV-VMGNWTDQWVYWVGPIVGGILAGAVYRL 235
H. irrituns exigua AVGLSIAAGHLCAIKLTGASMNPARSFGPAV-VQNMWIDHWVYWVGPIVGAIVAALLYKF 235
D. melanogaster (DRIP) AVGLAIAAGHLCAIKLSGASMNPARSFGPAV-VQGVWTYHWVYWVGPIAGGLLAGIIYRL 229
C. viridis AVGLAITCCHLAAIKYTGSSMNPARSFGPAVNGDDNWANHWVYWAGPIVGGVVAGITYRA 239
Bom1 ALRIKKAGDT-GSYDF 252
A. Aegypti FFKVRKGDEE--SYDF 57.7% 249
H. irrituns exigua VFKVRKGDDEANSYDF 52.8% 251
D. melanogaster (DRIP) IFKVRKGDDETDSYDF 52.5% 245
C. viridis LFRARKPEEEASSYDF 52.2% 255

B

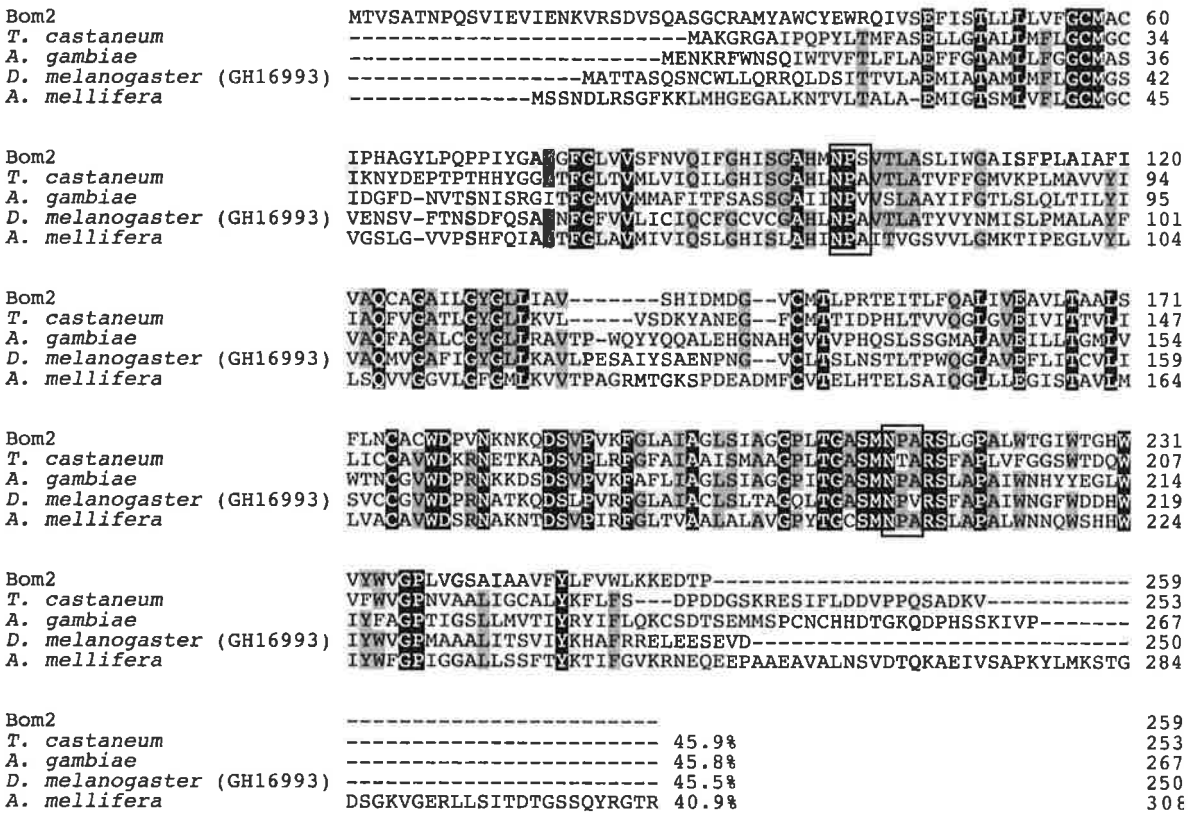

Bom2
T. castaneum
A. gambiae
D. melanogaster (GH16993)
A. mellifera
45.9%
45.8%
45.5%
40.9%

Fig. 12. Tissue specific expression of *Bommo* AQPs. 1$\mu$g of mRNA from several epithelial tissues were subjected to northern blot analysis. *Lane 1* posterior division of middle silk gland; *Lane 2*, anterior silk gland; *Lane 3,* anterior midgut; *Lane 4*, posterior midgut; *Lane 5*, colon; *Lane 6*, rectum; *Lane 7*, Malpighian tubules. Arrangement of the main tissues were indicated lower scheme of larva. (A) Each filter was hybridized with DIG labelled anti-sense RNA probe of AQP-Bom1 and (B) AQP-Bom2, respectively.

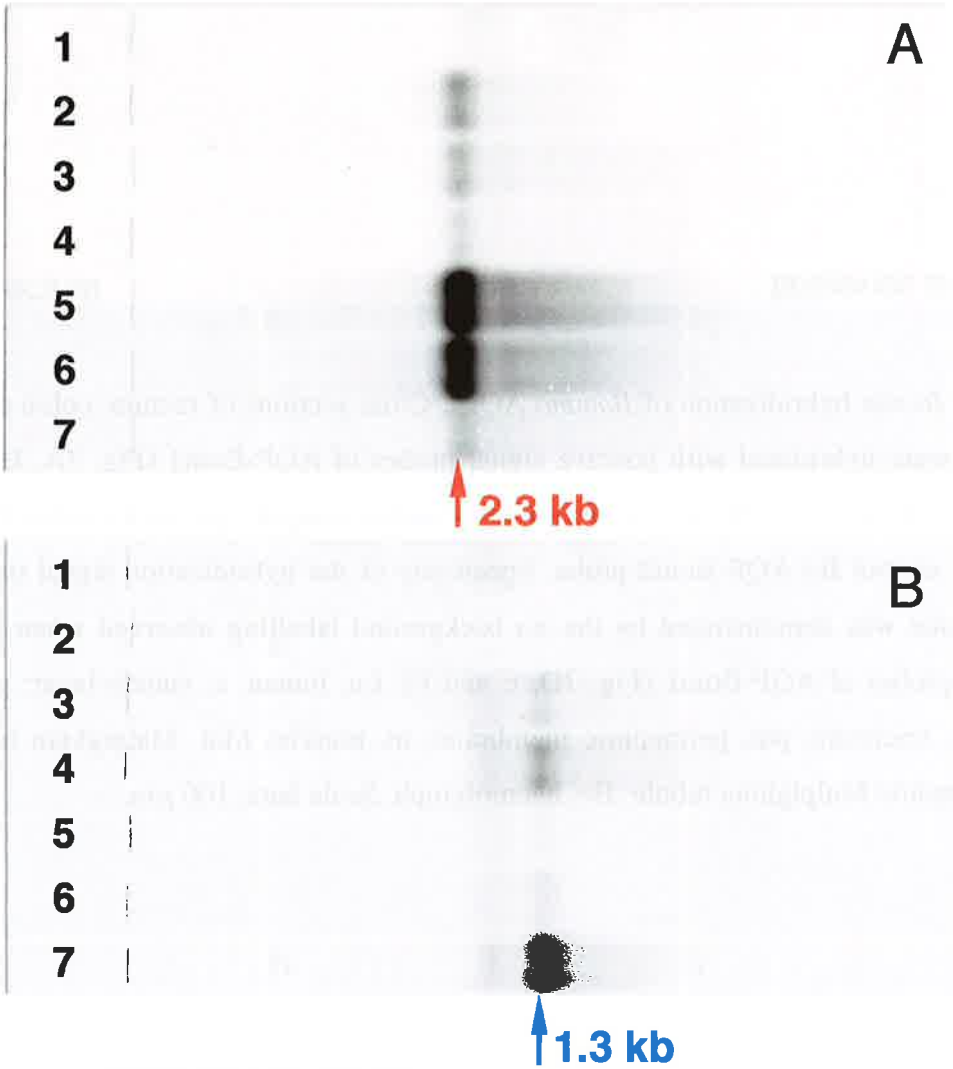

1
2
3
4
5
6
7
A
2.3 kb
1
2
3
4
5
6
7
B
1.3 kb
Malpighian tubule
(as an insect kidney) 7
hindgut
colon
5
rectum
6
anus
3
midgut
4
mouthpart

Fig. 13. *In situ* hybridization of *Bommo* AQPs. Cross sections of rectum, colon and posterior midgut were hybridized with positive signal probes of AQP-Bom1 (Fig. 7A, B and C) and AQP-Bom2 (Fig. 7G, H and I). Note that posterior midgut specimens utilized as a actual positive control for AQP-Bom2 probe. Specificity of the hybridization signal on sections of each tissue was demonstrated by the no background labelling observed when the negative control probes of AQP-Bom1 (Fig. 7D, E and F). Lu, lumen; c, cuticle layar; ps, perirectal space; t, tracheole; pm, perinephric membrane; m, muscle; Mal, Malpighian tubule; cMal, cryptonephric Malpighian tubule; He, haemolymph. Scale bars, 100 $\mu$m.

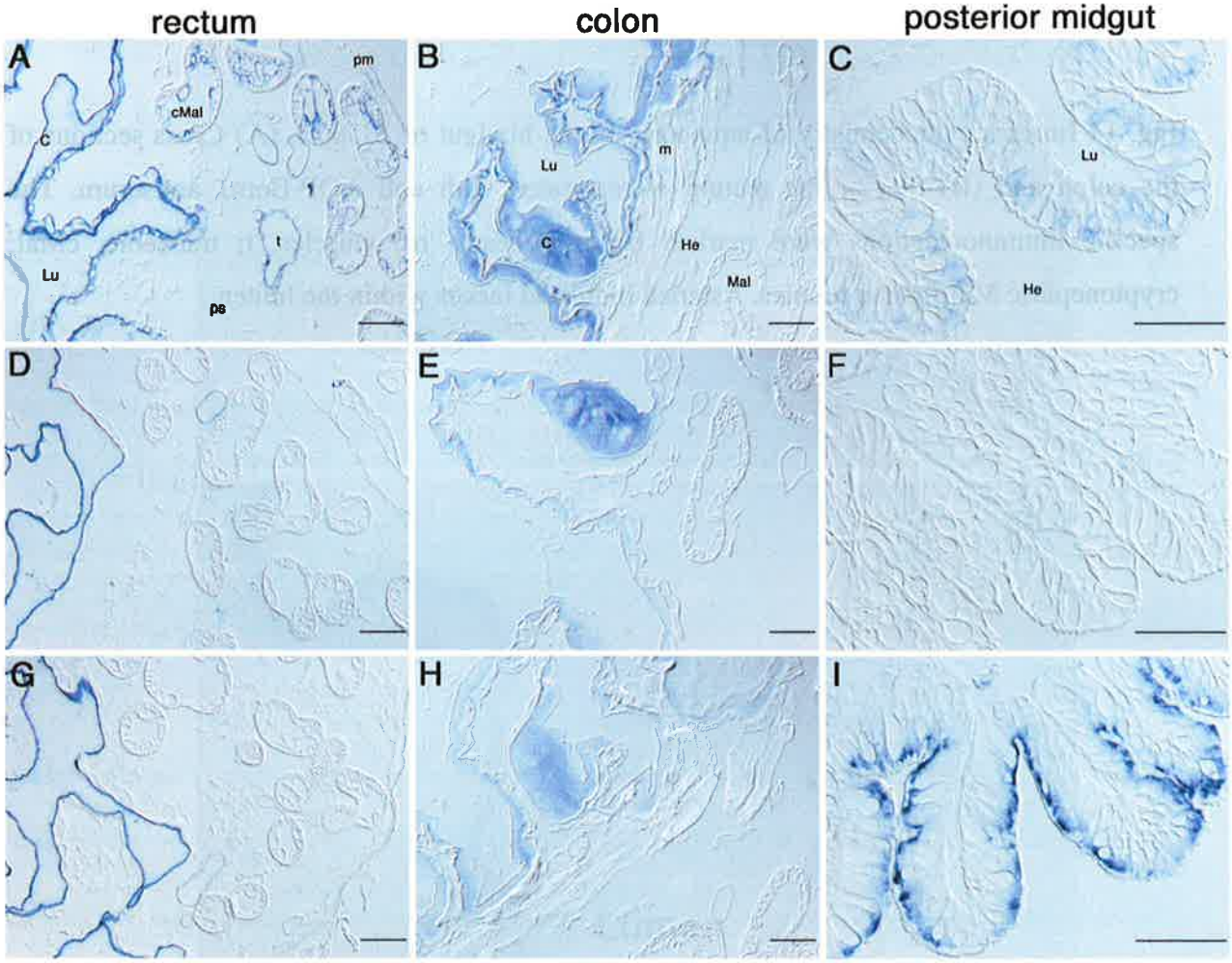
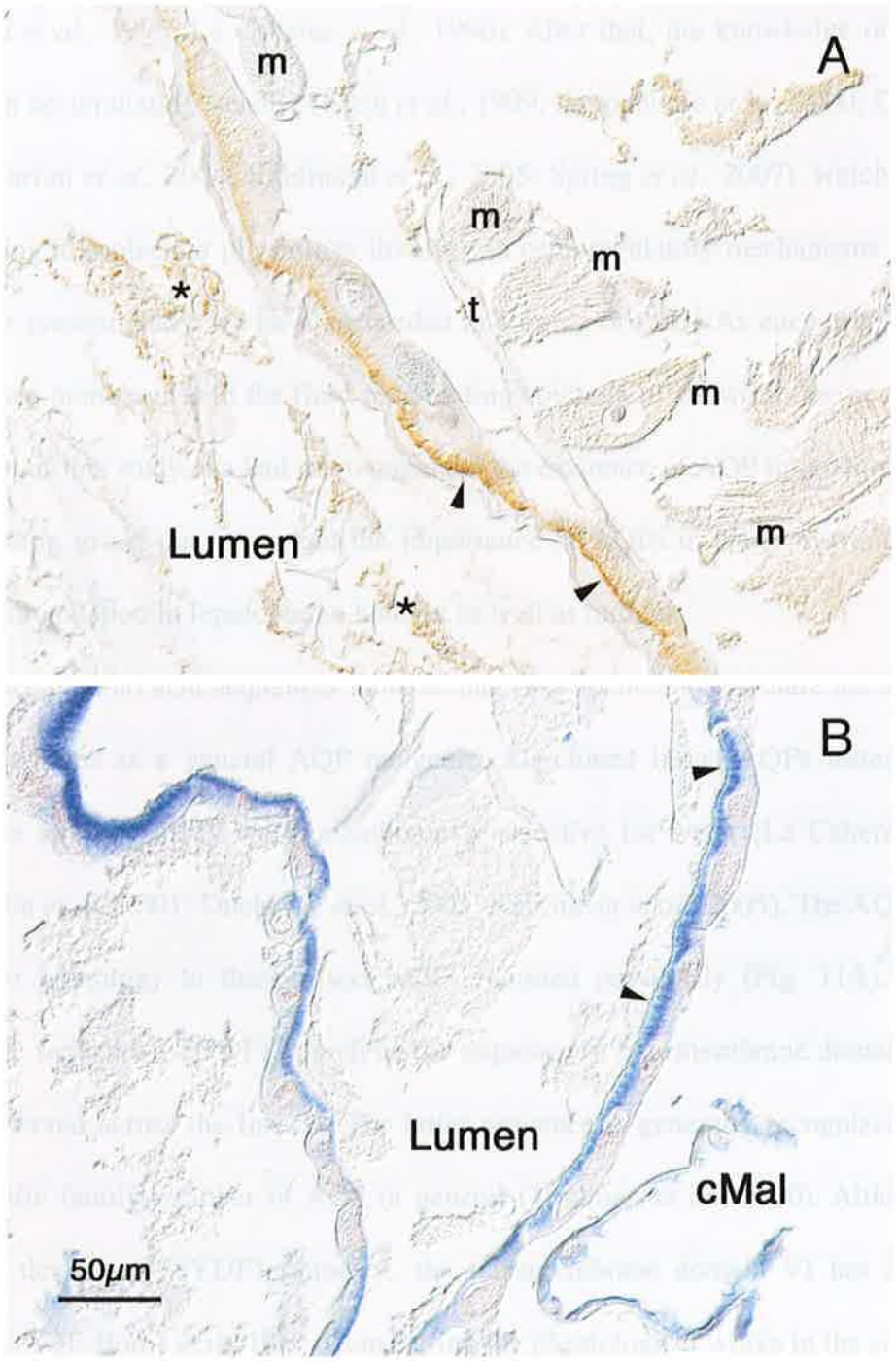

rectum
colon
posterior midgut
A
pm
cMal
c
t
Lu
ps
B
Lu
m
c
He
Mal
C
Lu
He
D
E
F
G
H
I

Fig. 14 Immunocytochemistry of aquaporin in the hindgut of *B. mori*. (A) Cross sections of the colon and (B) that of the rectum were treated with anti AQP-Bom1 antiserum. The specific immunoreactions were marked by arrowheads. m; muscles, t; tracheole, cMal; cryptonephric Malpighian tubules. Asterisk indicated faeces within the lumen.


A
m
m
m
t
m
m
*
*
Lumen
B
Lumen
cMal
50μm

# DISCUSSION

The functional insect AQP was first cloned and characterized by the pioneering study on the filter chamber as a water shunting complex in the midgut of homopteran insect, *Cicadella viridis* (Beuron *et al.*, 1995; Le Caherec *et al.*, 1996). After that, the knowledge of AQP in insects has been accumulating steadily (Elvin *et al.*, 1999; Pietrantonio *et al.*, 2000; Duchesne *et al.*, 2003; Martini *et al.*, 2004; Kaufmann *et al.*, 2005; Spring *et al.*, 2007), which leads to the understanding of molecular physiology involved in osmoregulatory mechanisms of insect epithelia. In the present study, we have succeeded in cloning two cDNAs encoding AQP and characterized two homologues in the fluid-transporting epithelia of silkworm larvae, *B. mori*. At the initiation of this study, we had not recognized the existence of AQP in the hindgut, but this study is going to set out to explain the importance of AQPs in water movements and associated osmoregulation in lepidopteran hindgut as well as midgut.

The deduced amino acid sequences indicate that two *Bommo* AQPs share the structural characteristic features as a general AQP molecule. All cloned insect AQPs tested by the *Xenopus* oocyte swelling assay were unambigously selective for water (Le Caherec *et al.*, 1996; Echevarria *et al.*, 2001; Duchesne *et al.*, 2003; Kaufmann *et al.*, 2005). The AQP-Bom1 shows a higher homology to these insect AQPs reported previously (Fig. 11A). The C-terminal peptide sequence (-SYDF) as well as the sequence in transmembrane domain VI are quite well conserved across the Insecta. The latter sequence is generally recognized among the water-specific family member of AQP in general (Verkman *et al.*, 2000). Although the AQP-Bom2 is devoid of "SYDF" sequence, the transmembrane domain VI has a similar sequence to the AQP-Bom1 (Fig. 10B). Considering the physiological works in the silk gland, hindgut, midgut and Malpighian tubules, we assume that these two clones serve as AQP functions for the respective transporting epithelia.

How many aquaporins does it need to be expressed in living insects? Eight homologues of AQP have been identified from the *Drosophila melanogaster*. Although DRIP was reported as a major functional AQP in *D. melanogaster*, the tissue specificity and the redundancy on the rest of putative AQPs remains unresolved (Kaufmann *et al.*, 2005). Although isolation of two AQP isoforms in the present study does not exclude further subtypes expressed in *B. mori,* the AQP-Bom1 seems to be major and shows a ubiquitous tissue distribution (Fig. 12). The midgut epithelia, alternatively, possess the AQP-Bom2 at the columnar cells of the posterior midgut (Fig. 13), but we detected the mRNA expression of AQP-Bom1 in the midgut tissues (including the visceral muscle and tracheoles on the analysis by the northern hybridization) (Fig. 12). Thus, we have to pay much attention to explore the expression pattern of these two AQPs at cellular levels along the length of midgut epithelia. More precise approach will shed light on the water movement in midgut epithelia at cellular and molecular levels, since the midgut with its contents in caterpillars seems to function a major reservoir of body internal water (Reynolds *et al.*, 1985).

The mRNA expression studies of *Bommo* AQPs indicate that the AQP dominates in the hindgut, indicating that not only the rectum but also the colon functions as a water gate. It is noteworthy that the colon is also the important region for water-absorbing process, where the faeces within the lumen are loosely packed as one or two excreta. This implies that the hindgut contents proceed a stepwise dehydration through defecation. This seems to be different from the assumption by the physiological study in *M. sexta* larvae (Reynolds and Bellward, 1989). Our preliminary experiment shows that the AQP-Bom1 strongly possesses the water transport ability in the *Xenopus* oocyte swelling assay which can be blocked by $HgCl_2$ (unpublished data). Taken together, the distribution and localization of AQP-Bom1 at the epithelia of the colon and rectum is physiologically indispensable for osmoregulation in

silkworm larvae. Future studies will no doubt resolve this issue in details including a functional analysis of AQP-Bom2.

Many larval lepidoptera as well as all Coleoptera develop a modified arrangement of the excretory system, named a cryptonephric complex, which is concerned with ionic regulation and water retrieval in response to the physiological stresses (Bradley, 1985; Schooley *et al.*, 2005). The rectum is closely associated with the distal ends of the Malpighian tubules and the complex is separated from the haemolymph by a water impermeable perinephric membrane (Liao *et al.*, 2000). It is generally believed that the complex is a fluid resorption and a water-recycling system to prevent cell and tissue dehydration among these Insecta (Bradley, 1985; Klowden, 2002). Very little is known about the molecular basis for the solute transporting mechanisms working in this complex, where only one excreta exists as a solid form, producing very dry faeces. We report here, for the first time, the molecular basis of this elaborate function for water balance in *B. mori* larvae.

For the caterpillar including *B. mori*, the water must be gained from food and taken up across the rectal epithelia to maintain internal water in tissues and cells. Daily expenditures of water are due to not only metabolic process but water loss as the faeces. There happens no physiological disorder by starvation for at least 3-4 days at the gluttonous feeding phase of the final stadium of *B. mori* larvae, being able to restart their larval growth by refeeding. This evidently explain that the *B. mori* larvae are rather tough against starvation. On the contrary, the homopteran insects such as *C. viridis* die ~10 h starvation (without water supply). A great water permeability is a chracteristic feature in the filter chamber as well as in the cryptonephric complex. The filter chamber functions a rapid elimination of water in order to avoid dilution of the circulating haemolymph (Le Caherec *et al.*, 1997). The cryptonephric complex functions a lower and minimum water loss by a water retrieval from the gut contents. Such a physiological work must be vital for terrestrial insects in maintaining water

homeostasis of the internal milieu by enduring an accidental water loss or an paucity of water from plant material in nature.

# Chapter 3

# Developmental expression and the physiological role of aquaporin in the silk gland of *Bombyx mori*

（カイコ絹糸腺のアクアポリンの生理的役割
―液状絹の水分調節に関わる絹糸腺細胞のアクアポリンの発現と分布―）

## Summary

We have explored the gene expression and localization of an aquaporin (AQP) in the silk gland of *Bombyx mori*. Whole mount *in situ* hybridization studies with digoxigenin-labelled RNA probes derived from an open reading frame of *B. mori* AQP (*Bommo* AQP: AQP-Bom1) showed its mRNA distribution strictly at the ASG from vigorously feeding larva. Its expression became undetectable from actively spinning larva. Northern hybridization analysis of *Bommo* AQP in the ASG demonstrated that a single transcript of 2.3 kb was abundantly present during the feeding phase of fifth instar stadium and its expression decreased after spinning. Immunocytochemical studies using an antipeptide antibody against the *Bommo* AQP molecules revealed that the positive reaction was localized at the apical surface of ASG cells from feeding larva and disappeared from spinning larva. Further the apical surface localization of *Bommo* AQP was found in the posterior division of the middle silk gland (posterior MSG) and very strong immunoreaction was also observed at the apically-opened vacuoles, cavities and pits predominantly existing in the most posterior MSG. The immunoreaction at the posterior MSG almost disappeared in the spinning phase. The occurrence and disappearance of AQP at the limited region of the silk gland are evidently coincided with that found in the $H^+$-translocating vacuolar-type ATPase. An osmoregulatory work along the length of the silk gland enables a silkworm larva to stabilize the liquid silk as a native state with entrained water during growth and development.



**Key words:** water, pH, V-ATPase, liquid silk, plasma membrane, *Bombyx mori*


**[Remarks]**
The AQP in the silk gland was designated as "*Bommo* AQP" in this chapter, but this type of AQP isoform is exactly the same as "AQP-Bom1" described in Chapter 2.

# INTRODUCTION

Silk proteins are stored in the silk gland as an aqueous solution (up to 30% wt/vol), which is called as liquid silk. It has long been argued that water as well as pH nature is a key factor for controlling the physico-chemical state of the liquid silk throughout development of the silk gland. The question has raised about how the liquid silk can be maintained in a concentrated silk dope without fear of irreversible precipitation or crystallization within the glandular lumen before spilling out of the spinneret. Recently, processing experiments in vitro have been performed with reconstituted silk solutions obtained from the silkworm, *Bombyx mori* (Jin and Kaplan, 2003). The existence of water as a plasticizing agent regulates in the hydrophobic/hydrophilic partitioning and chain folding of fibroin under the appearance of nanoscale colloidal-like particles (micelles), which aggregated into larger globules and gel-like states as the concentration of fibroin increased. Their findings mimic the actual behaviour of silk proteins in vivo (Akai, 1984, 1998). Jin and Kaplan (2003) implies that water molecules participates in stabilizing fibroin molecules within the glandular lumen where the silk proteins are accumulated. It seems to exist the cellular mechanism to circumvent the premature crystallization in the initial and intermediate stages of silk protein processing until the completion of spinning.

To elucidate the physiological mechanisms on constructing the supra-molecular structure of silk, it is necessary to explore the system regulating in water and pH nature in the silk gland. From this standpoint we have found the $H^+$-translocating vacuolar-type ATPase (V-ATPase), which locates at the apical plasma membrane and pumps $H^+$ into the glandular lumen (Azuma and Ohta, 1998; Azuma *et al.*, 2001). It is suggested that the physiology of V-ATPase is the luminal acidification during the gland development in feeding larvae, and at the spinning phase, when $H^+$-pumping by the V-ATPase is switched off, acidification stops and the glandular lumen becomes neutral. Furthermore, we have demonstrated that such a pH-

shift in the glandular lumen allows the cocoon-producing silkworms to preserve the liquid silk in vivo without any irreversible coagulation and denaturation until the completion of spinning (see Chapter 1). These previous studies indicate the indispensability of V-ATPase in the silk gland.

Only recently, we have identified and characterized the possible gene responsible for water transport (unpublished data), which is nowadays authorized as 'aquaporin' (Agre *et al.*, 1998; Agre, 2006). Not only pH shift in our previous studies but also water withdrawal and some other modifications must be critical for the conversion of liquid silk into solid fiber (Fedic *et al.*, 2002). Despite a wealth of data on the physical nature of silk proteins as a unique biopolymer (Vollrath and Knight, 2001; Shao and Vollrath, 2002; Jin and Kaplan, 2003), little information is available on the molecular mechanisms of water transport through the plasma membrane of silk gland epithelial cells. We here report the gene expression of aquaporin (AQP) and explore its physiological significance in the silk gland during larval growth and development.

## MATERIALS AND METHODS

### Insects and tissues

Hybrid races (Shunrei x Shogetsu and Kinshu x Showa) of the silkworm, *B. mori*, were reared on fresh mulberry leaves or an artificial diet (Aseptic Sericulture System Lab., Kyoto, Japan) at 24-26 °C. The final (fifth) larval instar stadium continues 7 days for feeding, followed by 3 days of spinning for cocoon production. Larvae were mostly used at 5- or 6-day-old fifth instar (vigorously feeding phase) and the stage in between one day and two days after the onset of spinning (actively spinning phase, after the gut purge).

For the whole mount in situ hybridization, the larvae were dissected with Dulbecco's phosphate-buffered saline (PBS). The specimens were quickly collected from (1) anterior silk gland (ASG) to the anterior division of middle silk gland (anterior MSG) and (2) the posterior division of middle silk gland (posterior MSG) to posterior silk gland (PSG), then immediately transferred to 4% paraformaldehyde (PFA) dissolved in PBS, and fixed for 5-6 h on ice. PFA-

fixed specimens were washed with PBSTw (PBS including 0.1% Tween 20) for overnight at 4 °C, then dehydrated through a graded methanol series, and finally kept in 100% methanol. Fixed tissues were stored at -20 °C when tissues were not processed immediately. For the northern hybridization, ASGs were dissected out and rinsed in an ice-cold sterile PBS. About 50 mg of aliquots (20 pieces of ASG from ten larvae) was quickly frozen in liquid nitrogen, and stored at -80 °C until utilized for mRNA preparation.

## mRNA expression studies

All reactions were performed with ribonuclease (RNase)-free reagents and all procedures described below were carried out under the RNase-free conditions at room temperature as long as not specified in the text.

By PCR-based cloning, we have identified the sequence encoding for the *B. mori* AQP designated as *Bommo* AQP in this report from the ASG of vigorously feeding larvae, which consists of a 2,246 bp of full-length cDNA (DDBJ/EMBL/GenBank accession no. AB178640). We carried out the hybridization with the single-stranded RNA (ssRNA) probe in order to increase the sensitivity and specificity as much as possible. Purified plasmid DNA of pGEM®-T Easy (Promega, Madison, USA) containing an open reading frame (756 bp) of the *Bommo* AQP was used as template for the generation of digoxigenin (DIG)-labelled ssRNA probes produced in vitro transcription by T7 and SP6 RNA polymerases using a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany). The DIG-labelled ssRNA probes were cleaned up with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Purity of ssRNA probes (antisense and sense strands) and the rough concentrations were determined by electrophoresis in a 2% agarose gel containing ethidium bromide. Precise probe concentrations were estimated using DIG Quantification Teststrips (Roche Diagnostics, Mannheim, Germany), and then the probes were diluted with the hybridization solution (50% formamide, 5x SSC, 1% SDS, 200 $\mu$g/ml tRNA, 50 $\mu$g/ml heparin) for either whole mount in situ hybridization (250~500 ng/ml) or northern hybridization (~25 ng/ml) as a working solution.

The distribution of AQP mRNA along the silk gland was investigated by whole mount in situ hybridization following the established procedure (Xu and Wilkinson, 1998) and the Roche DIG protocol in order to optimize the conditions for a better signal to noise ratio. Prior to hybridization, the specimens (kept under 100% methanol) were rehydrated, equilibrated with PBSTw, and then treated with Proteinase K (40 $\mu$g/ml, Roche Diagnostics) for 30 min at

37 °C. The proteinase K-treated tissues were fixed with PBS containing 0.2% glutaraldehyde and 4% PFA for 30 min at room temperature. After equilibrated with PBSTw, the specimens were prehybridized at 68 °C for 3 h in the hybridization solution and successively hybridized with the DIG-labelled RNA probe at 68 °C for 12-18 h in an Eppendorf® tube (Safe-Lock Tubes, 2.0 ml, Hamburg, Germany). To remove the excessive probe, high stringency washes were carried out at 68 °C for 30 min twice with the washing solution (50% formamide, 1x SSC, 0.1% Tween 20), followed by a wash with the mixture (1:1, v/v) of the washing solution and the maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl and 0.1% Tween 20, pH 7.5). The specimens were then washed three times for 5 min with the maleic acid buffer at 68 °C, followed by twice washes for 15 min in the maleic acid buffer at room temperature. The detection of hybridized probe was performed according to the Roche DIG protocol using alkaline phosphatase-conjugated anti-DIG antibody with the NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) colorimetrically. The specimens were kept overnight (~12 h) in a dark at room temperature. The color development was stopped by washing several times with PBSTw and the reaction deposits were finally fixed with 4% PFA in PBS.

For northern blotting and analysis, we prepared poly(A) RNA from the ASG with the QuickPrep Micro® mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Electrophoresis of equal amounts of mRNA (1 $\mu$g per lane) was performed on denaturing agarose/formaldehyde gels (1.2%) and blotted onto nylon membrane (positively charged, Roche Diagnostics) according to the manufacturer's instructions. After UV crosslinking, the poly(A) RNA was hybridized with a DIG-labelled ssRNA probe (antisense strand) overnight at 68 °C in 50% formamide, 5x SSC, 0.02% SDS, 0.1% N-lauroylsarcosine and 2% (w/v) blocking reagent (Roche Diagnostics). Stringency washing was performed at 68 °C in low salt buffer (0.1x SSC, 0.1% SDS). The detection of hybridized probe was performed colorimetrically in a similar way to whole mount in situ hybridization described above.

### Immunocytochemistry of aquaporin

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of most hydrophilic loop D region (Agre *et al.*, 1998), namely amino acid residues 165-179 (C165DPQRNDLKGSAPLA179) in *Bommo* AQP. The antigen peptide was conjugated via bovine serum albumin (BSA, Minimum 98%, Sigma, St. Louis, CA, USA) as a carrier, and was then injected into rabbits (Operon Biotechnology, Ltd., Tokyo, Japan). The

obtained antiserum for the *Bommo* AQP was further purified by passing through the column of IgG Purification Kit-A (Dojindo Laboratories, Kumamoto, Japan). The IgG fractions obtained were diluted with 1 : 1,000 by 1% BSA in PBS. We usually confirmed the significant reduction of positive reactions by those with the 1 : 10,000 dilution. Tissues used for immunocytochemistry were dissected and fixed for 5-6 h in Bouin's fixative. All procedures followed our previous studies (Azuma and Ohta, 1998; Azuma *et al.*, 2001; see also Chapter 1). The sections were stained using the avidin-biotin-peroxidase complex (ABC) method using commercial reagents (Vectastain® elite ABC kit, Vector Laboratories, Burlingame, CA, USA). The specificity of the immunostaining was also checked using an absorption test by preincubating the diluted IgG fraction (1:1,000 dilution) of anti-*Bommo* AQP with the antigen peptide (10 $\mu$g/ml). The sections were observed under a microscope (Olympus BX51) equipped with a differential interference contrast device (Olympus Corporation, Tokyo, Japan).

## RESULTS

### Whole mount in situ hybridization of aquaporin mRNA in the anterior silk gland

We have identified the AQP homologue from the ASG by PCR-based cloning. The spatial distribution of this AQP (*Bommo* AQP) mRNA was analyzed by the whole mount in situ hybridization along the silk gland. An intense *Bommo* AQP signal was clearly detected and limited to the ASG from vigorously feeding larvae (Fig. 15A, left). The signal intensity at ASG was significantly reduced in the control experiment using the sense probe (Fig. 15A, right). This positive signal totally disappeared in the specimens prepared from actively spinning larvae (Fig. 15B, left). As shown in Figure 1, the expression of *Bommo* AQP mRNA seemed to be absent in the anterior MSG at both physiologically different stages. No *Bommo* AQP signal was detected in the region from the posterior MSG to PSG (data not shown).

### Developmental mRNA expression and immunocytochemistry of aquaporin in the anterior silk gland

Next, we examined the mRNA expression of AQP at the ASG during the gland development by northern hybridization (Fig. 16). The explosive growth of silk gland at fifth instar stadium proceeds in the second half of feeding period (Kajiura and Yamashita, 1989; see also Chapter 1). Throughout the gland development, hybridized signals were detected as a single band of approximately 2.3 kb. It was confirmed that the size of the mRNA was in agreement with that of the cDNA for *Bommo* AQP (accession no. AB178640). The mRNA expression at the ASG started earlier before the vast growth of MSG and PSG, and the mRNA abundance reached a maximum at fully matured larvae (at the beginning day of spinning) and then declined. These expression pattern apparently coordinates with the massive growth of the silk gland, or with the deposition of liquid silk at the glandular lumen of MSG (Fig. 4 in Chapter 1). Although very faint signals were obtained from the specimens of spinning larvae, the *Bommo* AQP in the ASG is downregulated towards metamorphosis. The discrepancy to the whole mount in situ analysis at the spinning stage (Fig. 15B, left) seems to be due to the sensitivity of the techniques.

We further explored the cellular expression of AQP protein in the silk gland cell with the antipeptide antibody recognizing the *Bommo* AQP molecules specifically (Fig. 17). In the ASG from an active feeding larva (the same physiological stage with Fig. 15A), the *Bommo* AQP was detected at the apical surface of ASG cells and the positive signals was absent in the anterior MSG (Fig. 17A). This apical immunostaining existed underneath the cutilular layer (Fig. 17B). This indicates that the AQP is situated at the apical plasma membrane, which is identical with the location of V-ATPase (Azuma and Ohta, 1998). In the specimens treated with the antibody preabsorbed with the antigen peptide, the positive signal was almost eliminated at the apical surface of ASG from a feeding larva (Fig. 17C). The positive reaction disappeared at the sections from a spinning larva (Fig. 17D, the same physiological stage with Fig. 15B). The results shown in Figures 15, 16 and 17 suggest that the water permeability at

the ASG occurs actively at the growing phase of silk gland and that the water movement is closed at the spinning stage.

## Immunocytochemistry of aquaporin in the posterior division of the middle silk gland

In the posterior MSG from vigorously feeding larvae, we reported the presence of the plasma membrane V-ATPase at the apical surface of MSG cells. In addition, closer to PSG, we found the numerous vacuoles, cavities and pits, which also showed the positive immunoreaction of V-ATPase. These structures are unique and predominant near the border to PSG, but the physiological significance of these structures remains unknown (Azuma *et al.*, 2001).

In an active feeding larva (the same physiological stage with Figs. 15A and 17A), the strong reaction of *Bommo* AQP protein was observed at the apical surface including the above mentioned structures and the positive reaction was absent at PSG cells (Fig. 18A). The most prominent reactions were observed in the apically-opened vacuoles, which were stained conspicuously (Fig. 18B). The immunopositive regions were completely eliminated by preabsorption of the antiserum with the immunogen peptide (Fig. 18C). The intensity of immunostaining became faint at the apical surface towards the middle division of MSG, where the vacuoles, cavities and pits became few (Fig. 18D). In an active spinning larva (the same physiological stage with Figs. 15B and 17D), the positive immunoreaction decreased at the apical surface including the vacuoles, cavities and pits (Fig. 18E). These results suggest that the water transport through the AQP also exists at the posterior MSG during the gland development.

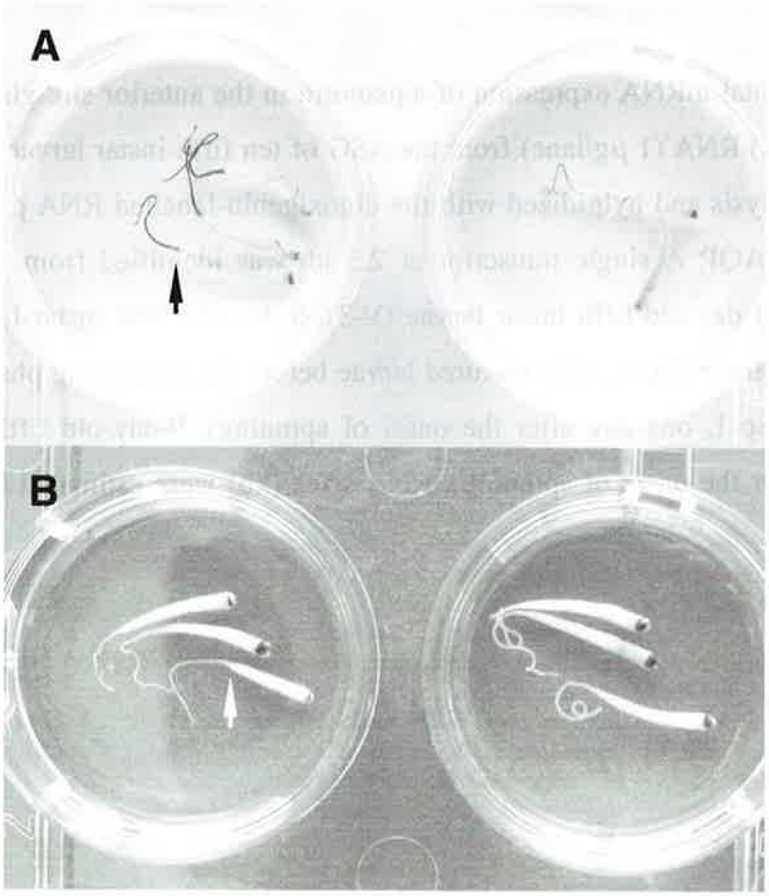

A
B

Fig. 15. Whole mount in situ hybridization of aquaporin mRNA in the silk gland of *Bombyx mori*. The anterior silk gland (ASG) and the anterior division of the middle silk gland (MSG) were probed with the digoxigenin-labelled RNA corresponding to the antisense strand (left photos) or the sense strand (right photos) for *Bommo* AQP. (A) 5-day-old fifth instar larvae. (B) one day after the onset of spinning. Note that the positive signal (A: left) was limited to the region of ASG. The arrows show the ASG-MSG junction.

Fig. 16. Developmental mRNA expression of aquaporin in the anterior silk gland of *Bombyx mori*. Pooled poly(A) RNA (1 $\mu$g/lane) from the ASG of ten fifth-instar larvae was subjected to northern blot analysis and hybridized with the digoxigenin-labelled RNA probe (antisense strand) for *Bommo* AQP. A single transcript of 2.3 kb was identified from 1-day-old fifth instar larvae (V-1), 3-day-old fifth instar larvae (V-3), 5-day-old fifth instar larvae (V-5), 7-day-old fifth instar larvae (Sp-0, fully matured larvae before the wandering phase), 8-day-old fifth instar larvae (Sp-1, one day after the onset of spinning), 9-day-old fifth instar larvae (Sp-2, two days after the onset of spinning). RNA sizes (kb) were estimated from the RNA standards.

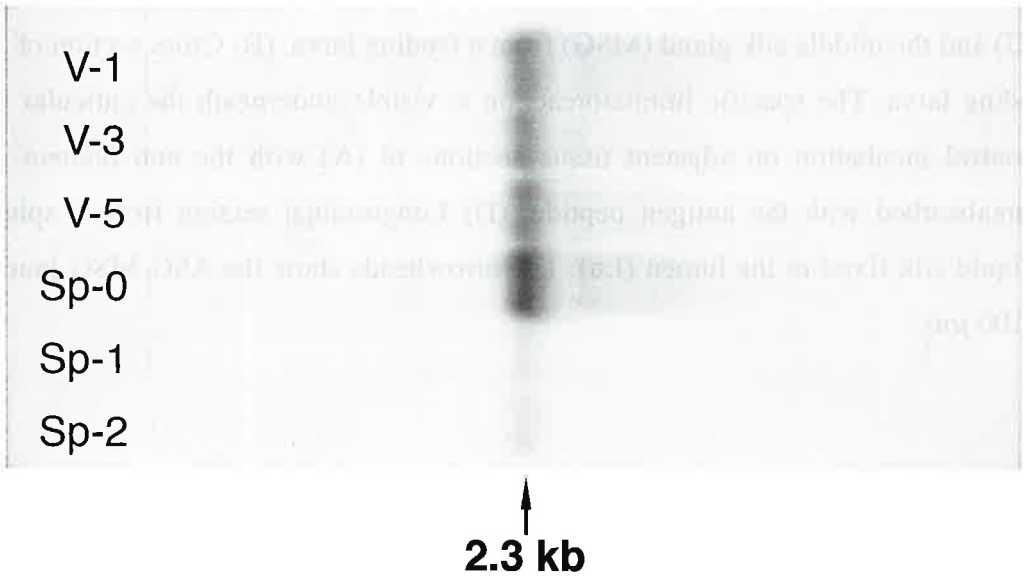

V-1
V-3
V-5
Sp-0
Sp-1
Sp-2
2.3 kb

Fig. 17. Immunocytochemistry of aquaporin in the anterior silk gland from the fifth instar stadium of *Bombyx mori*. (A) Longitudinal section at the boundary between the anterior silk gland (ASG) and the middle silk gland (MSG) from a feeding larva. (B) Cross section of ASG from a feeding larva. The specific immunoreaction is visible underneath the cuticular layer (c). (C) Control incubation on adjacent tissue sections to (A) with the anti-*Bommo* AQP antibody preabsorbed with the antigen peptide. (D) Longitudinal section from a spinning larva. Si: liquid silk fixed in the lumen (Lu). The arrowheads show the ASG-MSG junction. Scale bar, 100 $\mu$m.

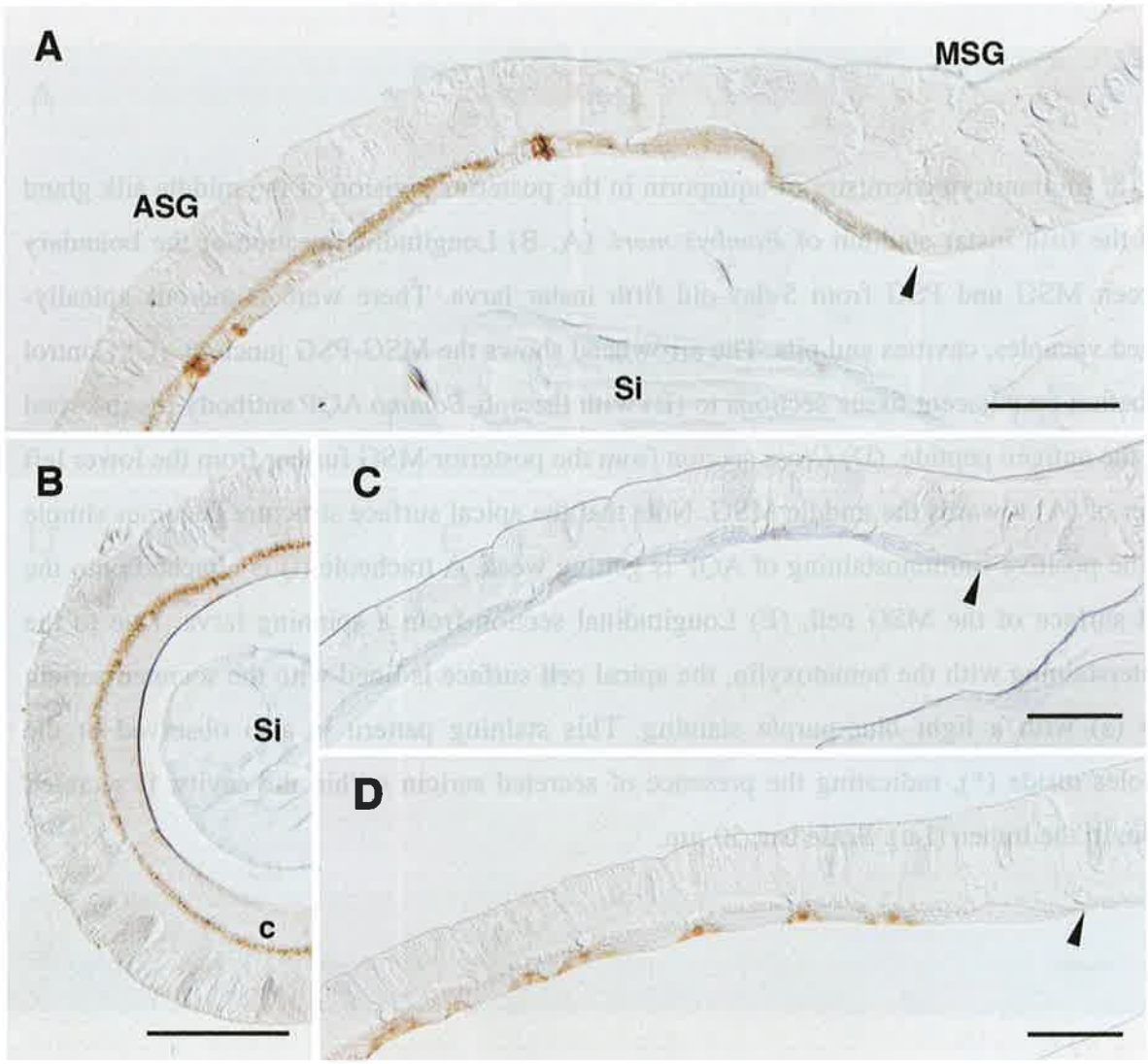
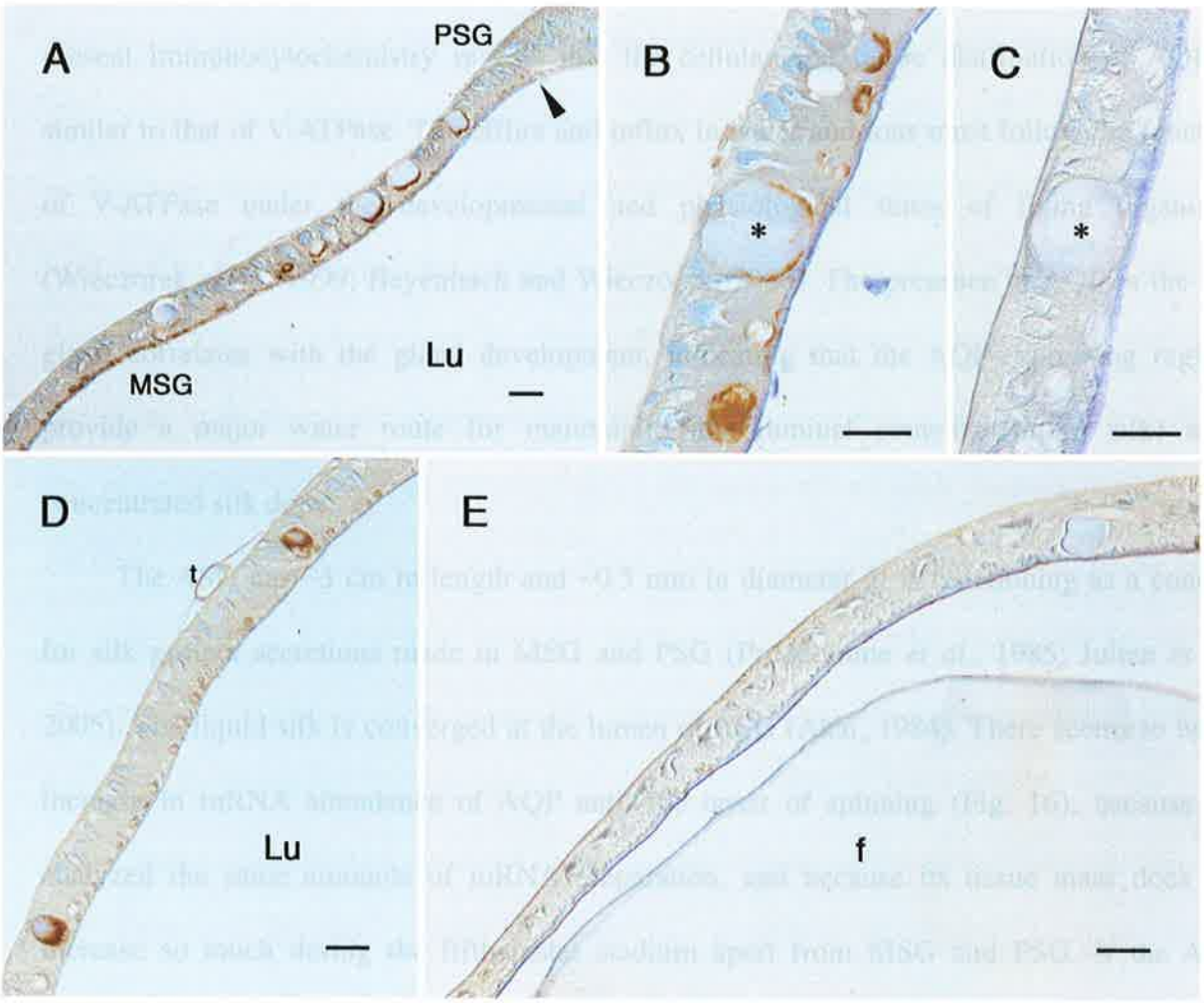

A
MSG
ASG
Si
B
Si
c
C
D

Fig. 18. Immunocytochemistry of aquaporin in the posterior division of the middle silk gland from the fifth instar stadium of *Bombyx mori*. (A, B) Longitudinal section at the boundary between MSG and PSG from 5-day-old fifth instar larva. There were numerous apically-opened vacuoles, cavities and pits. The arrowhead shows the MSG-PSG junction. (C) Control incubation on adjacent tissue sections to (B) with the anti-*Bommo* AQP antibody preabsorbed with the antigen peptide. (D) Cross section from the posterior MSG further from the lower left corner of (A) towards the middle MSG. Note that the apical surface structure becomes simple and the positive immunostaining of AQP is getting weak. A tracheole (t) is attached onto the basal surface of the MSG cell. (E) Longitudinal section from a spinning larva. Due to the counterstaining with the hematoxylin, the apical cell surface is lined with the secreted sericin layer (s) with a light blue-purple staining. This staining pattern is also observed at the vacuoles inside (*), indicating the presence of secreted sericin within the cavity. f: secreted fibroin in the lumen (Lu). Scale bar, 50 $\mu$m.


A
PSG
MSG
Lu
B
*
C
*
D
t
Lu
E
f

# DISCUSSION

The control of water in tissues and cells requires the contribution of the active transport system across the plasma membrane. In many insect epithelia, an osmoregulatory work is unequivocally energized by the plasma membrane V-ATPase (Harvey *et al.*, 1998; Wieczorek *et al.*, 2000). We have reported that the V-ATPase is localized at the apical plasma membrane of ASG cells and the posterior MSG cells (Azuma and Ohta, 1998; Azuma *et al.*, 2001). The present immunocytochemistry reveals that the cellular and tissue distribution of AQP is similar to that of V-ATPase. The efflux and influx in water and ions must follow the function of V-ATPase under the developmental and physiological states of living organisms (Wieczorek *et al.*, 1999; Beyenbach and Wieczorek, 2006). The presence of AQP in the silk gland correlates with the gland development, indicating that the AQP-expressing regions provide a major water route for maintaining the luminal contents (liquid silk) as a concentrated silk dope.

The ASG has ~3 cm in length and ~0.5 mm in diameter. It is functioning as a conduit for silk protein secretions made in MSG and PSG (Prudhomme *et al.*, 1985; Julien *et al.*, 2005). The liquid silk is converged at the lumen of ASG (Akai, 1984). There seems to be an increase in mRNA abundance of AQP until the onset of spinning (Fig. 16), because we analyzed the same amounts of mRNA preparation, and because its tissue mass does not increase so much during the fifth instar stadium apart from MSG and PSG. If the AQP functions in supplying water and maintaining a certain level of moisture in the glandular lumen of ASG, this might explain that the ASG from feeding larvae is mechanically flexible and strong, whereas that from spinning larvae is something brittle, fragile and easy to break into a few pieces. The closure of water movement after the onset of spinning reflects in such a texture of ASG.

We failed to demonstrate the mRNA expression of AQP at the MSG by northern hybridization, though it was absent at least in the anterior MSG (Fig. 15). Difficulty in mRNA expression studies at MSG seems to be due to the lower population of mRNA for AQP than that for sericin (Ser 1 mRNA, Garel *et al.*, 1997). The continuous expression of vast amounts of Ser 1 mRNA along the length of MSG (Matsunami *et al.*, 1998) may make the detection of *Bommo* AQP mRNA difficult. It was also practically impossible to obtain reliable data on the western blot analysis of *Bommo* AQP molecules due to the aggregation of silk proteins in membrane preparations (data not shown). Therefore, we adopt the immunocytochemical approach for exploring the occurrence of AQP in the MSG (Fig. 18). Localization of AQP in the posterior MSG coincided well with the V-ATPase immunoreaction reported previously (Azuma *et al.*, 2001). This suggests that the colocalization of both molecules in the apical membrane of MSG cells is important at this limited region of MSG. The secreted fibroin from the PSG first meets the sericin (Ser 1 gene product), which coats the fibroin for the first time and constructs the inner sericin layers (Akai, 1984, 1998). Here, the AQP must be necessary to maintain such a physical state of liquid silk during the enormous growing phase of the gland. This may be the reason why the liquid silk is evidently soft and jelly-like at this limited region.

Close to MSG-PSG junction, we have identified numerous huge vacuoles, cavities and pits, which also showed the AQP immnoreaction as well as the V-ATPase (Azuma *et al.*, 2001). The dense staining at the light microscopic level (Fig. 18B) indicates the numerous projections of the plasma membrane, just like goblet cell apical membranes of lepidopteran midgut epithelia (Cioffi, 1979). The liquid silk is truly moistened in this region, suggesting that the water transport from haemolymph is active and that the vacuoles function a certain pool for fluids (water, ions and small molecular solutes) probably including sericin. Since the sericin is a highly soluble protein in water (Fedic *et al.*, 2002), the most posterior MSG is a

critical region for coating the liquid fibroin with the first sericin. We conclude that not only the ASG but also the posterior MSG play an indispensable physiological work for pH and water regulation through V-ATPase as a proton pump and AQP as a water channel at the plasma membranes of silk gland cells during the silk protein production and accumulation.

Fully matured larvae often do not start spinning soon after the completion of feeding in rearing silkworms with an artificial diet. A number of researchers have experienced such a phenomenon that larvae spend some waiting hours (~12 h or more, before the wandering behavior). This must be rather complexed problem and consequentially happens on fully matured larvae fed on an artificial diet. These larvae are normally bigger than those reared with mulberry leaves, but look somewhat swollen with a bit of disorder in internal water economy (Reynolds *et al.*, 1985). Actually, the osmotic pressure of haemolymph in larvae reared on an artificial diet was ~10% more higher than those reared on mulberry leaves (Tsukada *et al.*, 1986). The significant delay of spinning leads us an idea that the larvae reared with an artificial diet suffer from overloading fluids from haemolymph and that the AQP-expressing cells at the limited region of the silk gland (the posterior MSG as well as ASG) impair a certain fluid-transporting function. We cannot ignore the control under the endocrine system, but the silk gland itself may have some imbalance of water metabolism. Study in AQP will shed light on the molecular and cellular basis for explaining such a common phenomenon encountered in rearing *B. mori* larvae with an artificial diet.

# おわりに

昆虫は様々な生物現象の宝庫であり，それぞれの昆虫がそのライフサイクルのなかで示す特異的な体のしくみについて，いかに生体を維持・発展させているか，分子レベル・細胞レベルあるいは個体レベルで，昆虫の「技」のロジックを研究する過程には，害虫制御へのヒントも隠されているかもしれない。陸上を主たる生活域としている多くの昆虫にとって体からの水分蒸散および体内水分の維持管理は，成長・変態・生殖・休眠などを行う昆虫の一生を支える基幹的な機能である。本研究では小型の無脊椎動物で開放血管系生物としてシンプルな体の中に仕組まれているホメオスタシス機構からみた昆虫の細胞生理機能について追究した。

昆虫の能動輸送機構や浸透圧調節に関する研究の歴史は古く，1950年代までさかのぼる（Maddrell, 2004参照）。しかし，昆虫細胞の構造と機能の両面から徹底的な研究が始まったのは1980年代後半以降である（東，1995）。それまでの多くの研究では，イオン濃度測定や浸透圧測定に基づいたもの，電気生理学的なアプローチを試みたもの，組織・細胞の電子顕微鏡観察に終始したもの，イオン依存性 ATPase 活性の性状調査を行ったものなど，それぞれの実験手法から組織の生理機能の解釈がなされ，多少の推論を交えての議論が数多く見受けられた。昆虫細胞の organellar membranes を用いた精度の高い研究の進展や，1990年代以降の昆虫の能動輸送機構に関する分子生物学的研究の進展が，タバコスズメガ（Manduca sexta）やネッタイシマカ（Aedes aegypti）を中心に精力的に進められて今日に至っている（Wieczorek et al., 2000；Beyenbach, 2003；Pullikuth et al., 2003；Beyenbach and Wieczorek, 2006）。昆虫のアクアポリン研究は，V-ATPase 研究と比べると後発であるが，ここ10年くらい遺伝子クローニングや組織特異的発現の証明など，アクアポリンが関わる細胞機能の実態が少しずつ報告されるようになった（東，2005）。

## 1．動物のアクアポリンファミリーは二つに分かれる　ーＡＱＰの多機能性ー

水という生命に直結する分子の細胞膜通過路が発見され，アクアポリンと命名され，水分子についてもプロトンや各種イオンのように，原形質膜を介して輸送を行う分子が確定した（Agre, 2006）。このアクアポリン分子が原形質膜に存在すると，水輸送が10～100倍早く進行し，また，そのチャネル分子の開閉（gating）やリン酸化の有無による調節によって，緻密な細胞制御が実行可能になっていることも哺乳類のアクアポリン研究から明らかにされている。全身のほぼすべての臓器に存在し，ヒトでは13種のアイソフォームがあり，

AQP0 ～ AQP12 と命名され遺伝子ファミリーを形成している（Fig. 19A）。水輸送が豊富な臓器には多数の，そして一つの細胞にも複数種の AQP が存在することも確認されている。これらの AQP アイソフォームは互いに協調・補完しながら機能しているのであろう。また水だけでなく，グリセロールや尿素のような小分子，ガス，イオンを通過させるものまで，その存在実体・多機能性が解明されつつある。これらの事実は動物の体成分の７割前後が水であり，体内ではダイナミックな水輸送が展開されていることを正に裏付けている。

昆虫研究の代表格であるショウジョウバエにおいてはゲノム情報から８ヶ推定されているが，現時点で cDNA クローニングからも同定されていた一種（DRIP, Kaufmann et al., 2005）のみが，水輸送機能が確認できているに過ぎない。カイコ幼虫を用いた本研究で少なくとも二種類の AQP（AQP-Bom1，AQP-Bom2）が，遺伝子産物・機能的膜タンパク質として，組織特異的な存在分布することが示された（**Chapter 2**）。この二つのカイコ AQP と一般の哺乳類の AQP との系統関係を Figure 19B に示した。アクアポリンは大きく二つの先祖型から分岐してきたと考えられており，一つは水選択性のもの（狭義の**アクアポリン水チャネル**，AQP０～２，AQP４～６，AQP８）並びに，水以外も通過可能なもの（**アクアグリセロポリン**，AQP３，７，９，10）に大別されている。アミノ酸の一次配列をベースにした相互関係であるが，**Chapter 2** においてクローン化したカイコ AQP はそれぞれのグループに分類された。特に，中腸・マルピーギ管で mRNA 発現が主に検出された AQP-Bom2 については，アクアグリセロポリンとしての機能を検証してゆくことが急務である。鱗翅目幼虫の中腸が，in vivo において水分子に対しては通過させない機構を有し，水分子はもう一方の AQP-Bom1 の主たる発現組織である後腸の働きに専ら委ねられているとするならば，幼虫個体としての水分管理機能を考える上でたいへん興味深い。

昆虫アクアポリン研究は植物汁液を吸汁する半翅目昆虫（Homoptera）の中腸（Filter chamber）からの cDNA クローニングで始まり，それ以降，吸血性の双翅目昆虫（蚊やハエの仲間）から遺伝子としていくつか同定されている。吸血行動は一過性であり，一度に大量の高濃度の動物血液が腸管内に流入してくるので，消化系・排泄系の機能と浸透圧調節のしくみを解明すること，および病原媒介性昆虫の害虫制御を開拓することの二つの視点からアクアポリン研究が進められている。カイコのような鱗翅目昆虫は堅く固形である植物葉を一過性ではなく断続的に，長期にしかも大量に摂取している（solid/plant feeder, Dow, 1986）。このような植食性の昆虫からアクアポリンを単離したのは本研究が初めてとなった。

## 2．後腸（hindgut）の細胞生理　ー昆虫の排泄機構ー

昆虫生物学の教科書には，古くから Cryptonephric complex が登場する（Klowden, 2002；Gullan and Cranston, 2005）。この存在が最初に報告されたのは今から約40年前で，釣り具店でも飼料として販売されている鞘翅目昆虫（甲虫類）のチャイロコメゴミムシダマシ（Tenebrio molitor）の幼虫からであった（Grimstone et al., 1968）。その後，鱗翅目幼虫の後腸でも同様な複合体の存在が確認された（Ramsay, 1976）。Cryptonephric complex 内のマルピーギ管では３Ｍにも達する KCl が水吸収に機能しているとされ（O'Donnell and Machin, 1991），チャイロコメゴミムシダマシの幼虫はほとんど水分を含まない食下物から直腸上皮細胞を介して水分を吸収するだけではなく，気管系・肛門から水蒸気までも水として利用しているといわれる（Bradley, 1985）。穀物の貯蔵倉庫では，いわゆる貯穀害虫（stored insects）がときどき大発生して厄介な問題になる。貯蔵倉庫内で害虫たちは，雨風や捕食者・天敵から身を守られた，たいへん安全な空間に棲んでいるが，そこは人工の乾燥地で，逆に一切の水補給は期待できないであろう。このような昆虫たちの多くは甲虫類で，植物葉を食する鱗翅目幼虫以上に水分含量の少ない固形物を食物としている。そのような環境下でも昆虫が生存可能である背景には，直腸において水分吸収を完璧に行い，しぶとく生き抜いている姿が想像される。

カイコ結腸においても直腸と同程度のアクアポリンの遺伝子発現が認められ，水輸送機能が活発であることが示唆された（**Chapter 2**）。タバコスズメガ幼虫では，体内の水分収支（water economy）に働いているのは直腸だけであり，回腸（ileum: カイコの結腸に相当）にはその上皮細胞の形態観察から水透過性がないと主張されていた（Reynolds and Bellward, 1989）が，カイコの結腸上皮細胞は直腸と同様に扁平であることや，ペースト状であった中腸内容物の水分率が結腸へ移動することで，次第に堅くなっていることから，結腸でも AQP-Bom1 による水分吸収が行われていると考えられる。結腸周辺をよく観ると，結腸の周辺部のマルピーギ管は，小腸から結腸にかけての領域だけが６本とも Cryptonephric complex 内のマルピーギ管のように，非常にジグザグ状でかつ複雑に入り組んでいる（Fig. 20）。さらに，この領域だけが前腸に始まる消化管系のなかで唯一大量の脂肪体で取り囲まれており，直腸の Cryptonephric complex のように閉鎖された系ではないものの，結腸，マルピーギ管，脂肪体の三者が連携して，脂肪体での尿酸顆粒の形成やイオン（$K^+$, $Mg_2^+$, $Ca_2^+$）調節に機能しているのではないかと想像される。後腸は外胚葉由来の器官であり，結腸と直腸の上皮細胞はともにクチクラによって裏打ちされている。結腸のそれは直腸よりも比較的厚く，あたかも上皮細胞を保護しているかのようである。後腸のクチ

クラ層の水透過性は比較的高いと言われていることから（Maddrell and Gardiner, 1980），おそらく結腸が圧搾機（squeezer）として機能することで内容物から水分を物理的に排除しているのかもしれない。

鱗翅目幼虫で中腸上皮組織（midgut epithelia）は，円筒細胞および杯状細胞から主に構成される（Cioffi, 1979）。杯状細胞の原形質膜で機能する V-ATPase による能動輸送機構によって中腸管腔内が強アルカリ性に維持されていること，円筒細胞が消化酵素の分泌センターで，分解されたアミノ酸や糖の吸収を行っていることは鱗翅目幼虫において広く認識理解されている（東，1995；2003）。しかし，後腸の組織形態学，そして大食漢（gluttony）の結果である大量排糞（東・三宅，2004）を支える生理学については不明な部分が多い。研究開始当初は，カイコ絹糸腺での水コントロールを焦点に当てていたが，研究を進める中で，鱗翅目幼虫の後腸の水分吸収におけるアクアポリンの存在意義を明らかにした。中腸研究と比較すると，昆虫生理学を追究する研究者でも今まであまり取り上げてこなかった組織である。カイコ幼虫では，食下物は中腸から結腸に送り込まれ，さらに結腸の収縮により直腸に移動し糞塊となり，結腸の内容物が２～３回送り込まれた後，体外に排泄される（Hukuhara and Satake, 1985）。一方，タバコスズメガ幼虫では糞塊は回腸においてすでに形成されており，それがそのままの形で直腸に運ばれ排泄される（Reinecke et al., 1973）。同じ鱗翅目幼虫でも寄主植物の栄養価や水分含量が組織の形態の違いや体内水分の調節機構，さらにはその排泄様式にも反映されていることを伺わせる。カイコ幼虫の結腸の内容物が完全に硬くならずにわずかに柔らかいことが，水分バランスを維持するために重要な意味を持っているのかもしれない。

カイコにはタバコスズメガと異なり絹糸腺が存在する。絹糸腺は終齢幼虫のクライマックス期（熟蚕期）には幼虫体重の４割にも達する（Kajiura and Yamashita, 1989；**Chapter 1 参照**）。幼虫個体の水分コントロールを議論する上で，営繭による液状絹の排出（吐糸行動）は，消化管系による排泄と並んで無視できない。また，絹糸腺が絹タンパク質を排出しているということは，窒素態化合物（アミノ酸の集合体であるタンパク質）の排出という意義もカイコ研究者の間で古くから，繭形成の生物学的意義として認識されている。絹糸昆虫は単に外敵を防ぐための通気性・保湿性に優れた「住まい」を造っているだけではないのである。吐糸営繭というやり方での窒素態の排泄という意味で，カイコなどの絹糸昆虫での絹糸腺の果たす役割は，個体の水分管理の枠内にとどまらず，過剰な窒素態制御のために重要であるに違いない（平山，2003）。

## 3．害虫制御への応用展開　ーみずみずしい農作物の保護を目指してー

安定した生物生産を望む農業現場にとって，病害虫の発生は減収に直結する。限られた耕地面積のなかで最大限の収量を得るためにも，安価で，利便性・即効性に特化した化学農薬は魅力的である。また，農業現場だけではなく，収穫後に穀物や飼料といった食品が保存されている精米所，貯蔵倉庫などにおいても，貯蔵穀物害虫が問題となっており，これらの防除にも化学農薬が長年使用されている。しかし，食の安心・安全や残留農薬の影響，さらには周辺作物への飛散（ドリフト）等に対しての関心が最近高まるようになった。このような背景のもとにポジティブリスト制度が施行（平成18年5月）され，すべての農薬に残留基準が設定されたことで，減農薬への取り組みが加速し，農薬の散布回数の削減や生物農薬（天敵昆虫・微生物農薬・ＢＴ剤）への転換が必要不可欠となっている。また，化学農薬の多用による慢性的な抵抗性病害虫の被害や地力の低下も懸念され，持続的農業を行うための打開策の一つという側面からも減農薬作物生産は時代の流れとなってきている。

農業害虫の多くは，鱗翅目および鞘翅目昆虫が圧倒的に多い。鱗翅目幼虫は主に植物葉を食害するが，鞘翅目昆虫の幼虫は地中生活で土壌中の根茎類を加害したり，貯蔵してある倉庫内の穀類といった生産者が見落としやすい場所に生息しているので，突発的な発生に対しては化学農薬に頼らざるを得ない。前述したように，これらの昆虫は Cryptonephric complex を備えており，特に鞘翅目昆虫は強力な水吸収機構を備えていることで，人工の乾燥地とも言える貯蔵倉庫内での生存をも可能にしている。もし，水代謝の中心的役割を果たす組織において水分調節に関わる遺伝子またはタンパク質をコントロールすることで代謝生理状態を破綻させ，排泄障害（例えば排泄困難な便秘状態など）を引き起こすことができれば，対象害虫に合わせたオーダーメイド防除技術の開発など，将来の応用研究への展開も可能となると期待される。

一般に昆虫の体は小さい。ヒトからみれば一滴の水が昆虫にとってはかけがえのない場合もあり，それが少なくても多過ぎても支障をきたすであろう。小さな個体においても水分過多や不意の絶食に耐える準備，さらには乾燥に耐えるように設計された体制の中で AQP は機能している。様々な昆虫にみられる休眠現象も長期にわたる乾燥耐性の象徴であるといえる。昆虫の AQP 研究は正常な細胞機能の説明が中心であるが，その遺伝的欠陥や分子機構の破綻による病理的症状も今後の研究で報告されるであろう。そこで昆虫研究者が思い起こすことは，ＢＴ毒素によって引き起こされるホメオスタシスの破綻（幼虫の嘔吐や下痢症状）である。これは正常な水分維持機構の不調や変調とみることもできる。カイコ幼虫でのアクアポリンの作用を通して，ＢＴ毒素の分子病理的側面についてのアプローチも興

味のあるところである。ハエや蚊では個体が小さいことも組織・細胞レベルでの研究の遅れの原因となっていたのかもしれないが，害虫制御などへの応用展開を目指すためにも，カイコ幼虫のような大型昆虫を研究の牽引役として用いることで，組織ごとや細胞タイプごとの解析を行い，一つの組織がどの様なアイソフォームの組み合わせによって機能しているのか，さらにそのアイソフォームそれぞれが何を輸送しているのかを明解にさせなければならない。昆虫でのアクアポリン研究も10年以上が経過し，遺伝子同定のあとの機能解析を証明するステップに達しており，研究の奥行きと幅を拡げてゆく努力がこれから必要とされている。

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2007年 12月 17日

三　宅　誠　司

Fig. 19. Members of the MIP family. (A) Biodistribution of human aquaporin. Thirteen aquaporins (AQP 0-12) have been identified to date, each with a distinct tissue distribution. (B) Phylogenic tree of AQP Z and GlpF from bacteria, *E. coli*, human aquaporins and published insect aquaporins. Using CLUSTAL W program, phylogenic analysis were conducted to establish the evolutionary relationships between *Bommo* AQPs and other members of the MIP family. Aquaporins are generally divided into four subfamilies: AQP-Z, AQP8, Super AQP and GlpF group. AQP-Bom1 belongs to a branch that contains water channels (AQP-Z group), and appears most related to insect aquaporins. In spite of its high degree of sequence identity with AQP-Bom1, AQP-Bom2 does not belong to either the aquaporins or the aquaglyceloporins but constitutes a separate group with Reduviid aquaporin.

(Aは佐々木，2005から転載)

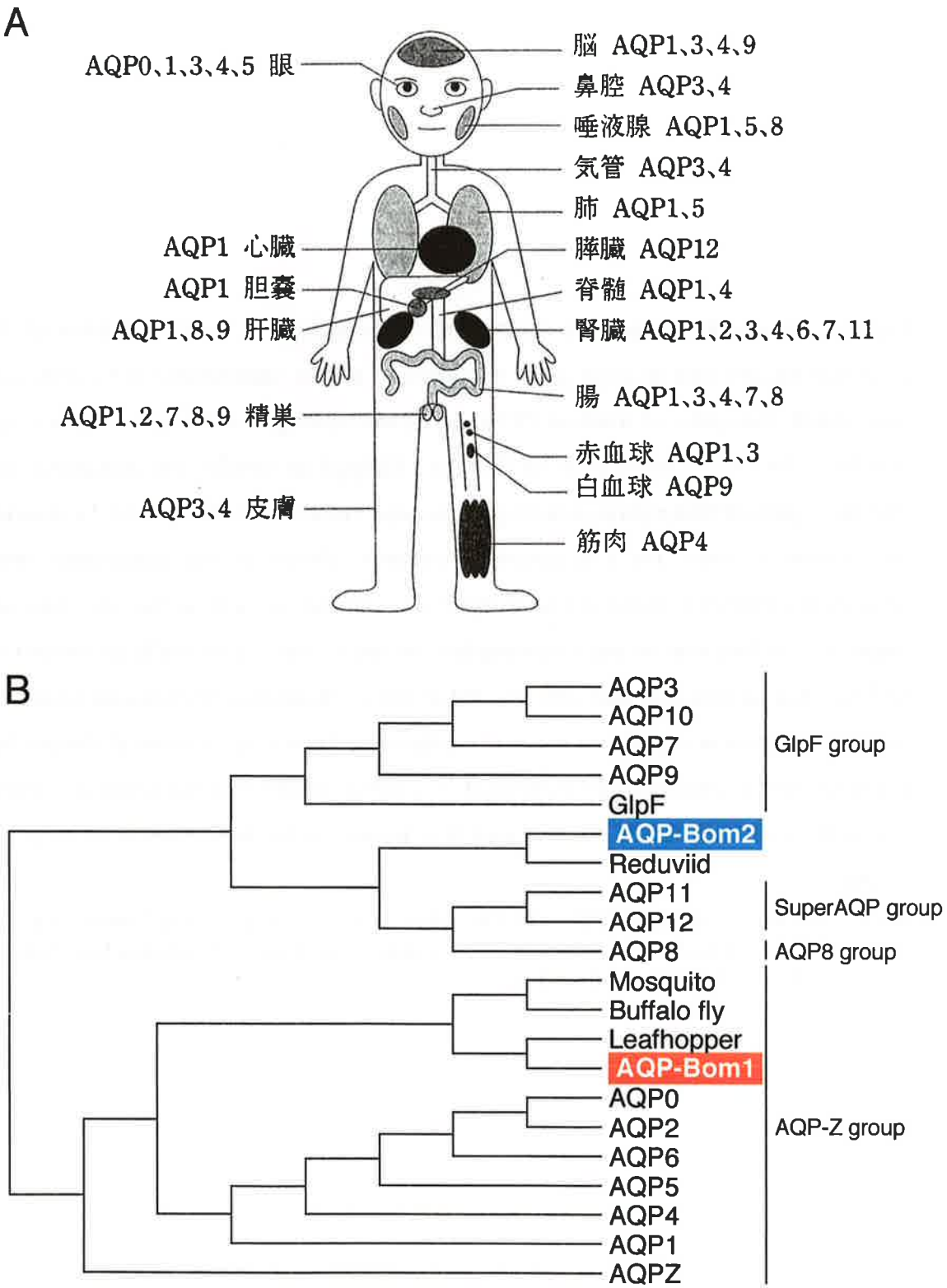
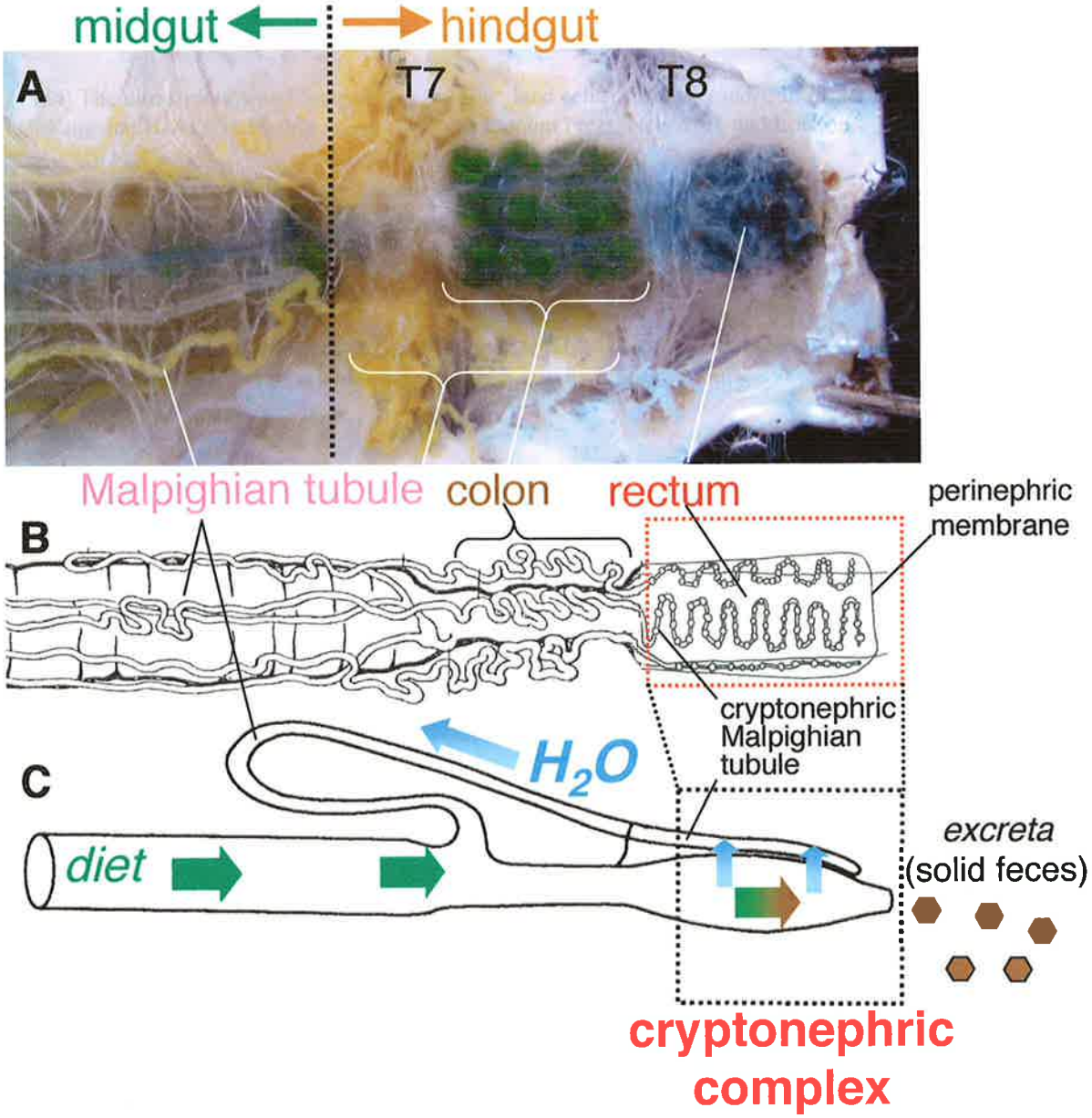

A
AQP0、1、3、4、5 眼
AQP1 心臓
AQP1 胆嚢
AQP1、8、9 肝臓
AQP1、2、7、8、9 精巣
AQP3、4 皮膚
脳 AQP1、3、4、9
鼻腔 AQP3、4
唾液腺 AQP1、5、8
気管 AQP3、4
肺 AQP1、5
膵臓 AQP12
脊髄 AQP1、4
腎臓 AQP1、2、3、4、6、7、11
腸 AQP1、3、4、7、8
赤血球 AQP1、3
白血球 AQP9
筋肉 AQP4
B
AQP3
AQP10
AQP7
AQP9
GlpF
AQP-Bom2
Reduviid
AQP11
AQP12
AQP8
Mosquito
Buffalo fly
Leafhopper
AQP-Bom1
AQP0
AQP2
AQP6
AQP5
AQP4
AQP1
AQPZ
GlpF group
SuperAQP group
AQP8 group
AQP-Z group

Fig. 20. Details of hindgut and cryptonephric complex of *B. mori*. (A) View of the intact posterior midgut and hindgut after dissection. Tubules surrounding the colon are highly convoluted. Two pairs of tracheae (T7 and T8) are running to colon and rectum, respectively (Locke, 1998). (B) Lateral view of the three Malpighian tubules and associated structures. The three pairs of Malpighian tubules pass through circular muscle layer of the anterior end of the rectum to enter the cryptonephric complex. Inside of the perinephric membrane, Malpighian tubules (cryptonephric Malpighian tubules) are also profusely convoluted. (C) Physiological function of the cryptonephric complex. Ions (principally potassium chloride, KCl) are transported into and concentrated in the cryptonephric Malpighian tubules, creating an osmotic gradient that draws water from the rectal lumen. As a result of dehydration from luminal content, solid feces were excreted. The tubule fluid is then transported forwards to the free portion of each tubule, from which it is passed to the haemolymph or recycled in the rectum.

(Bは森　精，カイコによる新生物実験，筑波書房，1970, p.20. とGrimstone et al, Further structure studies on the rectal complex of the mealworm Tenebrio molito, L. (coreoptera, Tenebrionidae), 1968, p.346の図をもとに作成。CはKlowden，2002から転載)


midgut
hindgut
A
T7
T8
Malpighian tubule
colon
rectum
perinephric membrane
B
cryptonephric Malpighian tubule
C
$H_2O$
diet
excreta (solid feces)
cryptonephric complex

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# 英 文 摘 要

The molecular aspects of pH and water regulation in living organisms have been greatly progressed in recent years. The control of pH and water in tissues and cells requires the contribution of the active transport system across the plasma membrane. In many insect epithelia, an osmoregulatory work is unequivocally energized the $H^+$-translocating vacuolar-type ATPase (V-ATPase) at the plasma membrane of fluid-transporting epithelia. Body water economy in silkworms largely depends on the metabolic activity of the silk gland as well as the midgut during the larval growth. The electrochemical gradients established by V-ATPase usually evoke several secondary active processes by transporters and channels. Silkworm larvae at the gluttonous feeding phase must carry out the active osmoregulatory work in such a high energy requiring tissue as the silk gland and midgut. The aim of this study focuses on the physiology of V-ATPase and aquaporin water channels in these transporting epithelia of silkworms. This doctoral dissertation consists of three frames of studies in molecular and cellular approaches as follows.

**(1) Acidification by the $H^+$-translocating vacuolar-type ATPase in the silk gland**

The silk gland of *Bombyx mori* and *Samia cynthia ricini* produces vast amounts of silk proteins and stores them in the glandular lumen as a liquid silk during the larval growth and development. We have explored the system regulating pH in the silk gland, because the gelation of fibroin is pH-dependent. By injecting the pH-sensitive dye (phenol red) into silkworm larvae, we have estimated the pH in the glandular lumen. Although the entry of dye was unsuccessful in the anterior silk gland (ASG) of *Bombyx*, the lumen of the middle silk gland (MSG: major reservoir for fibroin) and that of the posterior silk gland (PSG: the fibroin factory) were colored with phenol red. The coloration by phenol red indicated that the MSG was acidic (pH 5~6) in the vigorously feeding larvae leading to gelation of silk proteins at the MSG and that the PSG was neutral (pH 7~8). When the larvae started spinning, the lumen in the MSG became neutral. A similar pattern in the luminal pH shift was obtained in the silk gland of *Samia cynthia ricini* (Eri-silkworm) with a dye-injection experiment. In *Samia*, the V-ATPase locates at the apical surface of PSG, where the fibroin was produced, secreted and temporarily stored. The V-ATPase is also distributed at the apical surface of the anterior MSG as well as that in ASG. These V-ATPases became undetectable after the onset of spinning. The V-ATPase at the plasma membrane of silk gland cells regulates the physico-chemical state of liquid silk in the glandular lumen, in particular at the MSG of *Bombyx* and at the PSG of *Samia*, respectively.

**(2) Molecular cloning, tissue and cellular characterization of two isoforms of aquaporin**

Two different cDNAs encoding a homologue of aquaporin (AQP) were identified by a reverse transcription-polymerase chain reaction from the silkworm larva, *Bombyx mori*. The deduced amino acid sequence shares 40-60% homology with other insect AQP homologues. The first cDNA (AQP-Bom1) cloned from the anterior silk gland encodes a 25 900 Da protein

homologous to insect AQPs isolated from the liquid-feeding insect of *Aedes aegypti*, *Anophelles gambiae*, *Cicadella viridis* and *Haematobia irritans exigua* and from *Drosophila melanogaster* (DRIP). The second cDNA (AQP-Bom2) cloned from the posterior midgut encodes a 27 963 Da protein similar to putative AQPs identified in genome sequences of some insects such as a *Drosophila melanogaster* (GH16993 or Aqp17664). Northern blot analysis has revealed that the AQP-Bom1 mRNA (2.3 kb) is expressed predominantly in the hindgut (colon and rectum); moderate or lower expression in the silk gland, midgut and Malpighian tubules, while the AQP-Bom2 mRNA (1.3 kb) is mainly expressed in the posterior midgut and Malpighian tubules; lower expression in the hindgut. *In situ* hybridization studies confirmed the AQP-Bom1 mRNA expression, but no detectable AQP-Bom2 mRNA expression at the cryptonephric Malpighian tubules as well as the colonic and rectal epithelia. Immunocytochemistry using an antibody raised against a partial peptide of AQP-Bom1 protein could detect the positive reaction at the apical surface of the colonic and rectal epithelial cells. These results indicate that the AQP-Bom1 mRNA encodes an aquaporin working actively for the water-recycling machinery in the hindgut of lepidopteran larvae (solid/plant feeder), excreting even dried faeces during a gluttonous feeding phase.

**(3) Developmental expression and the physiological role of aquaporin in the silk gland**

The silk gland of *B. mori* divided into the PSG, MSG and ASG. The silk gland of *B. mori* produces vast amounts of silk proteins at PSG and temporarily stores them in the glandular lumen of MSG as a liquid silk. The ASG function as a conduit for silk protein secretions from MSG. The liquid silk converged at the lumen of ASG. In the feeding stage, mRNA distribution of AQP was strictly at the ASG and its expression became undetectable from in an active spinning larva. Immunocytochemical study revealed that AQP protein was found in not only the apical surface of ASG but also that of the posterior MSG in an active feeding larva. These positive reactions were disappeared in the spinning phase of silkworms. The occurrence and disappearance of AQP at the limited region of the silk gland are evidently coincided with that found in the V-ATPase. An osmoregulatory work along the length of the silk gland enables a silkworm larva to stabilize the liquid silk as a native state with entrained water during growth and development.

Insects employ a variety of structural and physiological adaptations to keep their water loss minimum. For the phytophagous caterpillars, which are often gluttonous habitants in an agricultural field, the gain and loss of body fluids and the water balance has to be strictly controlled throughout their growth and development. If the expression of AQP and V-ATPase in their epithelia were manipulated in a certain way for the successive agriculture, the molecular basis of water permeability and pH homeostasis would become a potential target for insect control.

# 和 文 摘 要

一般に昆虫の体は小さい。ヒトからみれば僅か1滴の水が昆虫1匹の全血液に相当する場合もあり，その微量な水分の維持が生死を分ける。脱皮・変態および休眠をも伴うドラマティックな一生のなかで，昆虫個体は摂食（吸汁）と排糞（排尿）の動的なバランスを維持している。昆虫はからだの成り立ちが開放血管系であるので，血液を介して組織間の相互作用だけでなく，細胞間の溶質交換つまり原形質膜を介したやりとりも，直接的で単純である。したがって，僅かな水分の漏出やpH・イオンに関わる細胞膜機能の変調や破綻が，全身の浸透圧維持に及ぼす影響は大きい。体内水分の適正な調節は，昆虫において正に水際作戦で行っているといえる。水という生命に直結する分子の通過路（水チャネル）が発見され，アクアポリン（AQP）と命名され，水分子についてもプロトン（$H^+$）や各種イオンのように原形質膜を介して輸送を行う分子が確定した。昆虫個体の生命維持の根幹に関わる細胞機能を解明するために，このAQPを中心にカイコ幼虫の浸透圧調節に関わる水分調節機構を検証し，水コントロールにおける鱗翅目幼虫（蛾・チョウ）の生理学的特徴をカイコ幼虫を用いて追究した。

（1）カイコ幼虫の成長の実体は絹タンパク質の生産を担う組織である絹糸腺にある。吐糸開始時に成長のクライマックスを迎え，幼虫体重（約5ｇ）の4割近くを占める絹糸腺では莫大量のシルク（液状絹）を貯留している。液状絹は30％にも達する高濃度タンパク溶液（ゲル状）であるので，そのpH調節が絹タンパク質の物性に必須であると考え，絹糸腺の成長発達過程における液状絹の実際のpHを調査した。盛食期（絹タンパク質生産期）には弱酸性（pH5～6）であったが，変態期（吐糸・繭形成期）には中性（pH7～8）に変化し，ゲル状態がゾル化（流動化）することが推定された。また，このpH調整機能に関わる能動輸送機構として，絹糸腺細胞には$H^+$の能動輸送機構（$H^+$ポンプ，V-ATPase）が存在関与していることを示した。カイコ幼虫が変態期に安定かつスムーズに吐糸営繭するために，pH環境維持機能と血液からの水輸送機能の双方が，絹タンパク質の溶液状態を規定する物理化学的要因として必須であると推論した。

（2）水輸送機能を担う2種類のAQP遺伝子を，カイコ幼虫の絹糸腺および消化管系の組織（中腸・後腸）からcDNAクローニングした。それぞれの配列から推定されるカイコAQPは，いずれもAQPの基本構造を包含しており，吸汁性昆虫（ヨコバイ）や吸血性昆虫（蚊・ハエ）で報告されているAQPとの相同類似性を示した。この2種類のカイコAQPは幼虫体内で組織特異的な分布を示し，1つは絹糸腺だけではなく排泄に与る後腸でたいへん強い遺伝子発現を示すタイプで，もう1つは消化吸収機能を担う中腸で発現するタイプで

あることがわかった。前者の AQP の主たる遺伝子発現組織が後腸であった事実は，カイコのようなイモムシの仲間（鱗翅目幼虫）が堅い固形粒状の糞を排泄することを説明し，後腸 AQP の生理的役割は食下物からの水吸収（水リユーズ）機能にあると推定された。鱗翅目幼虫は通常水飲み行動をとらないが，消化管末端，排泄前の腸管領域が AQP 分子を介した水リサイクルを実行することによって体内で必要とされている水を得ている，と説明することができる。

（3）絹糸腺における AQP の生理的役割を調査した。絹糸腺の大部分は絹タンパク質を生産する領域（後部絹糸腺）とそれを腺内腔で貯える領域（中部絹糸腺）が質・量共に圧倒的大部分を占め，口器で開口する吐糸口に連絡する導管部分（前部絹糸腺）は細管（長さ約3 cm）になっており，その領域で絹タンパク質分子の方向性が決定され，繊維としての物性を構築すると考えられている。その細管領域で後腸と同タイプの AQP の遺伝子発現は，絹糸腺全体の肥大成長と共に増大し，吐糸開始時（絹糸腺成長のピーク時）に極大となり，繭形成する過程で急減した。この絹糸腺 AQP は，AQP に対する特異的抗体を用いた免疫組織化学による細胞観察から，腺内腔に面した原形質膜に局在することがわかった。また，後部絹糸腺で大量生産された絹タンパク質が中部絹糸腺の腺内腔へ大量流入してくる領域でも AQP が原形質膜に分布することも示された。絹糸腺の肥大に伴って，絹タンパク質濃度が高まるので，それが腺内腔で非可逆的に固化することはカイコにとって吐糸不能に陥るので避けねばならない。絹糸腺では AQP が働いて血液より水供給することによって，液状絹の適正な水分維持管理がなされていると考えられた。つまり，先の（1）で示した pH と水の調節が絹糸腺にとって必要不可欠であると結論された。

哺乳類の AQP には13種類のアイソフォームがあり，臓器特異的な発現や水輸送だけにとどまらない多様な細胞生理機能が解明されつつある。カイコは比較的大型の昆虫であるので AQP の組織特異的解析が可能であった。昆虫の AQP 研究は植物汁液を吸汁する半翅目昆虫（ヨコバイ）の中腸からの cDNA クローニングで始まった（1996年）。それ以降，吸血性昆虫から遺伝子としていくつか同定されている。吸血行動は一過性であり，一度に大量の高濃度の動物血液が腸管内に流入してくるので，消化・排泄系の機能と浸透圧調節のしくみを解明すること，および病原媒介性昆虫の害虫制御を開拓することの2つの視点から研究が進められている。カイコのような鱗翅目昆虫は，幼虫時代には植物葉（時には農業作物）を断続的に長期に，しかも大量に摂取している（solid/plant feeder）。そのような飲水行動をとらない昆虫の水代謝研究について，これまで推測の領域を出なかった絹糸腺組織の細胞生理や，幼虫の排泄機構の分子生理について，長年昆虫生理学上の未解決であった課題に AQP のはたらきから説明付けることができた。

# 学位論文の基礎となる論文

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(2) Miyake, S. and Azuma, M. (2008) Developmental expression and the physiological role of aquaporin in the silk gland of *Bombyx mori*.
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# 参　考　論　文

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