이학석사학위논문

미나리아재비속 식물의 기공 발달 기작 연구

Molecular Analysis of Stomatal Development in *Ranunculus*

2016년 2월

서울대학교 대학원

생명과학부

경 진 슬

ABSTRACT

Molecular analysis of stomatal development in *Ranunculus*

Jinseul Kyung

School of Biological Sciences

The Graduate School

Seoul National University

Several aquatic or wetland plants including *R. trichophyllus*, have stomata mostly on the adaxial side. Such an adaxial-side-specific distribution of stomata is named epi-stomy. Epi-stomy is an adaptive strategy for wetland plants because stomata are the entry sites where pathogens infect plants and pathogens invade plants more easily if stomata were located at abaxial side. The molecular mechanism underlying epi-stomy is unknown yet. I hypothesized that epi-stomy in R. trichophyllus is mediated by auxin and leaf polarity genes. Here, I compared the stomatal densities and distributions of *R. trichophyllus* and 3 of its relative species. *R. trichopyllus* and *R. sceleratus* are epi-stomatous while *R. japonicus* is hypo-stomatous.

Then I treated auxin and auxin transport inhibitor, NPA, to *R. trichophyllus*. Stomatal distribution was significantly altered after the NPA treatment. To elucidate the molecular mechanism regulating stomatal distribution, I cloned *R. trichophyllus* genes which regulate stomatal development, including *RtSTO* and *RtSPCH*. Then I analyzed the spatial expression pattern of *RtSTO* and *RtSPCH*. Both of the genes are expressed specifically on adaxial side. In addition, *RtSTO* is negatively regulated by auxin. In addition, *A. thaliana* transformants overexpressing leaf polarity genes of *R. trichophyllus* showed altered stomatal density and distribution. Based on those findings, I concluded that (1) Auxin and leaf polarity genes function as signals with spatial information (2) Such patial signals induce the differential expression of *RtSTO* and *RtSPCH* and epi-stomy.

Keywords : stomatal development, leaf polarity, *SPEECHLESS, STOMAGEN*
Student Number : 2014-20314

CONTENTS

ABSTRACT ····· i

CONTENTS ····· iii

LIST OF FIGURES ····· v

LIST OF TABLES ····· vi

ABBREVIATIONS ····· vii

1. INTRODUCTION ····· 1

1.1 Molecular mechanism regulating stomatal development ····· 1

1.2 Stomatal distribution on adaxial and abaxial sides ····· 2

1.3 Molecular mechanism regulating leaf polarity ····· 4

1.4 Ranunculus as the study material ····· 5

1.5 Purpose of the study ····· 5

2. MATERIAL AND METHODS ····· 7

2.1 Plant materials and growth condition ····· 7

2.2 Observation of epidermis and measurement of stomatal density 9

2.3 Hormone treatment ····· 9

2.4 RNA sequencing and gene cloning ····· 10

2.5 *in-situ* RNA hybridization ····· 11

2.6 RNA extraction and qRT-PCR ····· 11

2.7 Construction of transgenic lines ····· 12
2.8 Phylogenetic analyses ····· 12

3. RESULTS ····· 13
3.1 Stomatal distributions of *Ranunculus* species are diverse ····· 13
3.2 Disruption of auxin distribution alters stomatal distribution ··· 16
3.3 Genes regulating stomatal development were cloned from *R. trichophyllus* ····· 19
3.4 Genes inducing stomatal development are expressed higher on adaxial side ····· 23
3.5 Auxin down-regulates genes inducing stomatal development ··· 25
3.6 Overexpression of leaf polarity gene alters stomatal density and distribution ····· 30

4. DISCUSSION ····· 33

REFERENCES ····· 38
ABSTRACT IN KOREAN ····· 47

LIST OF FIGURES

Figure 1. *Ranunculus* species used in this study

Figure 2. Epidermal morphology and stomatal density among *Ranunculus* species

Figure 3. Effect of 2,4-D and NPA treatment on the stomatal development in *R. trichophyllus*

Figure 4. Conservation of genes regulating stomatal development in *R. trichophyllus.*

Figure 5. Expression pattern of *RtSPCH* and *RtSTO*

Figure 6. Effect of auxin on the expression of the genes regulating stomatal development

Figure 7. Effects of overexpressing leaf polarity genes on distribution.

Figure 8. Working model on stomatal distribution in *R. trichophyllus*

LIST OF TABLES

Table 1. Candidate genes regulating stomatal development in *R. trichophyllus*

Table 2. *cis*-elements found in *RtSTO.*

Table 3. *cis*-elements found in *RtSPCH*

Table 4. Candidate genes regulating leaf polarity in *R. trichophyllus*

ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
3'	three prime end of DNA fragment
5'	five prime end of DNA fragment
bHLH	basic helix-loop-helix
ICE1	*INDUCER OF CBP EXPRESSION 1*
KAN	*KANADI*
NPA	1-N-Naphthylphthalamic acid
ORF	open reading frame
qRT-PCR	quantitative realtime polymerase chain reaction
REV	*REVOLUTA*
SPCH	*SPEECHLESS*
STO	*STOMAGEN*
UTR	untranslated region
YAB	*YABBY*

1. INTRODUCTION

1.1 Molecular mechanism regulating stomatal development

Stomata are epidermal valves regulating gas exchange (Wang *et al*., 2007). The early evolution of stomata before the evolution of plant organs suggests the physiological importance of stomata (Freeman, 2008). In *A. thaliana,* development of stomata is organized by sequential differentiation of a specialized epidermal cell lineage (Pillitteri and Torii, 2012). These stomatal lineages are classified into five major cell types : meristemoid mother cells, meristemoids, stomatal lineage ground cells, guard mother cells and guard cells (Lau and Bergmann, 2012).

Several genes required for the transitions between the cell types in the stomatal lineages are identified in *A. thaliana*. Three basic helix-loop-helix (bHLH) transcription factors, *SPEECHLESS (SPCH), MUTE* and *FAMA* are major examples. Expression of *SPCH* initiates the entry into the stomatal lineage (MacAlister *et al*. 2007; Pillitteri *et al*. 2007). SPCH induces the formation of meristemoid mother cells and the asymmetric entry division, as well as the subsequent asymmetric amplifying and spacing divisions. (MacAlister *et al*., 2007; Robinson *et al*., 2011; Pillitteri *et al*., 2007). MUTE induces the differentiation of meristemoids into guard mother cells. FAMA promotes the terminal cell division and differentiation of GMCs into GCs (Ohashi-Ito and Bergmann, 2006). In the recent studies using model monocots and basal land plants revealed that the evolution of

of SPCH, MUTE and FAMA predates the divergence of monocots and dicots and the function of these proteins are conserved in stomatal differentiation. (Peterson *et al*., 2010)

In addition to the transcription factors, several signaling peptides are known to regulate stomtal development. EPF1 and EPF2 are stomatal lineage-specific factors and they repress stomatal development (Hara *et al*., 2007; Hunt and Gray, 2009; Hara *et al*., 2009). Another EPFL-family member STOMAGEN is expressed in the mesophyll tissue below the epidermis and promotes stomatal production (Sugano *et al*., 2010; Kondo *et al*., 2010). Interestingly, *STOMAGEN* is negatively regulated by auxin through the binding of MONOPTEROS to the *STOMAGEN* promoter (Zhang *et al*., 2014)

1.2 Stomatal distribution on adaxial and abaxial sides.

Plants are classified into 3 categories based on the distribution of stomata. In amphi-stomatous plants, stomata are evenly distributed on the adaxial and abaxial side. In epi-stomatous plants, most of the stomata are distributed on the adaxial side. In contrast, most of the stomata re distributed on the abaxial side in hypo-stomatous plants (Guilioni *et al*., 2008). Most plants are either amphi-stomatous or hypo-stomatous (Mott *et al*., 1982). Some aquatic plants, like water lilies are epi-stomatous (Sreelakshmi *et al*., 2014). Several environmental or anatomical factors regulating stomatal distribution were suggested. Plants inhabiting under high light intensity tend to be amphi-stomatous. (Mott *et al*., 1982). In addition,

lower precipitation (Muir *et al*., 2015) and higher altitude (Woodwardt *et al*., 1982) are also associated with amphi-stomy. Plants with thicker leaves tends to be amphi-stomatous for better diffusion of carbon dioxide gas. (Parkhurst *et al*., 1978).

In *A. thaliana,* which is amphi-stomatous, molecular mechanism regulating stomatal density and distribution is well studied. Two genes which regulate stomatal development, *GTL1* and *STO,* are known to be regulated by leaf polarity. *GTL1,* which negatively regulates *SDD1* regulation, is expressed only in the abaxial epidermis (Yoo *et al*., 2010). In contrast, *STO* regulates light-induced stomatal formation only in the adaxial epidermis (Hronkova *et al*., 2015). Stomatal distribution on the adaxial and the abaxial side is also studied in species other than *A. thaliana*. In a genome-wide association studies (GWAS) in poplar tree (Mckown *et al*., 2014), candidate genes associated with hypo-stomy were suggested. They include adaxial-abaxial polarity gene (*PHABULOSA*), stomatal development gene (*BRASSINOSTEROID INSENSITIVE 2*) and disease/wound-response genes (*GLUTAMATE-CYSTEINE LIGASE*). From the study of chinese cabbage, the ectopic expression of BraYAB1-702, one of the leaf polarity genes in *A. thaliana,* significantly increased stomatal density (Zhang *et al*., 2013).

Hypo-stomy is suggested to be the ancestral characteristic compared to amphi- or epi-stomy (Willmer *et al*., 1996). The evolution from hypo-stomy to amphi-stomy is also shown in Ranunculaceae (Hoot *et al*., 1991) Since the intercellular diffusion in hypo-stomatous leaves is less efficient thant the intercellular diffusion in amphi-stomatous leaves (Parkhurst *et al*., 1990), amphi-stomy might evolved for better photosynthetic capacity.

However, there are trade-offs in adaxial stomata since the stomata are major point of entry for plant pathogenic bacteria (Huang *et al*., 1986). From the study of plant domestication, wild species tend to be more dependent to abaxial stomata compared to the domesticated species (Milla *et al*., 2013). In addition, study of wild tomatoes suggests that such trait transition during the domestication could have involved a few large-effect genetic changes, allowing rapid responses to new environmental conditions (Muir *et al*., 2014).

1.3 Molecular mechanism regulating leaf polarity

Leaf adaxial-abaxial polarity is established by leaf polarity genes. These genes encode transcription factors and small RNAs. The class III *HOMEODOMAIN - LEUCINE ZIPPER* (*HD-ZIP III*) genes function in adaxialization. The *HD-ZIP III* genes are expressed in adaxial domain and induces the development of adaxial side (Emery *et al*., 2003; Williams *et al*., 2005). In contrast, microRNAs 165 and 166 (MiR165/166) and *KANADI* (KAN) genes induces abaxial development.

In addition to leaf polarity genes, auxin functions in leaf adaxial-abaxial patterning. Several evidences supports this. (1) Auxin promotes abaxial cell fate in early leaf primordia. Transient low auxin zone is developed in the adaxial domain and this auxin signal induces MONOPTEROS-mediated abaxial cell fate (Qi *et al*., 2014). (2) *KANADI* controls the transport and auxin signaling through its target genes (Merelo *et al*. 2013). (3) *REV* induce auxin biosynthesis via the tryptophan-dependent

indole-3-Pyruvic acid (IPA) biosynthetic pathway (Brandt *et al*., 2012). (4) Auxin also regulates the activity of *ARF4,* which regulates leaf polarity at the post-translational level. (Kidner *et al*., 2010)

1.4 *Ranunculus* as the study material

Ranunculus L. (Ranunculaceae) is a well-studied genus which is a candidate model for the study of plant adaptations from terrestrial to aquatic habitats (Chen *et al*., 2015). Broad divergence resulted from the adaptation to various environment makes *Ranunculus* an interesting material for a research. The genus consists of approximately 360 species. Recent study suggests that aquatic *Ranunculus* clades are monophyletic (Horandl *et al*., 2012). *Ranunculus* species inhabits various terrestrial or aquatic habitats from lowlands to high alpine zones, and terrestrial species are often specialized in extreme conditions (Horandl *et al*., 2005). Various morphological adaptations and different reproductive strategies may be important for their capability to colonize habitats at higher altitudes and latitudes. (Horandl *et al*., 2005)

1.6 Purpose of the study

The purpose of this research is to elucidate the molecular mechanism underlying epi-stomy in *R. trichophyllus.* Although the evolutionary history and adaptative trade-offs of stomatal distribution have been studied for decades, molecular mechanism underlying stomatal

distribution was seldomly studied. This would be due to the fact that *A. thaliana* which is a model-species does not show differential distribution of stomata. Thus, my study will provide a model for a novel molecular mechanism regulating stomatal distribution which is also evolutionarily important.

2. MATERIAL AND METHODS

2.1 Plant materials and growth condition

Mature seeds of *R. trichophyllus* var. kadzusensis were collected from its native habitat at Ganghwa Island, South Korea by Insu Jo (2009). Seeds of *Ranunculus sceleratus, Ranunculus japonicus, Ranunculus chinensis* were donated from the Korea National Arboretum. *Ranunculus* species used in this study are shown in figure 1. *R. trichophyllus* and *R. sceleratus* seeds were sterilized for 1 min in 70% ethanol, then washed four times with distilled water. After washing, seeds were treated with 1% NaOCl and 0.5% Tween-20 (Sigma Aldrich, P9416) four times. Seeds were washed a further five times with distilled water, then sowed on half-strength Murashige-Skoog (MS) medium containing 50 μM carbenicilin, 75 μM cefotaxim, and 0.8% agar. R. *chinenesis* and *R. japonicus* seeds were sawed on the pot and placed in a 4℃ cold chamber for 3 weeks, and transferred to the growth room. For experiments involving *Arabidopsis thaliana,* Col-0 seeds were used. The growth room was maintained at 22℃, 60 ± 10% relative humidity in a long day photoperiod (16h light / 8h dark).

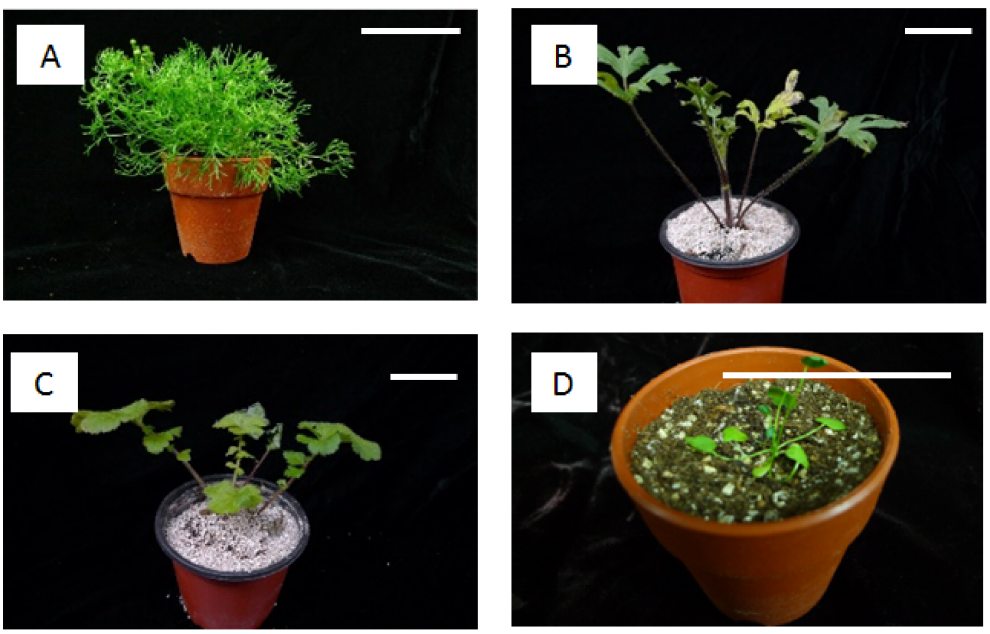


Figure 1. *Ranunculus* species used in this study (A-D) *R. trichophyllus* (A), *R. japonicus* (B), *R. chinensis* (C), *R. sceleratus* white bar = 5cm

2.2 Observation of epidermis and measurement of stomatal density

For epidermis observation and stomatal density measurement of *Ranunculus* species, cotyledons of about 1cm and first true leaves of about 2cm long harvested. For the observation and measurement of *A. thaliana,* mature rosette leaves were harvested. Harvested leaves were soaked in clearing solution (2.5g chloral hydrate; 0.3ml 100% glycerol; 0.7ml distilled water). After incubation for several hours under 55℃, epidermis was observed using an Axio Imager A1 microscope (Carl Zeiss) under DIC optics and were photographed using an AxioCam HRc camera (Carl Zeiss). Stomatal density was calculated from the number of stomata in each frame per area of each frame (mm^2)

2.3 Hormone treatment

Seeds or Seedlings of *R. trichophyllus* were used for hormone treatment. For the measurement of stomatal density in cotyledons, seeds were sawed to half-strength MS medium with 0.25μM, 0.5μM, 1μM 2,4-D, 20μM, 40μM NPA (1-N-Naphthylphthalamic acid). For the measurement of stomatal density in true leaves, seedlings were transplanted to the medium with 2,4-D (0.2μM, 0.4μM, 0.6μM), or NPA (2μM, 4μM, 6μM). For qRT-PCR, seedlings were transplanted to the medium with 0.1μM, 0.2μM, 0.4μM 2,4-D for 2 weeks.

2.4 RNA sequencing and gene cloning

The total RNA of *R. trichophyllus* leaves were subjected to transcriptome sequencing with the Illumina platform. A total of 189,241,664 clean reads with 19.11Gb nucleotides were generated. Based on those raw data, 183,520 transcripts were assembled and analysed.

Candidate genes of *Arabidopsis thaliana* were chosen, and the sequences from TAIR acquired (www.arabidopsis.org). The *Arabidopsis* sequences were compared to the Aquilegia formosa database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=aquilegia) and *Aquilegia coerulea* database (http://www.phytozome.net/search.php?method=Org_Acoerulea) because *Aquilegia* genus plants are closest to the *Ranunculus* genus among plants that have a sequenced database. Partial sequences of *Aquilegia* orthologs were obtained, and then primers were generated in a conserved region. Partial sequences of *R. trichophyllus* were amplified using Phusion® High-Fidelity DNA Polymerase (NEB, M0530) and cloned into pCR®2.1-TOPO® (Invitrogen, INV-45-0641) for sequencing.

To clone the total ORF sequence, a smarter race cDNA amplification kit (Clontech, 634923) was used. Primer sets were generated based on partial sequences of *R. trichophyllus* and *R. sceleratus.* For the study, 5’ and 3’ cDNA fragments were amplified using Ex Taq (TaKaRa, RR001A) and subsequently sequenced. To avoid any PCR errors, I prepared primers sets that recognized 5’ UTR and 3’ UTR, and then subsequent

cloning steps were processed.

2.5 *in-situ* RNA hybridization

To compare the transcription level of 3 genes regulating stomatal development (*RtSTO, RtSPCH, RtICE1*), *in-situ* RNA hybridization was performed. The whole experiment was performed based on the published protocol (Brewer *et al*., 2006). Each gene-specific dioxygenin-tagged RNA probe about 500bp long was used. Seedlings with 1st true leaf were prepared. Leaves were transverse-sectioned to compare the expression level on adaxial and abaxial side.

.

2.6 RNA extraction and qRT-PCR

For real-time quantitative polymerase chain reaction (qPCR), the total RNA was isolated using TRI reagent (Sigma Aldrich, T9424). cDNA was generated from 4 μg of total RNA using reverse transcriptase (Fermentas, EP0442) and oligo(dT). After reverse-transcription, the products were diluted with distilled water, and then the dilution ratios were 1/12 each. PCR reactions were performed with each 4 μl diluted cDNA as a template. 0.3 μl of synthesized cDNA was mixed with 2 μl of 5 μM primers and 10 μl of SYBR Green qPCR Master Mix (Bio-Rad), and ddH2O to 20 μl. Real-time-qPCR analysis was performed by CFX96 Real-Time PCR system (Bio-Rad). The relative transcript levels were

calculated according to the ΔΔCt method (Livak and Schmittgen, 2001).

2.7 Construction of transgenic lines

For the construction of transgenic lines over-expressing leaf polarity genes, ORF sequence of *RtREV* was cloned into myc-pBA vectors. *RtKAN-a, -b, -c, RtYAB1* were cloned into pCAMMBIA3300 vector. Those construct was transformed into *A. thaliana* Col-0. Transgenic lines were selected by proper antibiotics.

2.8 Phylogenetic Analyses

Multiple alignments of amino acid sequences were performed by ClustalX2.1 program (http://www.clustal.org/download/current/), generating aligned phy format files. These aligned files were passed through PHYLIP program (version 3.69), which was used for phylogenetic analyses (http://evolution.genetics.washington.edu/phylip.html). In the PHYLIP software, SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE program were sequentially run to generate draft unrooted phylogenetic trees and to obtain bootstrap values. The phylogenetic tree was drawn using the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3. RESULTS

3.1 Stomatal distributions of *Ranunculus* species are diverse

To confirm stomatal distribution of *Ranunculus* species, I measured stomatal density on adaxial and abaxial side of the cotyledon and true leaf (Figure 1). In *R. trichophyllus,* most of the stomata are distributed on the adaxial side. In the cotyledon, no stoma is observed on the adaxial side. In the true leaf, adaxial stomatal density is about 25-fold higher than the abaxial stomatal density. Other *Ranunculus* species showed diverse types of stomatal distribution. Similiar to *R. trichophyllus, R. sceleratus,* a sister specie of *R. trichophyllus,* adaxial stomatal density is significantly higher than abaxial stomatal density. However, a relative ratio of adaxial stomatal density to abaxial stomatal density of *R. sceleratus* is lower than that of *R. trichophyllus.* In *R. japonicus,* stomatal density was significantly higher on the abaxial side. In *R. chinensis,* stomatal density of adaxial and abaxial side was not significantly different, indicating they are more like amphi-stomatous

One interesting aspect of epidermis in *R. trichophyllus* was that the precursor of guard cells, meristemoid and guard mother cells, were not observed on the abaxial side. This suggests that the inhibition of stomatal development on abaxial side starts from the early stage.

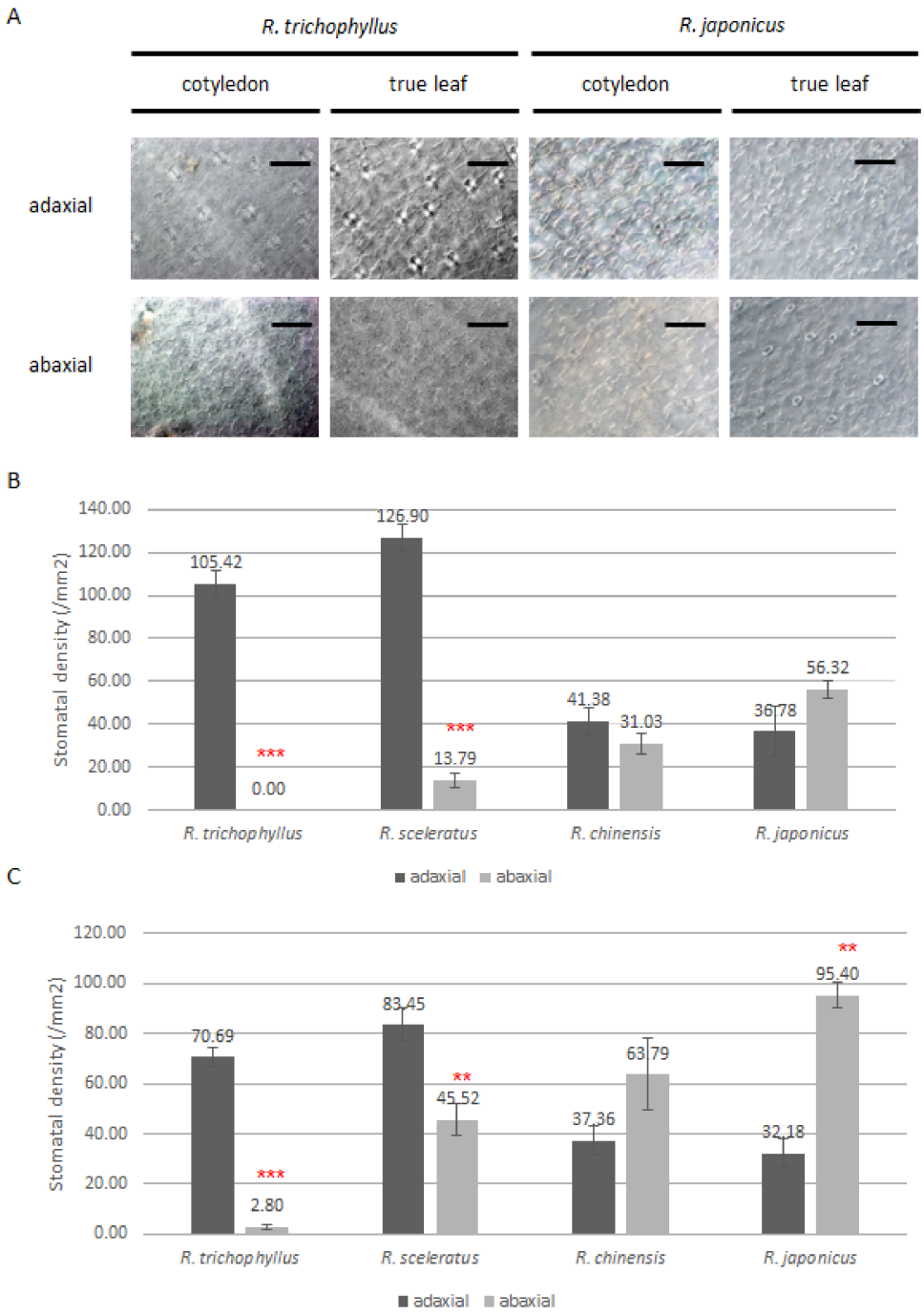

A
R. trichophyllus
R. japonicus
cotyledon
true leaf
cotyledon
true leaf
adaxial
abaxial
B
Stomatal density (/mm2)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
105.42
126.90
41.38
31.03
36.78
56.32
***
***
0.00
13.79
R. trichophyllus
R. sceleratus
R. chinensis
R. japonicus
adaxial
abaxial
C
Stomatal density (/mm2)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
70.69
83.45
37.36
63.79
32.18
95.40
***
**
**
2.80
45.52
R. trichophyllus
R. sceleratus
R. chinensis
R. japonicus
adaxial
abaxial

Figure 2. Epidermal morphology and stomatal density of *Ranunculus* species. (A) Epidermal morphology of *R. trichophyllus* and *R. japonicus*. black bar = 100ìm (B,C) Stomatal density of *Ranunculus* cotyledons (B) and leaves (C) species. **p<0.01,***p<0.001, error bar = standard error

3.2 Disruption of auxin distribution alters stomatal distribution

To test the effect of auxin on stomatal distribution, I treated 2,4-D (synthetic auxin) or NPA (auxin transport inhibitor) to *R. trichophyllus* (Figure 3) In the cotyledon, 2,4-D or NPA treatment did not alter stomatal distribution. However, NPA treatment significantly altered the stomatal distribution of a true leaf. Abaxial stomatal density was significantly increased while adaxial stomatal density was not much altered. This lead to the significant increase of stomatal ratio (abaxial stomatal density / total stomatal density). In contrast, 2,4-D treatment did not alter stomatal density either on the adaxial side or the abaxial side. Even if adaxial stomatal density decreased after 0.4μM 2,4-D treatment, this alternation was not replicated in 0.2μM or 0.6μM 2,4-D treatment. This result shows that proper distribution of auxin is important for developing epi-stomy.

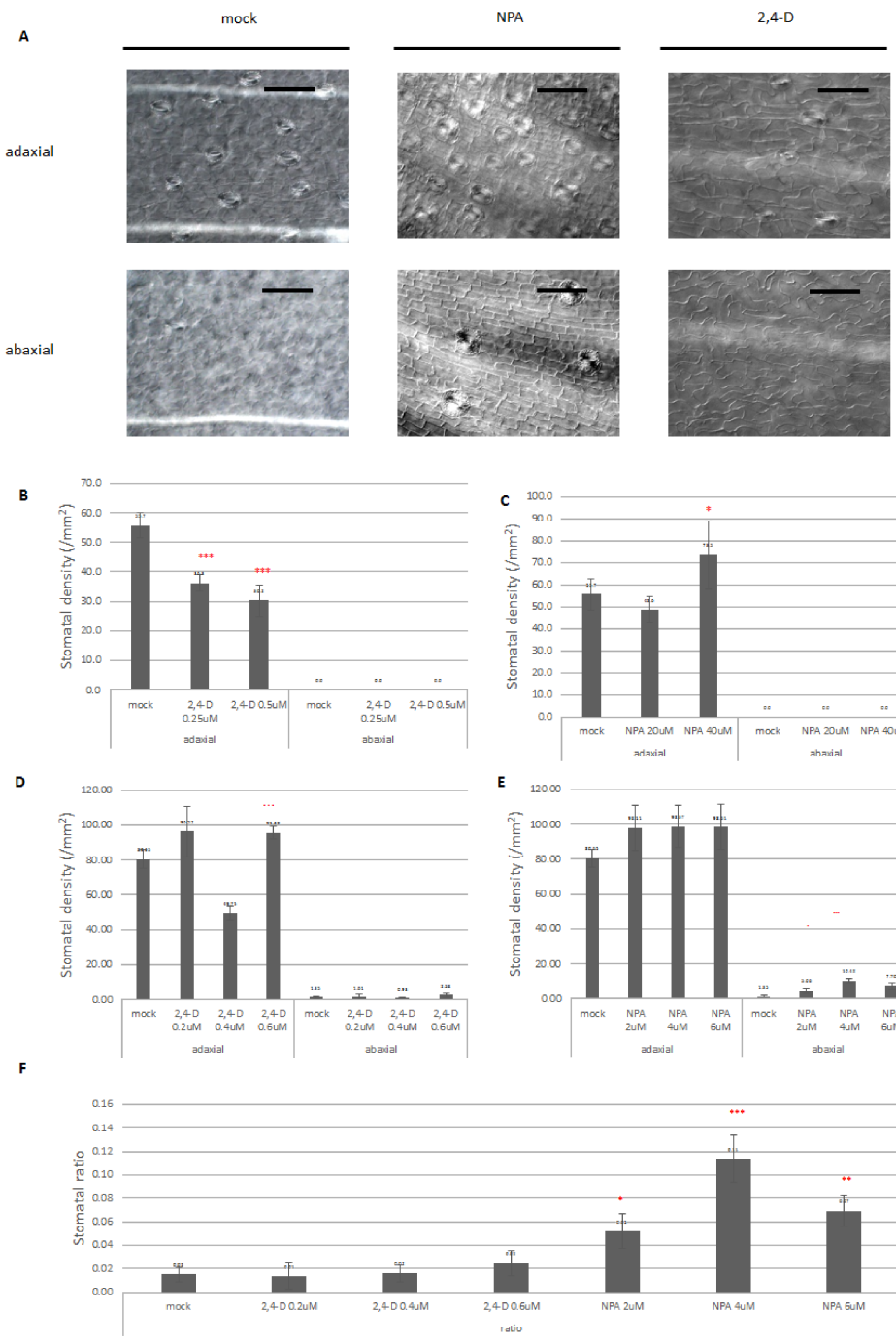

A
mock
NPA
2,4-D
adaxial
abaxial
B
Stomatal density (/mm²)
mock
2,4-D 0.25uM
2,4-D 0.5uM
adaxial
abaxial
C
Stomatal density (/mm²)
mock
NPA 20uM
NPA 40uM
adaxial
abaxial
D
Stomatal density (/mm²)
mock
2,4-D 0.2uM
2,4-D 0.4uM
2,4-D 0.6uM
adaxial
abaxial
E
Stomatal density (/mm²)
mock
NPA 2uM
NPA 4uM
NPA 6uM
adaxial
abaxial
F
Stomatal ratio
mock
2,4-D 0.2uM
2,4-D 0.4uM
2,4-D 0.6uM
NPA 2uM
NPA 4uM
NPA 6uM
ratio

Figure 3. Effect of 2,4-D and NPA treatment on the stomatal development in *R. trichophyllus*. (A) Epidermal morphology of NPA or 2,4-D treated leaves. black bar = 100ìm (B-E) stomatal density of 2,4-D treated cotyledons (B), NPA treated cotyledons (C), 2,4-D treated 1st true leaves (D), NPAtreated 1st true leaves (E). (F) Stomatal ratio of 2,4-D and NPA treated 1st true leaves. **p<0.01,***p<0.001, error bar = standard error

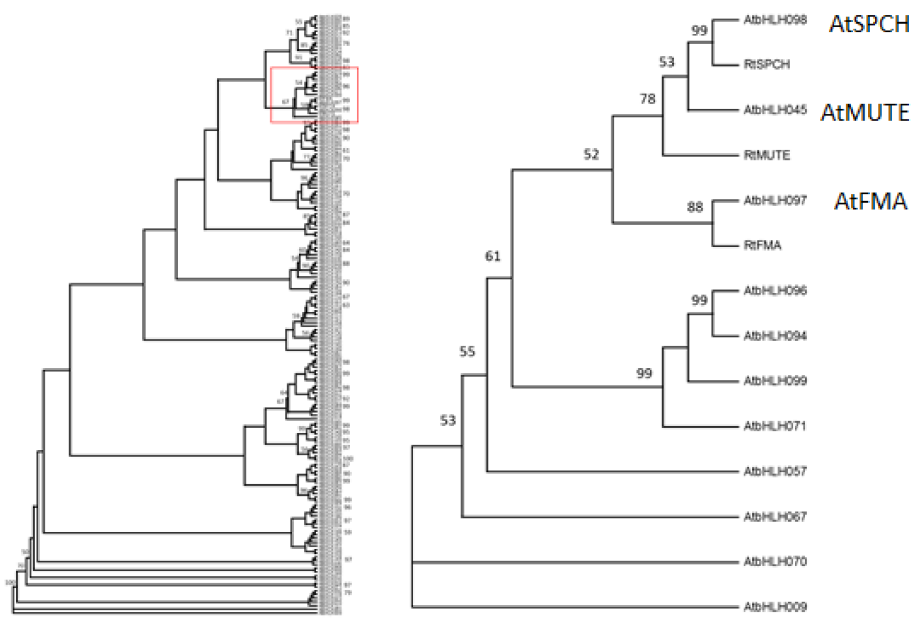
3.3 *R. trichophyllus* genes regulating stomatal development were cloned.

To elucidate the molecular mechanism regulating stomatal distribution of *R. trichophyllus,* I identified genes which would regulate stomatal development in *R. trichophyllus* (Table 1). Those genes are homologous to the known stomatal genes in *A. thaliana.* These newly identified genes are well conserved between *A. thaliana* and *R. trichophyllus*. Phylogenetic analysis between *RtSPCH, RtMUTE, RtFMA* and all the bHLH transcription factors in *A. thaliana* shows that *SPCH* and *FMA* are well conserved between *A. thaliana and R. trichophyllus* (Figure 4A). Functional STO peptide sequences are also conserved between *A. thaliana and R. trichophyllus* (Figure 4B).

category	sequence name	homologous gene of *A. thaliana*	full ORF	length (bp)
ligand signaling	comp612_c2_seq1	*GTL1*	X	2150
ligand signaling	comp75506_c0_seq1	*SDD1*	X	248
ligand signaling	comp22159_c0_seq1	*EPF1*	O	627
ligand signaling	comp29992_c0_seq1	*EPF2*	O	522
ligand signaling	comp29992_c0_seq1	*STO*	O	1357
ligand signaling	Rk_contig_16735	*ER, ERL1,2*	O	3523
MAPK pathway	comp9543_c0_seq1	*YDA*	X	2773
MAPK pathway	comp3048_c0_seq1	*MKK5*	O	1831
MAPK pathway	comp2951_c0_seq1	*MPK3*	O	1665
MAPK pathway	comp8379_c0_seq1	*MPK6*	O	1554
bHLH TF	comp11604_c0_seq1	*ICE1*	O	2315
bHLH TF	comp53764_c0_seq1	*SPCH*	O	3851
bHLH TF	Rk_contig_34067	*MUTE*	O	2224
bHLH TF	comp8658_c0_seq1	*FMA*	O	2027

Table 1. Candidate genes regulating stomatal development in *R. trichophyllus*

A


AtbHLH098
AtSPCH
RtSPCH
AtbHLH045
AtMUTE
RtMUTE
AtbHLH097
AtFMA
RtFMA
AtbHLH096
AtbHLH094
AtbHLH099
AtbHLH071
AtbHLH057
AtbHLH067
AtbHLH070
AtbHLH009
99
53
78
52
88
61
99
55
99
53

B

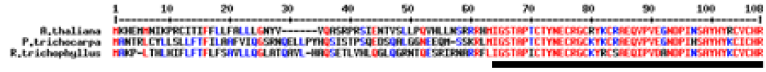

A.thaliana
P.trichocarpa
R.trichophyllus

Figure 4. Conservation of genes regulating stomatal development in *R. trichophyllus.* (A) bHLH transcription factors regulating stomatal development (SPCH, MUTE, FMA) is conserved in *R. trichophyllus*. Phylogenetic trees of SPCH, MUTE, FMA in *R. trichophyllus* with all the bHLH transcription factors in *A. thaliana.* Lineage marked with red box is shown in right. (B) STO is conserved in *R. trichophyllus*. Alignment of known stomagen orthologous of *Populus trichocarpa* and *A. thaliana*) proteins conducted by Multialign. Conserved amino acids among these 3 species are shown in red. Conserved amino acids among 2 of the 3 species are shown in blue. The final peptide sequence of stomagen in *Arabidopsis* is shown by a black bar..

3.4 Genes inducing stomatal development are expressed more on adaxial side.

To find the gene which regulate stomatal distribution of *R. trichophyllus*, expression pattern of 2 genes inducing stomatal development were analyzed. It is assumed that the early stage of stomatal development is blocked in abaxial side since no stomatal precursors were observed on the abaxial side (Figure 2). Thus, I analysed the expression of *SPEECHLESS,* which initiates the development of stomatal precursor. *RtSPCH* transcript is expressed higher on the adaxial epidermis, compared to abaxial epidermis (Figure 5A-C) This suggests that the differential expression of *RtSPCH* induces the differential distribution of stomata in *R. trichophyllus.*

To find the factor inducing the differential transcription of *RtSPCH,* I surveyed the factors regulating *SPCH.* The only known mechanism regulating transcription of *SPCH* is self-regulation. Several factors working on post-translational regulation of *SPCH* is also known. *STO* is one of the factor post-translationally up-regulates SPCH. Thus, I analyzed the expression pattern of *STO* (Figure 5D-F). *STO* was differentially expressed on the adaxial mesophyll, compared to the abaxial mesophyll. This result implies that the differential expression of *STO* induces the differential expression of *SPCH,* resulting in the differential distribution of stomata on the adaxial side.

\

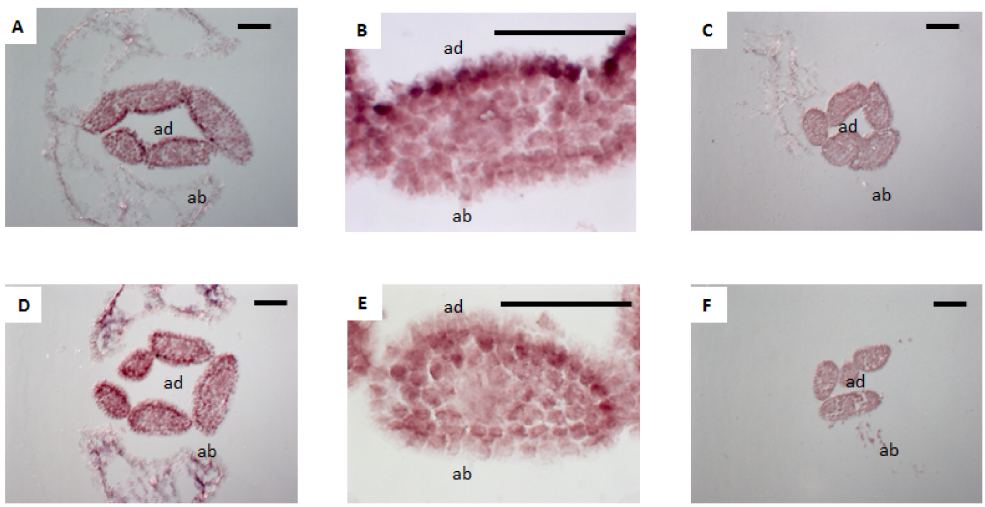


Figure 5. Expression pattern of *RtSPCH* and *RtSTO* in leaf section, detected by *in-situ* RNA hybridixation. (A, B) *RtSPCH* expression detected by RtSPCH-antisense probe. (C) Negative control with *RtSPCH*-sense probe. (D, E) *RtSTO* expression detected by *RtSTO*-antisense probe. (C) Negative control with *RtSTO*-sense probe. black bar = 100ìm ad = adaxial side, ab = abaxial side

3.5 Auxin down-regulates the genes inducing stomatal development.

To find the link between auxin gradient and differential expression of the genes regulating stomatal distribution, I tested whether auxin regulates stomatal genes (Figure 6). *STO* and *ICE1* level was significanly (> 2-fold change) and constitutively reduced after 2-weeks of auxin treatment This result suggests that auxin inhibits the genes inducing stomatal development, expecially *STO* and *ICE1.*

Supporting this result, auxin-responsive cis-elements are found on the promoter of *RtSTO* (Table 2). Interestingly, those elements were not found on the *AtSTO* promoter. This suggests that there would be some R. trichophyllus-specific regulations of *STO* by auxin. In addition, auxin-responsive elements are also found on the promoter of *RtSPCH* (Table 3). The existance of such elements suggests a possibility that auxin regulates *RtSPCH* expression.

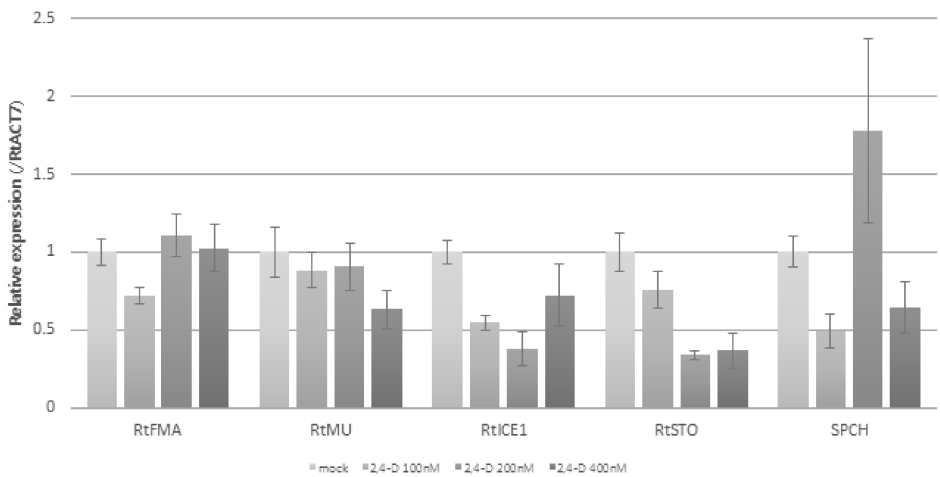


Figure 6. Effect of the auxin on the expression of the genes regulating stomatal development. error bar = standard error

site name	sequence	counts	function
GARE-motif	**TCTGTTG**	**1**	**gibberellin-responsive element**
Sp1	**CC(G/A)CCC**	**2**	**light responsive element**
TATC-box	**TATCCCA**	**1**	**cis-acting element involved in gibberellin-responsiveness**
TGA-element	**AACGAC**	**2**	**auxin-responsive element**
Box-W1	TTGACC	1	fungal elicitor responsive element
CAAT-box	CCAAT	12	common cis-acting element in promoter and enhancer regions
GATA-motif	GATAGGG	1	part of a light responsive element
I-box	GATAGGG	2	part of a light responsive element
MBS	CAACTG	1	MYB binding site involved in drought-inducibility
TATA-box	TAATA	12	core promoter element around -30 of transcription start
Unnamed__4	CTCC	6	
W box	TTGACC	1	

Table 2. cis-elements found in *RtSTO*. *cis*-elements which are found in *RtSTO* promoter sequence (612 bp) are described. Number of each element found in *RtSTO* promoter and the functions of each element on are also presented. gray background: elements which are not found in *AtSTO* promoter sequence of the same length

Site Name	sequence	counts	function
4cl-CMA2b	TCTCACCAACC	1	light responsive element
AC-I	TCTCACCAACC	1	
ATGCAAAT motif	ATACAAAT	1	cis-acting regulatory element associated to the TGAGTCA motif
AuxRR-core	GGTCCAT	2	cis-acting regulatory element involved in auxin responsiveness
CAT-box	GCCACT	1	cis-acting regulatory element related to meristem expression
CGTCA-motif	CGTCA	1	cis-acting regulatory elemen involved in the MeJA-responsiveness
EIRE	TTCGACC	1	elicitor-responsive element
GA-motif	ATAGATAA	1	part of a light responsive element
GCN4_motif	CAAGCCA	1	cis-regulatory element involved in endosperm expression
GT1-motif	GGTTAA	1	light responsive element
L-box	TCTCACCAACC	1	part of a light responsive element
MSA-like	TCCAACGGT	1	cis-acting element involved in cell cycle regulation
TATCCAT/C-motif	TATCCAT	1	
TGACG-motif	TGACG	1	cis-acting regulatory element involved in the MeJA-responsiveness
Unnamed__3	CGTGG	2	
Unnamed__6	taTAAATATct	1	
AAGAA-motif	GAAAGAA	1	
ARE	TGGTTT	2	cis-acting regulatory element essential for the anaerobic induction
Box 4	ATTAAT	2	part of a conserved DNA module involved in light responsiveness
Box I	TTTCAAA	1	light responsive element
CAAT-box	CAAT	22	common cis-acting element in promoter and enhancer regions
CATT-motif	GCATTC	1	part of a light responsive element
CCAAT-box	CAACGG	1	MYBHv1 binding site
circadian	CAANNNNATC	1	cis-acting regulatory element involved in circadian control
GAG-motif	AGAGATG	1	part of a light responsive element
G-box	CACATGG	1	cis-acting regulatory element involved in light responsiveness
HSE	CNNGAANNTTCNNG	2	cis-acting element involved in heat stress responsiveness
I-box	CTCTTATGCT	1	part of a light responsive element
MBS	CAACTG	1	MYB binding site involved in drought-inducibility
MRE	AACCTAA	1	MYB binding site involved in light responsiveness
P-box	CCTTTTG	2	gibberellin-responsive element
Skn-1_motif	GTCAT	2	cis-acting regulatory element required for endosperm expression
Sp1	CC(G/A)CCC	2	light responsive element
TATA-box	TTTTA	47	core promoter element around -30 of transcription start
TCA-element	GAGAAGAATA	1	cis-acting element involved in salicylic acid responsiveness
TGA-element	AACGAC	1	auxin-responsive element
Unnamed__1	CGTGG	2	
Unnamed__4	CTCC	7	

Table 3. cis-elements found in *RtSPCH*. cis-elements which are found in *RtSPCH* promoter sequence (1500 bp) are described. Number of each element found in *RtSPCH* promoter and the functions of each element on are also presented. gray background: elements which are not found in *RtSPCH* promoter sequence of the same length

3.6 Overexpression of leaf polarity gene alters stomatal density and distribution

Leaf polarity genes have important functions in leaf patterning. I tested whether the leaf polarity genes of *R. trichophyllus* regulates stomatal distribution. Table 4 shows the list of leaf polarity genes identified in *R. trichophyllus.* Then I constructed transgenic *A. thaliana,* overexpressing those genes. Similary to the transgenic line overexpressing leaf polarity genes of *A. thaliana*, those transgenic lines showed disruption of leaf development. Stomatal density and distribution were altered in some of those transgenic lines (Figure 7). Interestingly, *35S::RtYAB1* trangenic line showed altered stomatal distribution ($p < 0.05$). In addition, abaxial stomatal density *35S::RtKANs* transgenic line were significantly altered compared to the wildtype, even though stomatal distribution was not signlificanly altered. These results with altered stomatal density or distribution suggests that leaf polarity genes might regulate stomatal distribution of *R. trichophyllus.*

category	sequence name	homologous gene of *A. thaliana*	full ORF length (bp)		p-value
adaxial gene	RtREV	*REV*	O	465	9.70E-49
abaxial gene	RtKAN-b	*KAN1*	O	1407	3.40E-34
abaxial gene	RtKAN-a	*KAN2*	O	1041	4.50E-47
abaxial gene	RtKAN-c	*KAN4*	O	942	1.10E-42
abaxial gene	RtYAB	*YAB1*	O	552	8.60E-29

Table 4. Candidate genes regulating leaf polarity in *R. trichophyllus*

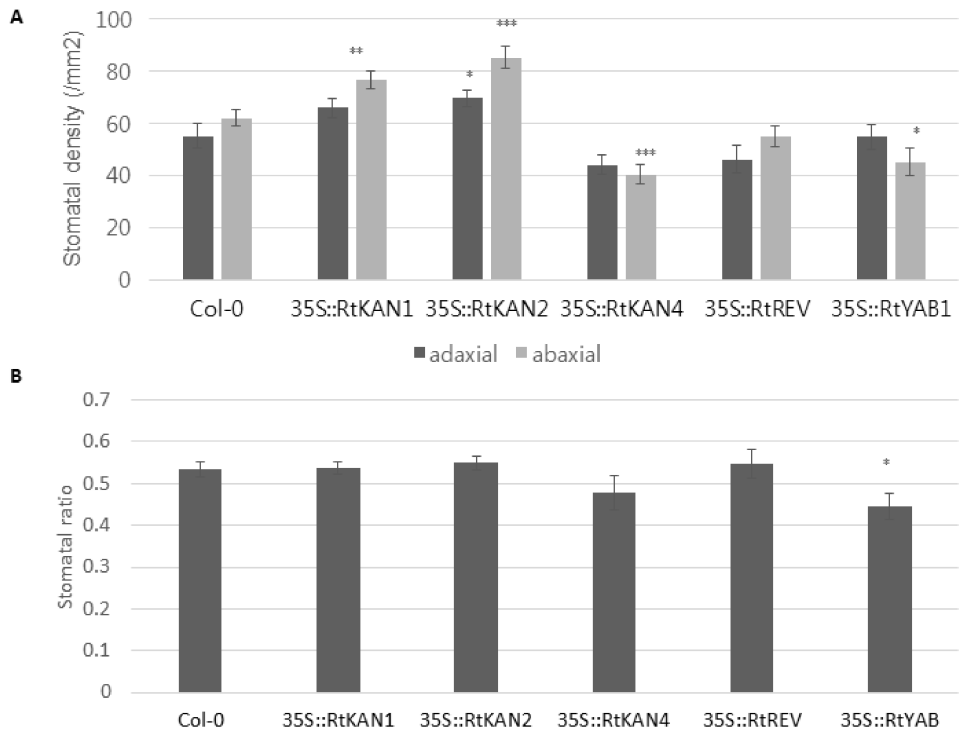


Figure 7. Effects of overexpressing leaf polarity genes on distribution. Leaf polarity genes of *R. trichphyllus* may regulate stomatal development. (A) Stomatal density of each transgenic *A. thaliana*. (B) Stomatal ratio of each transgenic line **p<0.01,***p<0.001, error bar = standard error

4. DISCUSSIONS

I found that auxin, the genes regulating stomatal development and the genes regulating leaf polarity contribute to the stomatal distribution of *R. trichophyllus.* In *R. trichopohyllus*, stomata are distributed more on adaxial side than abaxial side. Such a differential distribution called epi-stomy was weakened when auxin distribution was disrupted by auxin transport inhibitor (NPA) treatment. This result suggests that distribution of auxin is important for proper stomatal distribution. Consistently, auxin treatment down-regulated *RtSTO* expression. Collectively, I suggest that auxin regulates stomatal distribution by regulating spatial patterning of *RtSTO* expression.

In addition, I found that *RtSTO* and *RtSPCH* are differentially expressed on adaxial side compared to abaxial side. Since *RtSPCH* is a master initiator of stomatal development (Simmons and Bergmann, 2016), such a differential expression would induce the differential distribution of stomata in *R. trichophyllus*. Since the transcription of *RtSPCH* can be induced by SPCH protein itself (Lau *et al*., 2014), the differential transcription of *RtSPCH* would be a result of the differential post-transcriptional regulation including specialized phosphorylation and selective degradation on abaxial side. The differential expression of *RtSTO* on the adaxial side supports this mechanism. Since *STO* inhibits phosphorylation and degradation of *SPCH,* adaxial-specific expression of *RtSTO* would results the adaxial-specific expression of *RtSPCH* and consequently, stomatal development.

Based on those findings, I suggest the model of epi-stomy inducing mechanism in *R. trichophyllus* (Figure 8). In *A. thaliana*, auxin maxima is

formed in adaxial side and auxin minima is formed on abaxial side of leaf primordia (Qi *et al*, 2014). I assume that the same auxin gradient would exist in *R. trichophyllus*. Thus, auxin would be the signal with leaf spatial information. Since auxin down-regulates *RtSTO* expression, the adaxial-side specific expression of *RtSTO* would be a result of auxin gradient. The inhibition of *SPCH* protein degradation by *STO* and feed-foward loop of *SPCH* transcription would results in the adaxial-specific expression of *RtSPCH.* This would induce epi-stomy of *R. trichophyllus* In addition, overexpression of *R. trichophyllus* leaf polarity alters stomatal density or ratio. Thus, adaxial-specific or abaxial-specific expression of leaf polarity genes would also be the spatial signal regulating stomatal development and epi-stomy of *R. trichophyllus.*

One of the major problem interpreting the result of this study is that the proposed mechanism regulating *STO* expression by auxin is already existing in *A. thaliana,* in which stomata are not differentially distributed on the adaxial and abaxial side. The fact that auxin gradient along adaxial-abaxial axis also exists in *A. thaliana* also needs explanation. However, *AtSTO* is evenly distributed on the adaxial and abaxial side and so do stomata. Such inconsistency would be explained if the sensitivity to auxin is much stronger in *RtSTO* expression than in *AtSTO.* That hypothesis should be confirmed in the further study. In addition, the direct evidence that auxin gradient regulates spatial distribution of *RtSTO* expression is needed. The evidence would be obtained from *in-situ* RNA hybridization of *RtSTO* mRNA before and after the NPA treatment. Furthermore, Since I did not test the expression pattern of other genes regulating stomatal

development, including *EPF1, EPF2, ER, PPD*, the possibility that those genes also regulate stomatal distribution remains. Thus, experiment analyzing those genes should be done.

For the following study, I will test whether the mechanism inducing differential distribution of stomata that I suggests are common to phylogenetically diverse species. I hypothesize that the mechanisms underlying epi- or hypo-stomy are also analogous and relatively simple, since the evolution of stomatal distribution occured often in diverse lineage. To test this, I will analyze the expression pattern of *STO* before and after auxin treatment in various species.

Molecular mechanism of epi-, amphi-, and hypo-stomy was seldomly studied in this field before. My study using *R. trichophyllus* suggests a noble pathway linking leaf polarity and stomatal development. In a broad view, the result of the study shows an example linking spatial information and organogenesis. This noble mechanism that plant recognizes the spatial information (adaxial versus abaxial) and alters developmental process (whether to develop stomata or not) might be applied to developmental process other than epi-stomy, including trichome development. In addition, converting amphi- or hypo-stomatous plants to epi-stomous plants and generating plants with higher resistance to flooding would be capable. Similiary, the engineering of drought-resistant crop by altering stomatal conductance is performed (Franks *et al*, 2015).

In conclusion, I found that adaxial-specific stomatal distribution or epi-stomy of *R. trichophyllus* is correlated with differential expression of *RtSTO.* In addition, I suggests that auxin would regulate such a differential

expression of *RtSTO*. Furthermore, the possibility that leaf polarity genes regulate stomatal distribution is also suggested.

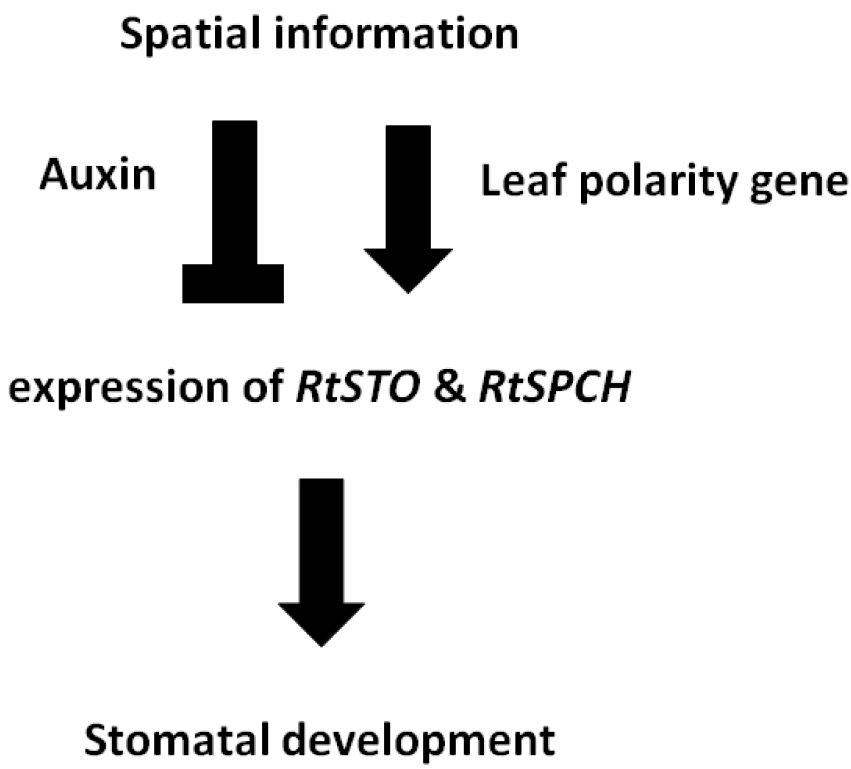


Figure 8. Working model of epi-stomaty in *R. trichophyllus*

REFERENCES

Brandt, R., Salla-Martret, M., Bou-Torrent, J., Musielak, T., Stahl, M., Lanz, C., Ott, F., Schmid, M., Greb, T., and Schwarz, M. (2012). Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses. The Plant Journal *72*, 31-42.

Brewer, P.B., Heisler, M.G., Hejátko, J., Friml, J., and Benková, E. (2006). In situ hybridization for mRNA detection in Arabidopsis tissue sections. Nature protocols *1*, 1462-1467.

Chen, L.-Y., Zhao, S.-Y., Wang, Q.-F., and Moody, M.L. (2015). Transcriptome sequencing of three Ranunculus species (Ranunculaceae) reveals candidate genes in adaptation from terrestrial to aquatic habitats. Scientific reports *5*.

Driscoll, S., Prins, A., Olmos, E., Kunert, K., and Foyer, C. (2006). Specification of adaxial and abaxial stomata, epidermal structure and photosynthesis to CO2 enrichment in maize leaves. Journal of Experimental Botany *57*, 381-390.

Eshed, Y., Izhaki, A., Baum, S.F., Floyd, S.K., and Bowman, J.L. (2004). Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development *131*, 2997-3006.

Franks, P.J., W Doheny-Adams, T., Britton-Harper, Z.J., and Gray, J.E. (2015). Increasing water-use efficiency directly through genetic manipulation of stomatal density. New Phytologist.

Guilfoyle, T.J., and Hagen, G. (2007). Auxin response factors. Current opinion in plant biology *10*, 453-460.

Guilioni, L., Jones, H., Leinonen, I., and Lhomme, J.-P. (2008). On the relationships between stomatal resistance and leaf temperatures in thermography. Agricultural and Forest Meteorology *148*, 1908-1912.

Hörandl, E., and Emadzade, K. (2012). Evolutionary classification: A case study on the diverse plant genus Ranunculus L.(Ranunculaceae). Perspectives in Plant Ecology, Evolution and Systematics *14*, 310-324.

Hörandl, E., Paun, O., Johansson, J.T., Lehnebach, C., Armstrong, T., Chen, L., and Lockhart, P. (2005). Phylogenetic relationships and evolutionary traits in Ranunculus sl (Ranunculaceae) inferred from ITS sequence analysis. Molecular phylogenetics and evolution *36*, 305-327.

Hara, K., Kajita, R., Torii, K.U., Bergmann, D.C., and Kakimoto, T. (2007). The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Genes & Development *21*, 1720-1725.

Hara, K., Yokoo, T., Kajita, R., Onishi, T., Yahata, S., Peterson, K.M.,

Torii, K.U., and Kakimoto, T. (2009). Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis leaves. Plant and Cell Physiology *50*, 1019-1031.

Hoot, S.B. (1991). Phylogeny of the Ranunculaceae based on epidermal microcharacters and macromorphology. Systematic Botany, 741-755.

Hronková, M., Wiesnerová, D., Šimková, M., Skůpa, P., Dewitte, W., Vráblová, M., Zažímalová, E., and Šantrůček, J. (2015). Light-induced STOMAGEN-mediated stomatal development in Arabidopsis leaves. Journal of experimental botany, erv233.

Huang, J.-s. (1986). Ultrastructure of bacterial penetration in plants. Annual review of phytopathology *24*, 141-157.

Hunt, L., and Gray, J.E. (2009). The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. Current Biology *19*, 864-869.

Kanaoka, M.M., Pillitteri, L.J., Fujii, H., Yoshida, Y., Bogenschutz, N.L., Takabayashi, J., Zhu, J.-K., and Torii, K.U. (2008). SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to Arabidopsis stomatal differentiation. The Plant Cell *20*, 1775-1785.

Kang, C.-Y., Lian, H.-L., Wang, F.-F., Huang, J.-R., and Yang, H.-Q.

(2009). Cryptochromes, phytochromes, and COP1 regulate light-controlled stomatal development in Arabidopsis. The Plant Cell *21*, 2624-2641.

Kidner, C.A., and Timmermans, M.C. (2010). Chapter Five-Signaling Sides: Adaxial–Abaxial Patterning in Leaves. Current topics in developmental biology *91*, 141-168.

Kim, T.-W., Michniewicz, M., Bergmann, D.C., and Wang, Z.-Y. (2012). Brassinosteroid regulates stomatal development by GSK3-mediated inhibition of a MAPK pathway. Nature *482*, 419-422.

Kondo, T., Kajita, R., Miyazaki, A., Hokoyama, M., Nakamura-Miura, T., Mizuno, S., Masuda, Y., Irie, K., Tanaka, Y., and Takada, S. (2010). Stomatal density is controlled by a mesophyll-derived signaling molecule. Plant and cell physiology *51*, 1-8.

Lau, O.S., and Bergmann, D.C. (2012). Stomatal development: a plant's perspective on cell polarity, cell fate transitions and intercellular communication. Development *139*, 3683-3692.

Lau, O.S., Davies, K.A., Chang, J., Adrian, J., Rowe, M.H., Ballenger, C.E., and Bergmann, D.C. (2014). Direct roles of SPEECHLESS in the specification of stomatal self-renewing cells. Science *345*, 1605-1609.

MacAlister, C.A., Ohashi-Ito, K., and Bergmann, D.C. (2007). Transcription

factor control of asymmetric cell divisions that establish the stomatal lineage. Nature *445*, 537-540.

McKown, A.D., Guy, R.D., Quamme, L., Klápště, J., La Mantia, J., Constabel, C., El-Kassaby, Y.A., Hamelin, R.C., Zifkin, M., and Azam, M. (2014). Association genetics, geography and ecophysiology link stomatal patterning in Populus trichocarpa with carbon gain and disease resistance trade-offs. Molecular ecology *23*, 5771-5790.

Merelo, P., Xie, Y., Brand, L., Ott, F., Weigel, D., Bowman, J.L., Heisler, M.G., and Wenkel, S. (2013). Genome-wide identification of KANADI1 target genes. PloS one *8*, e77341.

Milla, R., de Diego-Vico, N., and Martín-Robles, N. (2013). Shifts in stomatal traits following the domestication of plant species. Journal of experimental botany *64*, 3137-3146.

Mott, K.A. (1982). PHYSIOLOGICAL ECOLOGY OF AMPHISTOMATOUS LEAVES.

Mott, K.A. (2007). Leaf hydraulic conductivity and stomatal responses to humidity in amphistomatous leaves. Plant, Cell & Environment *30*, 1444-1449.

MOTT, K.A., GIBSON, A.C., and O'LEARY, J.W. (1982). The adaptive

significance of amphistomatic leaves. Plant, Cell & Environment *5*, 455-460.
Muir, C.D. (2015). Making pore choices: repeated regime shifts in stomatal ratio. Paper presented at: Proc R Soc B (The Royal Society).

Muir, C.D., Pease, J.B., and Moyle, L.C. (2014). Quantitative genetic analysis indicates natural selection on leaf phenotypes across wild tomato species (Solanum sect. Lycopersicon; Solanaceae). Genetics *198*, 1629-1643.

Ohashi-Ito, K., and Bergmann, D.C. (2006). Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. The Plant Cell *18*, 2493-2505.

Parkhurst, D.F. (1978). The adaptive significance of stomatal occurrence on one or both surfaces of leaves. The Journal of Ecology, 367-383.

Parkhurst, D.F., and Mott, K.A. (1990). Intercellular Diffusion Limits to CO2 Uptake in Leaves Studies in Air and Helox. Plant Physiology *94*, 1024-1032.

Peterson, K.M., Rychel, A.L., and Torii, K.U. (2010). Out of the mouths of plants: the molecular basis of the evolution and diversity of stomatal development. The Plant Cell *22*, 296-306.

Pillitteri, L.J., Sloan, D.B., Bogenschutz, N.L., and Torii, K.U. (2007). Termination of asymmetric cell division and differentiation of stomata.

Nature *445*, 501-505.

Pillitteri, L.J., and Torii, K.U. (2012). Mechanisms of stomatal development. Annual review of plant biology *63*, 591-614.

Qi, J., Wang, Y., Yu, T., Cunha, A., Wu, B., Vernoux, T., Meyerowitz, E., and Jiao, Y. (2014). Auxin depletion from leaf primordia contributes to organ patterning. Proceedings of the National Academy of Sciences *111*, 18769-18774.

Robinson, S., de Reuille, P.B., Chan, J., Bergmann, D., Prusinkiewicz, P., and Coen, E. (2011). Generation of spatial patterns through cell polarity switching. Science *333*, 1436-1440.

Saibo, N.J., Vriezen, W.H., Beemster, G.T., and Van Der Straeten, D. (2003). Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. The Plant Journal *33*, 989-1000.

Serna, L. (2014). The role of brassinosteroids and abscisic acid in stomatal development. Plant Science *225*, 95-101.

Simmons, A.R., and Bergmann, D.C. (2016). Transcriptional control of cell fate in the stomatal lineage. Current opinion in plant biology *29*, 1-8.

SREELAKSHMI, V., SRUTHY, E., and SHEREENA, J. RELATIONSHIP BETWEEN THE LEAF AREA AND TAXONOMIC IMPORTANCE OF FOLIAR STOMATA.

Sugano, S.S., Shimada, T., Imai, Y., Okawa, K., Tamai, A., Mori, M., and Hara-Nishimura, I. (2010). Stomagen positively regulates stomatal density in Arabidopsis. Nature *463*, 241-244.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., and Zhang, S. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. The Plant Cell *19*, 63-73.

Wang, M., Yang, K., and Le, J. (2015). Organ-specific effects of brassinosteroids on stomatal production coordinate with the action of TOO MANY MOUTHS. Journal of integrative plant biology *57*, 247-255.

Willmer, C., and Fricker, M. (1996). Stomata, Vol 2 (Springer Science & Business Media).

Woodward, F. (1986). Ecophysiological studies on the shrub Vaccinium myrtillus L. taken from a wide altitudinal range. Oecologia *70*, 580-586.

Yoo, C.Y., Pence, H.E., Jin, J.B., Miura, K., Gosney, M.J., Hasegawa, P.M., and Mickelbart, M.V. (2010). The Arabidopsis GTL1 transcription factor

regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. The Plant Cell *22*, 4128-4141.

Zhang, J.-Y., He, S.-B., Li, L., and Yang, H.-Q. (2014). Auxin inhibits stomatal development through MONOPTEROS repression of a mobile peptide gene STOMAGEN in mesophyll. Proceedings of the National Academy of Sciences *111*, E3015-E3023.

Zhang, X.-L., Yang, Z.-P., Zhang, J., and Zhang, L.-G. (2013). Ectopic Expression of BraYAB1-702, a Member of YABBY Gene Family in Chinese Cabbage, Causes Leaf Curling, Inhibition of Development of Shoot Apical Meristem and Flowering Stage Delaying in Arabidopsis thaliana. International journal of molecular sciences *14*, 14872-14891.

국문 초록

미나리아재비속 식물의 기공 발달 기작 연구

경진슬

자연과학대학 생명과학부

서울대학교 대학원

매화마름(*Ranunculus trichophyllus*)은 논을 비롯한 습지에 분포하는 미나리아재비속의 양서식물이다. 매화마름을 비롯한 여러 수생 및 습지 식물에서 기공은 잎 윗면에 분포한다. 기공이 잎 윗면에 특이적으로 발달하는 현상은 epi-stomy라고 불린다. 기공은 병원체가 식물을 침입하는 입구가 되기 때문에, 습지 식물에게 epi-stomy는 진화적으로 유리하다. epi-stomy를 유도하는 분자적 기작은 지금까지 알려져 있지 않다.

이 연구에서 나는 매화마름을 포함한 미나리아재비속 4종의 식물의 기공 분포를 비교하고, 매화마름에서 epi-stomy가 나타나는 기작을 연구하였다. 매화마름과 개구리자리 (*R. sceleratus*)는 기공이 주로 잎 윗면에 분포한 반면 미나리아재비 (*R. japonicus*)는 주로 아랫면에 분포하였다. 나는 매화마름에서 잎의 윗면과 아랫면 이라는 위치 정보가 식

물 호르몬인 옥신과 잎 축 발달 유전자들을 통해 기공 발달 유전자에 전달되어 epi-stomy가 나타날 것 이라는 가설을 제시하였다. 이러한 가설을 검증하기 위해서 식물체에 옥신 수송 억제제인 NPA를 처리하면 기공 분포가 변화함을 확인하였다. 다음으로 나는 기공 발달 유전자인 *SPEECHLESS*와 *STOMAGEN*을 매화마름에서 클로닝하였고, *in-situ* RNA hybridization을 통해 이들 유전자가 잎 윗면에 특이적으로 발현하며, *STOMAGEN*의 발현이 옥신에 의해 저해됨을 확인하였다. 또한, 매화마름의 잎 축 발달 유전자를 과발현하는 애기장대 형질전환체를 제작하여 이들 형질전환체에서 기공 분포가 변화함을 확인하였다. 이 연구를 통해 (1) 기공 발달 유전자들이 잎 윗면에 특이적으로 발현하기 때문에 epi-stomy가 생기며, (2) 기공 발달 유전자의 발현 패턴은 옥신과 잎 축 발달 유전자들에 의해 조절됨을 확인하였다.

주요어 : 기공 발달, 잎 축 형성, *SPEECHLESS, STOMAGEN*
학번 : 2014-20314