

**The Roles of Cotton (*Gossypium hirsutum*)  
Aquaporins in Cell Expansion**

by

**Youmin Zhu**



Thesis submitted for the degree of Doctor of Philosophy

in

Biological Sciences

School of Environmental and Life Sciences

The University of Newcastle

Australia

March, 2016.



## DECLARATION

### *Statement of Originality*

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968.

---

Mr. Youmin Zhu



## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Professor Yong–Ling Ruan, Emeritus Professor John Patrick and Dr. Lu Wang for their academic guidance during my whole program of Ph.D study. Their passion of plant molecular biology and physiology has been a strong driving force during the project. They offered me key ideas, inspiration and encouragement in tackling with my thesis problems, which I deeply appreciate. Their critical reading and sharp comments made this dissertation more rigorous. Their enthusiasm and encouragement helped me get through the most difficult time of the program.

My thanks are extended to the other committee members for their careful reading and valuable comments on this dissertation.

Special thanks to Lei Ru, Yonghua Liu, Astija, Jun Li, Shengjin Liao, Jiashuo Yang, Xiaoguang Shang and Akiko Cook for their invaluable technical advice.

I am very much grateful to Conjoint Professor Christina Offler, Professor Christopher Grof, Associate Professor David McCurdy, Dr. Andy Eamens, Dr. Huiming Zhang, Jessie Hou, Suong Nguyen, Thomas Wu and Xue Xia for their help in technical advice.

I would like to express my gratitude to all other academics and students, too many to name who helped making this dissertation possible.

Finally, I thank my family and friends for their endless and unselfish support throughout. Particularly, I am grateful for the love and concerns of my wife Zhenhuan Liu, my parents Yuanhou Zhu and Shizhen Lu, my sisters Lihua Zhu and Lihui Zhu. Completing the Ph.D would have been impossible without their love and encouragement.



## TABLE OF CONTENTS

<b>DECLARATION.....</b>	<b>3</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>5</b>
<b>TABLE OF CONTENTS .....</b>	<b>i</b>
<b>ABBREVIATIONS .....</b>	<b>v</b>
<b>ABSTRACT .....</b>	<b>vii</b>
<b>CHAPTER 1: GENERAL INTRODUCTION .....</b>	<b>1</b>
<b>1.1 INTRODUCTION.....</b>	<b>2</b>
<b>1.2 TRANSCELLULAR PASSWAY MEDIATED BY AQPS IS THE MAJOR WATER     TRANSPORT PATHWAY .....</b>	<b>3</b>
<b>1.3 COTTON FIBRE .....</b>	<b>4</b>
<b>1.3.1 Cotton Fibre is an Ideal Model to Study Cell Elongation .....</b>	<b>5</b>
<b>1.3.2 Cotton Fibre is an Ideal Model to Study AQPs .....</b>	<b>6</b>
<b>1.4 AQPS ARE INVOLVED IN THE REGULATION OF FIBRE ELONGATION.....</b>	<b>7</b>
<b>1.5 AQUAPORINS.....</b>	<b>8</b>
<b>1.5.1 Classification of the Plant AQPs.....</b>	<b>8</b>
<b>1.5.2 Plant AQPs Expression .....</b>	<b>8</b>
<b>1.5.3 Subcellular Localization of AQPs.....</b>	<b>9</b>
<b>1.5.4 Hour–Glass Structure of AQPs for Water Transport .....</b>	<b>10</b>
<b>1.5.5 Water is the Major Substrate of AQPs .....</b>	<b>11</b>
<b>1.5.6 Water Transport Activity of AQPs .....</b>	<b>11</b>
<b>1.6 CELL EXPANSION .....</b>	<b>12</b>
<b>1.6.1 Determination of the Cell Expansion Rate .....</b>	<b>12</b>
<b>1.6.2 Potential Roles of AQPs in Cell Expansion.....</b>	<b>14</b>
<b>1.6.3 The Role of Solutes in Cell Expansion by Regulating AQPs.....</b>	<b>14</b>
<b>1.7 OTHER PHYSIOLOGICAL FUNCTIONS OF PLANT AQPs .....</b>	<b>14</b>
<b>1.8 ROLES OF AQPS IN ROOT HAIR EXPANSION AND POLLEN TUBE     ELONGATION .....</b>	<b>15</b>
<b>1.9 AIMS AND HYPOTHESES .....</b>	<b>16</b>
<b>1.9.1 Hypothesis.....</b>	<b>18</b>

1.9.2 Aims.....	18
<b>CHAPTER 2: <i>GhAQP</i> EXPRESSION ANALYSES AND INTRACELLULAR LOCALIZATION.....</b>	<b>19</b>
2.1 INTRODUCTION.....	20
2.2 MATERIALS AND METHODS .....	22
2.2.1 Plant Materials and Growth Conditions.....	22
2.2.2 Relevant Software Used.....	23
2.2.3 Media, Buffers and Solutions (see Appendix Page 175–177) .....	23
2.2.4 EST Numbers Analysis (from Jones <i>et al.</i> , unpublished) .....	23
2.2.5 Arabidopsis DNA Extraction .....	24
2.2.6 RNA Isolation .....	24
2.2.7 Semi–Quantitative PCR .....	25
2.2.8 Fibre Elongation Rate Measurement .....	25
2.2.9 Construct Design.....	26
2.2.10 Transformation (Heat Shock) for <i>E. coli</i> /Agrobacteria.....	32
2.2.11 Plant Transformation .....	32
2.2.12 Confocal Laser Scanning Microscopy (CLSM).....	33
2.2.13 Microscope Slide Preparation and Plasmolysis.....	33
2.3 RESULTS .....	33
2.3.1 Pre–analyses to Identify Candidates .....	33
2.3.2 Bioinformatics Analysis of Nucleotide and Amino Acid Sequences .....	34
2.3.3 Expression Patterns of <i>GhPIPs</i> and <i>GhTIPs</i> .....	39
2.3.4 The Sub–cellular Localization of GhPIP2;3 and GhTIP1;2 in Arabidopsis Roots .....	44
2.3.5 The Sub–cellular Localization of GhPIP2;3 and GhTIP1;2 in Tobacco Leaves.....	47
2.4 DISCUSSION .....	52
2.4.1 Bioinformatics Analyses Identified Candidate GhAQPs.....	52
2.4.2 Candidate <i>GhAQPs</i> may Play Roles in Fibre Elongation.....	54
2.4.3 Intracellular Localization Reinforces GhAQPs’ Fibre Expansion Role .....	56
<b>CHAPTER 3: OVEREXPRESSION AND COMPLEMENTATION OF <i>GhAQPs</i> IN ARABIDOPSIS..</b>	<b>59</b>
3.1 INTRODUCTION.....	60
3.2 MATERIALS AND METHODS .....	62
3.2.1 Plant Material and Growth Conditions .....	62



3.2.2 Website Resources and Useful Software .....	63
3.2.3 Standard PCR .....	63
3.2.4 qPCR .....	63
3.2.5 Gene Constructs and Transformation .....	64
3.2.6 Screening for Homozygous T–DNA Insertion Lines.....	65
3.2.7 Root, Root Cell and Pollen Tube Length Measurements .....	66
3.2.8 Water Loss Rate Measurement.....	67
3.3 RESULTS .....	67
3.3.1 The Role of GhPIP2;3 in Cell Expansion.....	67
3.3.2 The Role of GhTIP1;2 in Cell Expansion .....	96
3.4. DISCUSSION .....	105
3.4.1 GhPIP2;3 Functions as a PIP <i>in Planta</i> .....	105
3.4.2 GhPIP2;3 Played Important Roles in Cell Expansion.....	105
3.4.3 GhPIP2;3 Played a Role in Cell Expansion by Increasing Water Transport	106
3.4.4 Na <sup>+</sup> and Cl <sup>-</sup> Toxicity were All Involved in Root Length Reduction .....	107
3.4.5 GhPIP2;3 might be Required for NaCl Resistance.....	108
3.4.6 GhTIP1;2 Functioned as a TIP <i>in Planta</i> .....	109
3.4.7 GhTIP1;2 Function on Cell Expansion .....	109
3.4.8 GhTIP1;2 may Transport both Water and Urea .....	110
3.4.9 GhTIP1;2 and GhPIP2;3 Play different Roles .....	111
<b>CHAPTER 4: THE CELLULAR AND MOLECULAR BASIS OF THE OBSERVED GhAQPS–MEDIATED PHENOTYPES .....</b>	<b>115</b>
4.1 INTRODUCTION.....	116
4.2 MATERIALS AND METHODS .....	119
4.2.1 Plant Material and Growth Conditions .....	119
4.2.2 Fresh and Dry Weight Determination.....	119
4.2.3 Measurement of Root Hair Length and Density .....	119
4.2.4 Tissue Collection for RNA Extraction and qPCR Analyses.....	119
4.2.5 RNA Extraction and cDNA Synthesis.....	120
4.2.6 qPCR.....	120
4.2.7 Co–expression Analyses.....	121
4.3 RESULTS .....	121
4.3.1 Overexpression of <i>GhPIP2;3</i> Increased Fresh and Dry Weights .....	121

4.3.2 Overexpression of <i>GhPIP2;3</i> Increased Root Hair Length.....	122
4.3.3 Overexpression of <i>GhPIP2;3</i> Increased Root Hair Density .....	128
4.3.4 Screening Sugar and K <sup>+</sup> Transporters .....	133
4.3.5 Overexpression of <i>GhPIP2;3</i> Increased the Expression of Sugar and K <sup>+</sup> Transporters in Roots.....	138
4.3.6 Overexpression of <i>GhTIP1;2</i> Increased the Expression of Sugar Transporters in Pollen Tubes .....	140
4.3.7 Overexpression of <i>GhPIP2;3</i> Increased Salt Tolerance.....	141
4.4 DISCUSSION .....	144
4.4.1 Overexpression of <i>GhPIP2;3</i> and <i>GhTIP1;2</i> Increased Root and Pollen Tube Elongation Respectively by Increasing Water and Solute Transport .....	144
4.4.2 Overexpression of <i>GhPIP2;3</i> Increased Biomass probably by Enhancing Water Transport, Transpiration and Photosynthesis .....	147
4.4.3 Overexpression of <i>GhPIP2;3</i> Promoted Root Hair Elongation, Probably by Regulating Some Root Hair Elongation Related Genes .....	149
4.4.4 Overexpression of <i>GhPIP2;3</i> Increased Root Hair Density Possibly by Regulating Root Hair Density Related Genes .....	151
4.4.5 Overexpression of <i>GhPIP2;3</i> Increased Salt Tolerance Possibly by Reducing Salt Toxicity Through Facilitating Water Influx, Increasing Root Hair Length and Density and Regulating some Salt Tolerance Genes.....	151
<b>CHAPTER 5: GENERAL DISCUSSION .....</b>	<b>155</b>
5.1 <i>GhPIP2;3</i> AND <i>GhTIP1;2</i> PLAY IMPORTANT ROLES IN CELL EXPANSION .....	156
5.2 <i>GhPIP2;3</i> AND <i>GhTIP1;2</i> FUNCTION AS AQPs <i>IN PLANTA</i> .....	159
5.3 THE ROLES OF <i>GhPIP2;3</i> IN REGULATING ROOT HAIR ELONGATION AND DENSITY.....	160
5.4 OVEREXPRESSION OF <i>GhPIP2;3</i> INCREASED SALT TOLERANCE .....	161
5.5 <i>GhTIP1;2</i> AND <i>GhPIP2;3</i> PLAY DIFFERENT ROLES .....	163
<b>APPENDICES .....</b>	<b>165</b>
<b>REFERENCES.....</b>	<b>179</b>

## ABBREVIATIONS

Ψ <sub>g</sub>	Gravitational force	PD	Plasmodesmata
Ψ <sub>p</sub>	Hydrostatic pressure (turgor)	PFN1	Profilin 1
Ψ <sub>s</sub>	Osmotic (solute) potential	PIP	Plasma membrane intrinsic protein
Ψ <sub>w</sub>	Water potential	PRP3	Proline-rich protein 3
ABA	Abscissic acid	PVP	Polyvinylpyrrolidone
AQP	Aquaporin	Q-PCR	Quantitative PCR
AUX1	Auxin transporter protein 1	Rd29A	Responsive to desiccation 29A
C	Cotyledon	RHD6	Protein ROOT HAIR DEFECTIVE6
CBF3	CRT-binding factor 3	RNA	RiboNucleic Acid
cRNA	Complementary RNA	SAT32	Protein salt tolerance 32
CTAB	Cetyl Trimethyl Ammonium Bromide	SDS	Sodium dodecyl sulphate
DEPC	Diethyl parocarbonate	STP1	Sugar transporter 1
DAA	Days after anthesis	SUT	Sucrose transporter
DNA	Deoxyribose nucleic acid	Taq	Thermus Polymerase
dNTP	DeoxynucleoSide Triphosphate	T-DNA	Transfer DNA
DTT	Dithiothreitol	TE	Tris-EDTA-NaCl buffer
EDTA	Ethylene diaminetetraacetic acid	TIP	Tonoplast intrinsic protein
EST	Expressed Sequence Tags	Tris	Tris (hydroxymethyl) aminomethane
ETR1	Ethylene receptor 1	UTR	Untranslanton region
ETO1	Ethylene-overproduction protein 1	X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside
GFP	Green fluorescent protein	YEP	Yest-extract peptone
GUS	β-Glucuronidase	β-Me	β-Mercaptoethanol
ICAC	International cotton advisory committee	bp	Base pair
KEA5	K <sup>+</sup> efflux antiporter 5	g	Gram
KUP5	K <sup>+</sup> uptake permease 5	h	H(s)
LB	lysogeny broth culture medium	L	Litre
<i>L<sub>p</sub></i>	Hydraulic conductivity	M	Molar
MIP	Major Intrinsic Protein	min	Minute(s)
mRNA	Massager RNA	rpm	revolutions per minute
NCBI	National Center for Biotechnology Information	k	kilo 10 <sup>3</sup>
NIP	NOD26-like Intrinsic Protein	c	centi 10 <sup>-2</sup>
OD	Optical density	m	milli 10 <sup>-3</sup>
ORF	Open Reading Frame	μ	micro 10 <sup>-6</sup>
PCR	Polymerase Chain Reaction	n	nano 10 <sup>-9</sup>



## ABSTRACT

Aquaporins (AQPs), with a major function as water channels, may play important roles in cell expansion. However, the roles of GhAQPs (*Gh*, *Gossypium hirsutum*) in cell expansion have not been clearly clarified. To address the roles of GhAQPs in plant cell expansion, this study aimed at identifying candidate *GhAQPs* involved in cotton fibre expansion by exploring their expression patterns and sub-cellular localization, examining their physiological roles *in vivo*, and exploring the cellular and molecular basis of the observed *GhAQPs*-mediated phenotypes.

EST contigs were analysed by searching *GhAQPs* with high EST numbers in the cotton EST database ([www.cottondb.com](http://www.cottondb.com)) to generate a preliminary selection of eight candidate *GhAQPs* possibly involved in cotton fibre elongation. These were four *GhPIPs* (*GhPIP1;2*, *GhPIP1;3*, *GhPIP2;3*, *GhPIP2;4*) and four *GhTIPs* (*GhTIP1;1*, *GhTIP1;2*, *GhTIP1;4*, *GhTIP2;1*). Semi-quantitative transcript analyses reviewed that *GhAQPs* were highly expressed during fibre expansion (5–15 days after anthesis, DAA) and relatively lower during the post expansion phase (~20 DAA onwards), which was generally consistent with temporal changes in rates of fibre elongation. These *GhAQPs* were also highly expressed in other expanding organs and more lowly expressed in expanded organs. *GhPIP2;3* and *GhTIP1;2* exhibited the highest transcript levels of all candidates, suggesting that *GhPIP2;3* and *GhTIP1;2* played a major role in cell expansion of cotton fibre and other organs, and thus were targeted as fibre expansion representatives for more detailed study. *GhAQPs* fused with green fluorescent protein (GFP) constructs were transformed into WT Arabidopsis by floral dipping to determine the intracellular localization of GhAQPs. In addition, a parallel experiment was designed in which *GhAQPs* fused with red fluorescent protein (RFP) constructs were transformed into WT Arabidopsis and Arabidopsis transformed by a tonoplast marker *ShMTP* (magnesium transporter proteins)-*GFP*. For *GhPIP2;3* and *GhTIP1;2*, a putative plasma membrane or tonoplast intracellular localization was discovered, respectively. There is a strong link between AQP localization and their functions. The cell membrane localization of *GhPIP2;3* and *GhTIP1;2* implied that these water channels would increase the permeability of the cell membranes, which reinforces their role in cell expansion.

To examine the physiological role of identified *GhAQPs in vivo*, a complementation and an overexpression experiments were performed by transforming *GhPIP2;3/GhTIP1;2* into Arabidopsis (a time-saving model to study GhAQPs' function) T-DNA insertion lines *atpip2;3-1/attip1;3* and WT respectively. The mutants *atpip2;3-1* and *attip1;3* exhibit reduced root length compared to WT when grown in the standard ½ MS medium or a medium containing 100 mM NaCl and reduced pollen tube length when grown in the standard medium or a medium without nitrogen (NO<sub>3</sub><sup>-</sup>) respectively. In this study, overexpression of *GhPIP2;3* in *pip2;3-1* and overexpression of *GhTIP1;2* in *tip1;3* respectively complemented the mutants' short root and pollen tube lengths respectively in the standard medium or in other media (osmotic/salt stress and without nitrogen), which demonstrated that GhPIP2;3 and GhTIP1;2 really functioned as a PIP and a TIP respectively *in planta* (translated into functional proteins after transformation). Overexpression of *GhPIP2;3* and *GhTIP1;2* in WT Arabidopsis exhibited phenotypes of increased root/root cortex cell and pollen tube lengths respectively in ½ MS medium alone as well as modified ½ MS media causing osmotic/salt stress and nitrogen deficiency, suggesting that GhPIP2;3 and GhTIP1;2 played important roles in cell expansion. During cell expansion, GhAQPs were found to confer salt tolerance by increasing water transport and diluting salt ions. It was also observed that the Arabidopsis root length didn't show significant differences between their growth under NaCl and KCl stresses except the mutant showed shorter root length in 100 mM NaCl medium compared with in 100 mM KCl medium, suggesting Cl<sup>-</sup> might be the major ion performing toxicity and leading to the root reduction while Na<sup>+</sup> toxicity also played some roles in root reduction. Three lines overexpressing *GhPIP2;3* in WT showed significantly less reduced root length compared with WT in ½ MS + 100 mM NaCl medium compared with in ½ MS + 200 mM sorbitol medium, suggesting overexpression of *GhPIP2;3* increased salt tolerance.

In exploring the molecular basis of the observed increased root and pollen tube elongation in the lines overexpressing *GhPIP2;3* and *GhTIP1;2* respectively, a new phenomenon was found that overexpression of *GhPIP2;3* and *GhTIP1;2* could increase the expression of some sugar transporters (*ERD6* for *GhPIP2;3*, *ERDL6* and *STP11* for

*GhTIP1;2*) and K<sup>+</sup> transporters (*KCI* and *SKOR* for *GhPIP2;3*) (except that *SWEET17* was decreased in overexpression of *GhPIP2;3* lines, as decreasing the expression of *SWEET17* might help to maintain cytoplasmic sugar homeostasis or maintain the solutes concentration in the vacuole during cell expansion). The roles of *GhPIP2;3* and *GhTIP1;2* in cell expansion might be due to their water transport activity combined with their impact on elevating sugar and K<sup>+</sup> transporter expression that lowered cell water potentials with the overall effect of increasing turgor pressure to drive cell expansion. Another new discovery was that root hair length of the lines overexpressing *GhPIP2;3* in WT were significantly increased compared with WT grown on MS medium containing 100 mM NaCl (½ MS + 100 mM NaCl). Meanwhile, three lines overexpressing *GhPIP2;3* in *pip2;3-1* background complemented the short root hair phenotype in the mutant in the ½ MS medium, and three lines overexpressing *GhPIP2;3* in WT increased root hair length compared with WT in the ½ MS medium. At a molecular level overexpression of *GhPIP2;3* was found to increase expression of root hair elongation related genes including *AUX1* (auxin transport), *ETR1* (ethylene receptor), *Myosin XIK* (Myosin XI) and *EPC1* (Glycosyltransferase), and some hair density related genes such as *IAA17* (Repressor of auxin-responsive transcription), *PRP3* (Proline-rich cell wall protein), *RHD6* (Protein ROOT HAIR DEFECTIVE6) and *RHL1* (Topoisomerase subunits). These findings suggested that overexpression of *GhPIP2;3* might increase root hair elongation and hair density by inducing expression of these root hair elongation related genes (in addition to increasing cell turgor for hair elongation by enhancing water transport) and root hair density related genes. The molecular basis for the role of *GhPIP2;3* on salt tolerance was also explored. Among the salt tolerance genes chosen from NCBI and published papers, three salt tolerance genes, *Rd29A* (Responsive to desiccation 29A), *SAT32* (Protein salt tolerance 32) and *SOS1* (Sodium/hydrogen exchanger 7) were found to be higher expressed in the lines overexpressing *GhPIP2;3* than in WT. It was proposed that overexpression of *GhPIP2;3* increased salt tolerance probably by a combination of increased water transport, increased root hair length and density, overlaid by enhancing expression of salt tolerance genes. Furthermore, one interesting discovery was that overexpression of *GhPIP2;3* increased stomatal density, which might increase CO<sub>2</sub> assimilation, then contributed to transpiration and photosynthesis.

