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양서식물 *Ranunculus trichophyllus* 에서 식물 호르몬과 잎 축 형성 유전자에 의한 잎 이형성 결정

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2014 년 12 월

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Heterophylly determination by phytohormones and leaf polarity genes in *Ranunculus trichophyllus,* an amphibious plant

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ABSTRACT

Heterophylly determination by phytohormones and leaf polarity genes in amphibious plant, *Ranunculus trichophyllus*

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Aquatic plants have evolved from various taxa of land plants. In spite of the convergent evolution, aquatic plants share common traits to adapt water environment. One of them, aquatic plants produce specialized leaves called aquatic leaves. Many investigators have been focused to theses specialized leaves, but the molecular mechanisms of aquatic leaf development have not yet been investigated intensively. *Ranunculus trichophyllus,* an amphibious aquatic plant, produces thin, cylindrical leaves if grown under water, and thick, broad leaves if grown on land. Physiological study showed that the heterophylly of amphibious *R. trichophyllus* is mediated by two plant hormones, abscisic acid (ABA) and ethylene. ABA mediates terrestrial leaf morphologies, ethylene mediates aquatic leaf morphologies. In addition, the expression

of ABA biosynthetic and responsive genes was decreased in aquatic leaves compared to terrestrial leaves. On the other hand, the expression of ethylene in biosynthetic and responsive genes was increased in aquatic leaves compared to terrestrial leaves.

Under aquatic conditions, the increased ethylene causes induction of the expression of abaxial genes, RtKANADIs (RtKANs) and decreased ABA causes attenuation the expression of adaxial genes, RtHD-ZIPIIIs. These leaf polarity genes control leaf morphology, thus cylindrical leaves are produced through abaxialization. In contrast, under terrestrial conditions, ABA upregulates the expression of *RtHD-ZIPIIIs*, thus leaf polarity is established. The traits of aquatic leaves such as cylindrical shapes, lack of stomata and reduced xylem development can be produced by abaxialization. Therefore, I propose a model for heterophylly determination of *R. trichophyllus*. Hormonal regulation such as ethylene increase and ABA suppression and leaf polarity regulation such as accumulation of *RtKAN* and reduction of *RtHD-ZIPIIIs* by submergence, which are required for abaxialization, are two key evolutionary steps for aquatic plants.

Keywords: amphibious plant, heterophylly, ethylene, ABA, leaf polarity

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ABBREVIATIONS

3’	three prime end of DNA fragment
5’	five prime end of DNA fragment
AAO3	ABA-aldehyde oxidase 3
ABA	abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACT	actin
BIN2	brassinosteroid insensitive2
BR	brassinosteroid
DMSO	dimethyl sulfoxide
CNA	corona
CYP86	cytochrome P450, family 86
EBL	epi-brassinolide
GA	gibberellin
GLUR	glutamate receptor
HD-ZIP III	Class III homeodomain leucine zipper protein
KAN	kanadi
MS	Murashige-Skoog
NAA	1-naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
PBZ	paclobutrazol
PHB	phabulosa

PHV	phavoluta
PP2A	protein phosphatase 2A
RACE	rapid amplification of cDNA ends
RAP2.2	related to apetala2.2
REV	revoluta
RNA	ribonucleic acid
qPCR	quantitative polymerase chain reaction
TAIL-PCR	thermal asymmetrical interlaced PCR
TAS3	transacting siRNA3
XCP	xylem -specific papain-like cysteine peptidase
YAB	yabby

INTRODUCTION

In the mid-Paleozoic era, a new plant appeared. All of its relatives lived in the freshwater, but it lived in land; it was one of the first plants to grow on land. Since then, its descendants have spread out many inland places. Its descendants are called " land plants" or "embryophytes"; indeed, all land plants are monophyletic(Kenrick and Crane, 1997; Steemans et al., 2009). Embryophytes have adapted themselves to water-deficient environments, e.g. stomata, structural tissues, and vascular tissues (Kenrick and Crane, 1997). As a result, land plants cover various land habitats from alpine biomes to deserts.

Some lineages in embryophytes returned to a water-rich environment; they are called aquatic plants. Since most embryophytes live in land, the evolution of aquatic plants has considered a rare event in the history of embryophytes. However, the first aquatic vascular plants already appeared in early angiosperms and free-sporing ferns during the early Cretaceous(Martín-Closas, 2003). Moreover, aquatic plants are abundant among varioustaxa in embryophytes (Table 1, Cook, 1999). Phylogenetic trees show that not only the ancestors of aquatic plants lived on land, but aquatic plants are also polyphyletic which means that evolutionary events of aquatic plants have happened several times independently.

In spite of the frequent evolutionary events that have led to aquatic plants, the evolutionary challenge from terrestrial plants to aquatic plants is not easy. Terrestrial plants cannot survive in a submerged environment for a long time, because the

chemico-physical properties of water are so different from those of the air. First, gas solubility and diffusion rates in the water are very low compared to those in air. Even more, gas solubility is highly influenced by temperature, turbulence, and other dissolved molecules. Therefore, the supply of oxygen is not sufficient in water to land plants. Lack of oxygens induces hypoxia stress in terrestrial plants. The supply of carbon dioxide is also decreased in water, which reduces the carbon fixation rate (Bailey-Serres and Voesenek, 2008). Second, light quality and quantity in the water is lower than in the air because reflection and refraction occur on water surface and in the water. Reduction of light energy decreases the efficiency of plant photosynthesis (Mommer and Visser, 2005; Colmer et al., 2011). A compromised photosynthesis rate cannot support sufficient nutrients and cellular building blocks to maintain the life of terrestrial plants. Third, toxic molecules are accumulated in the tissues during anaerobic metabolism, and pollutants from floodwater contaminate terrestrial plants (Blom and Voesenek, 1996; Jackson and Colmer, 2005). These molecules threaten the lives of terrestrial plants. Therefore, uniqueness of aquatic plants is originated from their specific traits to resist these inconvenient environments.

Table 1. The number of aquatic plants among angiosperm taxa

Taxa		Subclass	Superorder	Order	Family
MAGNOLIOPSIDA	total (no.)	11	32	83-112-40	460
(angiosperms)	aquatic (no.)	10	25	35-41 -24	83
	aquatic (%)	91	78	37-42 -60	18
MAGNOLIIDAE	total (no.)	6	25	64-84 -30	363
(basal & dicots)	aquatic (no.)	5	18	23-27 -18	46
	aquatic (%)	83	72	31-36 -60	13
LILIIDAE	total (no.)	5	7	19-25 -10	97
(monocots)	aquatic (no.)	5	7	12-14 - 7	37
	aquatic (%)	100	100	63-56 -70	38

The table adapted from Cook (1999).

1. The adaptive mechanisms of aquatic plants allow them to survive in water.

Aquatic plants have several adaptive mechanisms to cope with these unfavorable underwater conditions. First, aquatic plants use bicarbonate as a carbon source. The diffusion rate of carbon dioxide in water is about 10000 times less than its rate in air. The equilibrium concentration of carbon dioxide in water is also below than that of air, which means that the quantity of carbon dioxide in water is less than that of air (Jackson and Colmer, 2005; Bornette and Puijalon, 2011). Under this condition, tissues are suffered from low concentration of carbon dioxide, which causes a low rate of photosynthesis. However, ionized form of carbon dioxide are abundant in water, the total fixable carbon is much more than aerial condition if plants can use such ionized form (Prins and Elzenga, 1989; Colmer et al., 2011). The use of bicarbonate compensates for the slow diffusion of carbon dioxide, and supplies enough carbon for photosynthesis. Moreover, some aquatic plants can actively uptake bicarbonate. This mechanism increases the concentration of carbon dioxide in tissues, and elevates the photosynthesis rate in aquatic plants (Lucas, 1983; Raven et al., 1985). In addition, the oxygen produced by photosynthesis increases the pH concentration. This alkaline condition increases conversion rate of carbon dioxide into bicarbonate. Therefore, the concentration of carbon dioxide is not only reduced by photosynthesis, but also alkalization. Even in an alkaline condition, aquatic plants can maintain a steady photosynthesis rate using bicarbonate (Prins and Elzenga, 1989).

Second, aquatic plants are more tolerant to anoxia stress. In anaerobic conditions, plants start to accumulate lactate. Lactate drops the cellular pH, which

causes cytoplasmic acidosis so that is harmful to cell. Fermentation process reduces the cellular acidification by the conversion of pyruvate, a precursor of lactate, into ethanol (Ricard et al., 1994; Vartapetian and Jackson, 1997), but accumulated ethanol is also toxic to plants. Indeed, the process is also existed in terrestrial plants; it is just prepared for standing short time emergency. Aquatic plants spout ethanol to extracellular regions or convert it to innocuous compounds such like malate and alanine (Kennedy et al., 1992). Also, aquatic plants reserve more carbohydrate for fermentation. They have fast metabolic converting system between aerobic respiration and anaerobic fermentation, which helps reducing energy consumption in prolonged submergence (Summers et al., 2000; Colmer and Voesenek, 2009).

Third, aquatic plants also have specialized tissues to adapt to underwater environments. Aerenchyma is gas-filled tissues in the intracellular regions of plants. Well-developed aerenchyma are important and common in aquatic plants (Jung et al., 2008). Aerenchyma provides a passage for gas exchange, and the gas reservoirs that are required for photosynthesis and respiration. It is also used as a chimney for excreting unnecessary volatiles. Moreover, it makes a flexible plant body, which reduces the physical resistance and friction force. Aerenchyma-bearing tissues require less energy and resources per volume for growth compared to tissues that lack it (Jackson and Armstrong, 1999; Evans, 2004). Adventitious root is another specialized organ to deals with hypoxia. Normally, adventitious roots can arise on the base of the shoot or on stem nodes. Since arenchyma is well developed in adventitious roots, this root system can provide other gas exchange routes in root tissue.

Lastly, aquatic plants possess shoot elongation abilities when they are exposed to submergence. The stems, petioles, and leaves of aquatic plants can be elongated. By accelerating growth, parts of aquatic plants can reach the air. Aquatic plants diffuse oxygen and carbon dioxide to other plant tissues via aerenchyma. The growth acceleration mechanism resolves hypoxia and carbon dioxide deficiencies. The elongation mechanism allows another advantage because aquatic plants can use much light source on water surface than under deep water. Therefore, fast shoot elongation not only solves respiration problems, but also those of photosynthesis. Many aquatic plants have the capacity for strong shoot elongation when they are submerged (Vartapetian and Jackson, 1997; Voesenek et al., 2006), which minimizes hypoxia damage.

In conclusion, aquatic plants have developed unique adaptive traits to survive in submerged condition. Some traits, like using bicarbonate and aerenchyma developments, are extraordinary mechanisms for aquatic plants. Other traits, like fermentation and hyponastic growth, is common process to deal with submergence in plant but aquatic plants acquired efficient form compared to terrestrial ones. These traits show that water adaptation is challenge to aquatic plants not only the level of cellular metabolism but whole plant architecture.

2. Properties and benefits of aquatic leaves.

As mentioned above, some structural differences are observed in aquatic plants compared to terrestrial plants. As submerged root produces adventitious root

and submerged stem is elongated, leaf structure can be affected by submergence. Leaf is a main place of photosynthesis and it needs proper gas exchange for carbon fixation and respiration. Since prolonged submergence disturbs both of them, aquatic plants need another type of leaf to survive water environment. Indeed, aquatic plants produce specialized leaves to survive in underwater environments, called aquatic leaves. The aquatic leaves of aquatic plants have similar shapes and share common traits. They show thin and filamentous shapes. At the microscopic level, they show thin cuticles, chloroplasts in their epidermis cells, and few or no stomata phenotypes, etc. (Wells and Pigliucci, 2000; Colmer et al., 2011). These universal traits among aquatic leaves emphasize that these specialized leaves have advantageous aspects for underwater life. First, the narrow shape of aquatic leaves reduces the diffusion length for gas exchange. If the length is high, insufficient gas is burned up near leaf epidermal cells and cannot penetrate to center of leaves then it causes anoxia. Moreover, the total mass of aquatic leaves is less than that of terrestrial leaves, therefore, the total demands of oxygen and carbon dioxide in aquatic plants is also less. Thus, aquatic plants can maintain metabolism relatively low oxygen and carbon dioxide concentrations compared to terrestrial plants. Lastly, viscosity of water is higher than that of air so that water flow prevents the growth of aquatic plants. The cylindrical shape of aquatic leaves reduces drag stress in water (Bal et al., 2011), which is another advantage of their unique shapes. Thin cuticle layers on aquatic leaves correspond to the low resistance rates of carbon dioxide and oxygen. Thick cuticle layers on leaf surface slow down the transpiration rate thus carbon dioxide and oxygen

can penetrate the inner layers of leaves easily in thin cuticle layer of aquatic leaves (Frost-Christensen and Floto, 2007; Colmer and Pedersen, 2008). Moreover, aquatic leaves have a higher leaf area per leaf dry mass and they are thinner than terrestrial ones. Increasing the ratio of surface to volume elevates the gas exchange rate rates of aquatic leaves (Mommer et al., 2005). The fast uptake of carbon dioxide and oxygen supply the substrates for carbon fixation and support cellular metabolism. These advantages make aquatic plants avoiding oxygen-deficiency stress by elevating carbon assimilation rates and dropping the photorespiration rates.

Aquatic leaves show reduced vein densities. The number of veins in aquatic leaves is reduced, and their venation patterns are monotonous (Deschamp and Cooke, 1985; Kane and Albert, 1989). Because the shape of aquatic leaves is filamentous and most cell layers are under the supporting sphere of mid-vein, they do not need complex vein systems. Moreover, water transport is the main function of xylem, but aquatic leaves are soaked in water. Therefore, in spite of the reduced vein system, aquatic leaves do not succumb to water-deficiency.

Aquatic leaves have either no stomata or non-functional stomata (Deschamp and Cooke, 1985; Kane and Albert, 1989), because stomatal degeneracy in aquatic leaves is advantageous in water. The role of stomata is the regulation of the gas exchange between the interior of a plant's body and its exterior environment. In terrestrial plants, the surfaces of leaves are covered by thick cuticle layers to prevent water loss, but the layer also prevents the exchange of oxygen and carbon dioxide. The stomata keep the balance between water loss by transpiration and gas exchange

for photosynthesis and respiration (Schulze et al., 1987; Hetherington and Woodward, 2003). Aquatic plants do not have thick cuticle layers, and both evaporation and transpiration are absent in aquatic leaves. Therefore, aquatic leaves do not need a porous structure. In addition, stomata are historically considered as pathogen entrances(Melotto et al., 2008), thus, the loss of stomata could mean closing the entrance of pathogen.

In aquatic leaves, chloroplasts are developed in the epidermal cells (Rascio et al., 1999; Ronzhina and P'yankov, 2001). The chloroplasts of terrestrial leaves are oriented around the intercellular air spaces and absent from the walls facing neighboring cells because carbon dioxide is diffused through intercellular air spaces in terrestrial leaves. By contrast, the chloroplasts of aquatic leaves are oriented toward the epidermal layers (Mommer et al., 2005). This corresponds to the uptake of carbon dioxide that occurs on a leaf's surface in aquatic leaves; therefore, the different location of chloroplast in leaves of aquatic plants is the mechanism by which chloroplasts reach close to the carbon source. In terrestrial plants, only guard cells on the epidermis have chloroplasts that regulate stomatal opening; the perception of blue lights, the sensing of carbon dioxide and the mechanisms of photosynthesis are important for controlling stomatal opening, and these processes occur in the chloroplasts of guard cells (Zeiger et al., 2002).

Adaptive traits of aquatic leaves can be reaffirmed by amphibious plants. "Amphibious plant" refers to plants that inhabit both standard and submerged conditions. As amphibious plant lives in dual habitats, the plant produces two types of

leaves (Figure 1). If amphibious plant grows in the water, the plant makes aquatic leaves that share similar morphological and anatomical traits (Wells and Pigliucci, 2000; Minorsky, 2003; Colmer et al., 2011). Some amphibious plants generate both types of leaves simultaneously in their developmental phase. In the juvenile phase, when the plant grows in the water, it produces aquatic leaves. In the adult phase, shoot apical meristem (SAM) can emerge out of the water, and produce terrestrial leaves (Winn, 1999). The others do not have such transition capabilities, thus they can end their lives as terrestrial plants or aquatic plants, generating only one type of leaf (Winn, 1999; Wells and Pigliucci, 2000). In both cases, there is no doubt that the amphibious plants have mechanisms which perceive environmental changes so that he plant chooses adequate leaf types. The leaf dimorphism of amphibious aquatic plants is called “heterophylly,” and aquatic plants are typical examples of this trait. Heterophyllous phenotypes of amphibious plants indicate that respective leaf types are essential or increase the plant fitness in each environment because amphibious plants should prepare dual suits and change according to the occasion and their location.

Figure 1

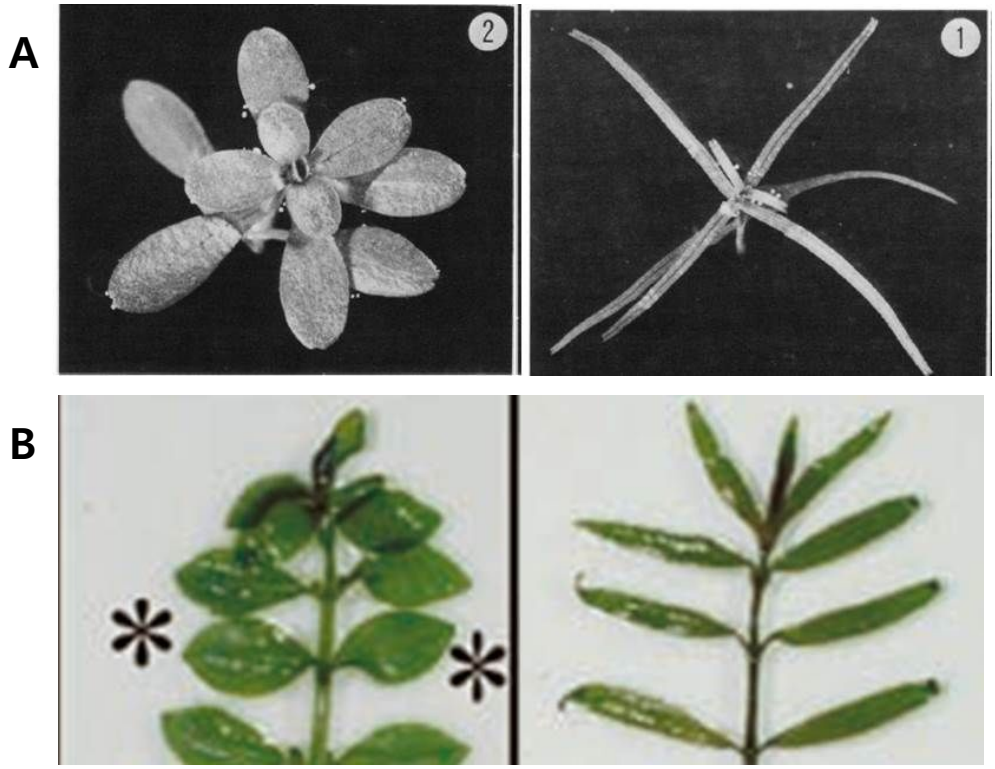


Figure 1. Amphibious plants with terrestrial leaf and aquatic leaf.

(A) *Callitriche heterophylla*

(B) *Ludwigia arcuata*

Left panel shows terrestrial forms and right panel shows aquatic forms for each species. These figures adapted from Deschamp and Cooke, (1985) and Kuwabara *et al.*, (2003)

3. Endogenous and exogenous factors to regulate heterophylly

Generating aquatic leaves could have common mechanisms among amphibious plants that generate aquatic leaves, in spite of their convergent evolution. Amphibious plants should be able to perceive similar environmental cues, whether or not they are submerged, and the plants need to produce proper leaf types that share similar traits. As they have developed to deal with similar evolutionary pressures, physiological and molecular solutions to deal with these natural selections can be convergent, similar to their morphological phenotypes (Li et al., 2010). Some phytohormones and environmental cues are already known to be common heterophyllous inducers beyond taxa.

Abscisic acid (ABA) is a prominent example of heterophyllous regulator. ABA is well-known hormone related to water-deficiency because ABA causes stomata closure and prevent water loss. As ABA signaling can be activated in terrestrial environment, it is possible that amphibious plants recognize ABA as a terrestrial sign. Therefore, exogenous ABA induces terrestrial leaves on submerged shoots in all amphibian plant species investigated so far (Zeevaart and Creelman, 1988; Wells and Pigliucci, 2000). The terrestrial traits of aquatic shoots induced by ABA are identical to those of original terrestrial leaves on both an anatomical and morphological level. ABA treatment changes thin and filamentous aquatic leaves into broad shapes similar to terrestrial leaves. Other aspects of the aquatic leaves turn into those of terrestrial leaves, such as stomata density, the shapes and sizes of the epidermis and mesophyll cells, and chlorophyll content (Anderson, 1982; Deschamp and Cooke, 1985; Young et al., 1987;

Kane and Albert, 1989; Lin et al., 2005). The ABA concentrations of terrestrial shoots are also more abundant than those of aquatic shoots (Goliber and Feldman, 1989; Kuwabara et al., 2003). Previous literatures corresponded to the terrestrial traits that are induced by exogenous ABA treatment and amphibious plants could rearrange their ABA function for the induction of signaling for terrestrial leaf production.

Gibberellin (GA) is considered a growth-accelerating agent that promotes the processes of seed germination, stem elongation, leaf expansion, etc. ABA inhibits these processes, and it is regarded that there is antagonistic crosstalk between GA and ABA (Razem et al., 2006). Therefore, it is reasonable to assume that GA has the opposite function to ABA in heterophyllous development. GA can mimic aquatic leaves on the terrestrial shoots of amphibious plants (Allsopp, 1962; Deschamp and Cooke, 1984), which may be another example of an antagonistic effect between these two phytohormones. However, although ABA is a general inducer of terrestrial leaf production, GA is not widely used as a signal for aquatic leaf production. For example, GA has no effect on heterophylly in *Ludwigia arcuata* (Kuwabara et al., 2003) and cannot induce terrestrial leaf growth in *Proserpinaca palustris* (Kane and Albert, 1987). Therefore, GA does not play a major role for generating aquatic leaves in amphibious plants..

Ethylene also can be a key factor for amphibious traits. Ethylene is a gaseous phytohormone that is diffused steadily from tissues to the external environment. When plant tissues are covered by water, the diffusion rate of ethylene is also decreased like other gas molecules. Thisphenomenon is called entrapment process , which increases

the ethylene concentration in the leaves, even if the ethylene biosynthesis rate is unaltered (Jackson, 1985). In *Ludwigia arcuata*, the ethylene signaling is necessary and required for the induction of aquatic leaves, which indicates that this species employs ethylene signaling as an alarm for submergence (Kuwabara et al., 2003).

Other factors such as temperature, photoperiod, light intensity, light quality, osmotic stress, and humidity are also related to heterophyllous development (Wells and Pigliucci, 2000; Kuwabara et al., 2003; Minorsky, 2003). These factors and parameters are changed by submergence, either directly or indirectly. The mechanism that temperature and photoperiod regulate heterophylly in some amphibious plants may reflect their ecological niches; for instance, if a periodical inundation is occurs in their habitat, their responses to temperature or photoperiod fluctuation by seasonal changes increase their fitness (Wells and Pigliucci, 2000).

In summary, several endogenous phytohormones and environmental cues act together or in parallel, and change the internal mechanisms of amphibious plants, and then the pathways finally converge for heterophyllous development.

4. The importance of leaf shape in plant

Leaves are an organ for photosynthesis. Photosynthesis is a process by which light energy is converted into chemical energy to produce organic carbon compounds for growth and life. The shape and size of the leaves are important to for receiving light energy, and those are linked to the plant fitness. To maximize absorbance of light energy, flat, wide leaves are advantageous. Thin leaves are favorable to promote gas

exchange for carbon fixation and respiration. However, thin, wide leaves are vulnerable to desiccation and overheating. Therefore, the terrestrial plants need to balance between maximization of light absorbance and minimization of drought stress and the trade-off of them is an important factor to determine leaf shape. As a result, the thin, flat leaves of terrestrial plants with a high density of veins and stomata have evolved to increase their fitness and optimize their photosynthesis efficiency in land environments (Tsukaya, 2006).

As a consequence of different trade-off mechanisms, there are many types of leaf shape in the world. Thesevarious shapes of leaves reflect environmental adaptation to respective niches. For example, the genus Hakea has terete leaves, which means they are needle-like (Groom et al., 1997). This needle-like leaves have advantages to drought stress, which increases fitness to Hakea in Mediterranean drought habitat (Groom et al., 1994). Peltate leaves are round or shield-like leaves, and their petioles are located underneath the center of the leaf; this type of leaf minimizes total mass of stem and vein (Givnish, 1987; Gleissberg et al., 2005). Peltate leaves can cover broad field, which maximize sunlight harvest. In extreme cases, climbing plants produce tendrils to support themselves and carnivorous plants can produce a pitcher to catch small animals (Fukushima and Hasebe, 2014). These specialized leaves are essential for their behavior and survivor. Therefore, proper leaf structure is one of pivotal points to survive each plant species.

5. The relationship between leaf shape and leaf polarity

In spite of variety of leaves among plant species, leaf development is started from similar dome-like structure, SAM. Leaf primordia start as protrusions from SAM. After subsequent cell proliferation, outer cells lose the identity of SAM and acquire leaf traits. Meantime, adaxial and adaxial pattering arises based on its vicinity to a SAM (Kerstetter et al., 2001; McConnell et al., 2001; Emery et al., 2003); the proximal SAM cell layers become the adaxial domain, and distal cell layers differentiate to the abaxial domain. With the exception of unifacial leaves, most leaves are composed of these two domains. Leaf blades are generated in the boundary region between the adaxial and abaxial domains (Nicotra et al., 2011; Fukushima and Hasebe, 2014). Finally, the size and shape of leaves are determined by leaf blade outgrowth (Rodriguez et al., 2014) Adaxial-abaxial leaf patterningplays an important role in leaf shape because the position of leaf blade is controlled by leaf polarity. If leaf polarity is altered, the growth of leaf blade is also attenuated or diminished (McConnell and Barton, 1998); therefore, the patterning of leaf polarity is a main issue of leaf development.

Leaf polarity is also linked to other leaf development. Each side of leaf is functionally diversified. The adaxial domain has relatively thick cuticle layers and compact palisade mesophyll cells except for unifacial and equivocal leaves. The palisade structure maximizes its ability to harvest light. In contrast, the abaxial domain has loose, spongy mesophyll cells surrounded by an intercellular air space. Porous structure provides the diffusion space for gas exchange and transpiration. Vascular tissue is also divided into adaxial xylem and abaxial phloem (Yamaguchi et al., 2012;

Fukushima and Hasebe, 2014) and in non-dorsiventral leaves such as unifacial leaves, they show radially symmetrical or compressed cylindrical veins (Nelson and Dengler, 1997), which reflects that vascular patterning is regulated by leaf polarity. The development of trichomes and stomata and their densities are also differed along with leaf polarity. In arabidopsis, stomata are dominantly developed in abaxial side of cotyledons and leaves but the differentiation of trichomes are restricted in adaxial side of early produced leaves (Bowman, 2000). Moreover, cellular metabolism can be regulated by leaf polarity. *Egeria densa* and *Potamogeton lucens* are aquatic plants; they can utilize carbonate as carbon source for photosynthesis by inducing acidification in abaxial side. In this way, carbon dioxide is released and concentrated on abaxial surface of leaves, and then it diffuses into the leaves (Prins and Elzenga, 1989; Lara et al., 2002).

Leaf polarity is setup from early leaf .development. The axis of adaxial-abaxial patterning is a basis of leaf blade position and leaf outgrowth. Other leaf components such like mesophyll cells, vascular cells, and epidermis cells are also regulated by leaf polarity. Therefore, leaf polarity is a good target for leaf growth and shape.

5. Molecular mechanism for leaf adaxial–abaxial specification

Leaf polarity is established by leaf polarity genes. There are several gene families which have function to set-up leaf adaxial identity. The *class III HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIP III*) genes belong to adaxial determinant group. The name of HD-ZIP-III is originated from *N*-terminal DNA-binding

homeodomain and leucine-zipper motif for dimerization. They also have putative sterol binding START domain and another protein interacting MEKHLA motif in C-terminal (Husbands et al., 2009; Yamaguchi et al., 2012). In *Arabidopsis*, there are five *HD-ZIP III* genes; *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *AT-HB8*, and *CORONA* (*CNA*). The *HD-ZIP III* genes are expressed in adaxial domain of the leaf (Emery et al., 2003; Williams et al., 2005). Loss-of-function mutants of *HD-ZIP III* genes show abaxialized cotyledon (Emery et al., 2003). Conversely, in gain-of-function mutants, ectopic expressions of them are raise in entire leaf primordia and mutants show adaxialized curling leaves(McConnell et al., 2001). These phenotypes indicate that *HD-ZIP III* genes establish adaxial identity. In other species, homologs of *HD-ZIP III* genes are expressed in adaxial side and ectopic expressions of them produce adaxialized leaves (Juarez et al., 2004; Floyd et al., 2006; Itoh et al., 2008). These results suggest that the function of *HD-ZIP III* genes is conserved among plant kingdom, which control adaxial fate in the leaf (Floyd et al., 2006; Husbands et al., 2009; Yamaguchi et al., 2012).

ASYMMETRIC LEAVES1 (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*) are distinct adaxial identity genes. *AS1* encodes MYB transcription factor. It belongs to ARP protein family which is composed of ortholog genes of *AS1*, *ROUGH SHEATH2* in maize and *PHANTASTICA* (*PHAN*) in *Antirrhinum* (Waites et al., 1998; Timmermans et al., 1999; Byrne et al., 2000). *AS2* encodes a plant specific AS2/LOB domain protein with leucine-zipper domain (Iwakawa et al., 2002). Both loss-of-function and gain-of-function mutants of *ARP* genes do not show obvious defects in adaxial-abaxial

patterning except of *PHAN* (Timmermans et al., 1999; Bowman et al., 2002; Xu et al., 2003). The ARP genes are expressed throughout developing leaf primordial (Waites et al., 1998; Timmermans et al., 1999; Byrne et al., 2000). By contrast, *AS2* is expressed in adaxial domain and overexpression of *AS2* results in adaxialized leaf even though *as2* mutant do not show defect phenotypes in leaf polarity like as1 mutant (Xu et al., 2003; Iwakawa et al., 2007). AS1 protein interacts with AS2 protein, which suggests that AS1 can contribute to adaxial fate through interaction with AS2(Xu et al., 2003). In maize, the ortholog gene of *AS2* is expressed in adaxial domain and the mutant shows defect in leaf polarity pattern(Evans, 2007). Therefore, the function of *AS1–AS2* pathway in leaf polarity is partially conserved in angiosperm (Yamaguchi et al., 2012).

On the other hand, there are also several families for establishing abaxial fate determination. *KANADI* (*KAN*) genes belong to plant specific transcription factors which have GARP domain (Yamaguchi et al., 2012; Fukushima and Hasebe, 2014). This gene family is expressed in abaxial side. The expression patterns of *KAN* are conserved among angiosperm (Kerstetter et al., 2001; Candela et al., 2008; Zhang et al., 2009). There are four *KAN* genes (*KAN1-KAN4*) in Arabidopsis (Yamaguchi et al., 2012). Each loss-of-function mutant of *KANs* does not show remarkable phenotypes in aspect of leaf polarity but progressive losses of them represent severe abaxialized leave phenotypes. Conversely, in gain-of-function mutant of *KAN1* and *KAN2*, ectopic expressions of them are occurred in entire leaf primordial, which produce abaxialized leaves (Eshed et al., 2004). These phenotypes indicate that *KAN* genes function in abaxial fate specification. Homologs of *KAN* genes in other species are also related to

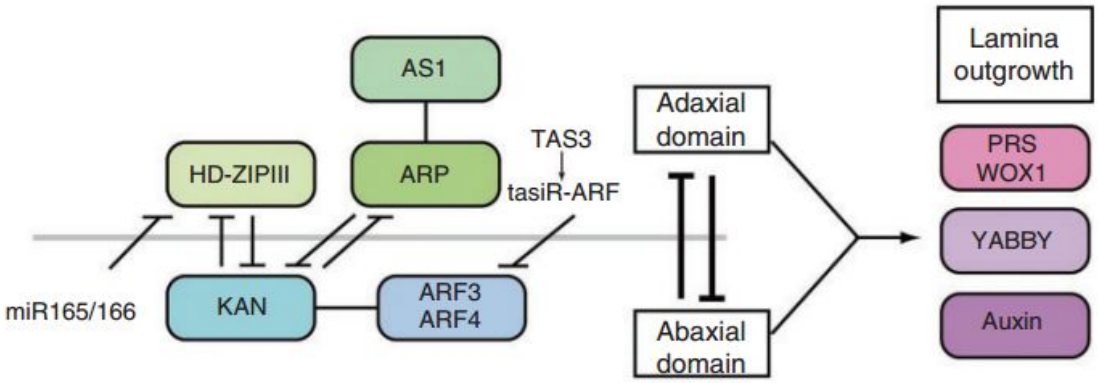
abaxial cell fate (Candela et al., 2008; Zhang et al., 2009). These results suggest that the determination mechanism of abaxial identity by *KAN* genes is conserved among angiosperm.

AUXIN RESPONSE FACTOR 3/ETTIN (*ARF3/ETT*) and *ARF4* also regulate leaf abaxial specification. ARF genes encode transcription factors which are downstream targets of auxin (Husbands et al., 2009). Individual loss-of-function mutants do not show severe phenotypes in leaf polarity(Hunter et al., 2006), but double mutant of ARF3/4produce adaxialized leaves (Pekker et al., 2005). ARF genes are expressed in abaxial domain, which is consistent with their roles in leaf polarity. The severe phenotype of *ARF3* overexpression lines is similar to *as2* mutant and they produce leaves with partial abaxialized trichomes (Husbands et al., 2009). In addition, ARF3 interacts with KAN protein(Kelley et al., 2012), which suggests that ARF3 controls abaxial fate identity.

Small RNAs also involve in leaf polarity through interfering leaf polarity genes. The messenger RNAs of *HD-ZIP III* genes have complementary sequences which are recognized by miR165/166. The messenger RNA of *HD-ZIP IIIs* are cleaved by miR165/166, therefore, the stability of them is affected by miR165/166 (Emery et al., 2003). miR165/166 also induce DNA methylation on *PHB* and *PHV* loci, which suggests that miR165/166 leads transcriptional gene silencing of *HD-ZIP III* genes (Bao et al., 2004). The messenger RNAs of ARF3/4 is another target of small RNA. They are recognized by trans-acting small interfering RNA (ta-siRNA). TAS3 is a protein noncoding gene and target of miR390. Cleaved TAS3 mRNA by miR390

become to template of RNA-dependent RNA polymerase, then produce ta-siRNA which targets to ARF3/4 (Allen et al., 2005; Fahlgren et al., 2006). The transcription of TAS3 is occurred only adaxial side of leaves, thus the expression of ARF3/4 is restricted to abaxial side of leaves (Garcia et al., 2006). The mutants of small RNA biogenesis genes show defects in leaf shape and leaf polarity, which also represents the relationship between small RNAs and leaf polarity (Garcia et al., 2006; Nogueira et al., 2007).

Figure 2



Model for molecular mechanism of leaf adaxial–abaxial patterning

These figures adapted from Yamaguchi *et al.* (2012).

5. Purpose of study

Even though the underwater environment is not friendly conditions to land plant, transitions from land plant to aquatic plant have occurred in various taxa. As a result, aquatic plants cover 1–2% of total angiosperm species (Cook, 1999).In early angiosperm evolution, aquatic plants were dominant, and some investigators argued that the first angiosperms were aquatic plants (Sun et al., 2002; Doyle, 2012). This is consistent with the aquatic habitat of basal angiosperms including Nymphaeales, Hydatellaceae, and *Ceratophyllum*. Moreover, it has been proposed that monocots' ancestors were aquatic plants. The acorales, the most basal monocots, are also regarded as aquatic plants, which emphasizes the aquatic origins of monocots (Cook, 1999).Therefore, aquatic plants are noteworthy objects to study.

However, direct investigations of true aquatic plants are not easy. First, true aquatic plants need aquarium systems that are quite expensive, heavy, and hard to maintain. Second, circulation systems are also needed, and isolation of each individual plant is seldom possible for practical reasons. This problem makes it vulnerable to pathogen and seed mixing. Therefore, amphibious plants are good alternative substitutions because they can be cultivated and harvested on land, which minimizes of the need for aquarium work. Moreover, if true aquatic plants are compared with related terrestrial species, it is necessary to find each homologous gene for respective species. In the study of amphibious plants, only one set of gene information is needed. Therefore, amphibious plants can be easy and simple materials for studying aquatic leaves.

Amphibious plants also can be used for heterophylly study itself. These heterophyllous plants have evolved mechanisms that produce proper leaf types according to environmental or developmental cues. This means that new cross-talks have arisen that connect from external and internal signaling to networks for plant architecture. The study of amphibian plants can reveal these new cross-talks or networks.

The *Ranunculus* genus is one of the biggest genera in angiosperm clade. Matching its size, it covers highly diverse habitats and life types, including aquatic environment. The batrachium subgenus contains about 30 aquatic species, which have evolved from related terrestrial species (Hörandl and Emadzade, 2012). If comparative studies are achieved among these abundant examples of aquatic species, they give many clues about how aquatic leaves have evolved. I chose an amphibious species, *Ranunculus trichophyllus* var. *kadzusensis*, as material for the present study. With this species, I built up primary physiology data for morphological heterophylly. Then, I cloned several homolog genes that they were known as essential genes for specific metabolism or developmental factors in model plants, especially studied in Arabidopsis. I tested whether these homolog genes showed differential expression patterns under terrestrial and aquatic conditions. This approach would give information and insights for fundamental mechanisms of aquatic leaves and hetorophyllic phenomenon.

MATERIALSAND METHODS

1. Plant materials and growth conditions

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* were donated from the Korea National Arboretum, which collected from Namyangju City. Seeds of Ranunculus sceleratus, collected from Namyangju City, were donated from the Korea National Arboretum. 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. The petri dish was placed in a 4ºC cold chamber for 1 week, then transferred to the growth room. For experiments involving Arabidopsis thaliana, Col-0 seeds were used. The *pAS1-AS2* transgenic line was donated by Dr. C. Machida (Chubu University, Japan) (Iwakawa et al., 2007). The growth room was maintained at 22ºC, 60 ± 10% relative humidity in a long day photoperiod (16h light/8h dark).

2 Microscopicanalyses

One week after germination, the seedlings were transferred to experiment conditions—like aquatic condition or hormones containing medium—for 1 week. The seedlings were fixed by Karnovsky's fixative method (Karnovsky, 1965). Fixed samples were treated with 0.05 M cacodylate and 0.05 M osmium tetroxide for 2 h. The samples were dehydrated by ethanol and then immersed in isoamyl acetate solution. Finally, the samples were dried by critical point drying method. Dried samples were coated with platinum particles, and then their epidermis shapes were observed using SEM (Carl Zeiss, SUPRA 55VP).

3. Hormone treatment and whole mount clearing

Seedlings of *R. trichophyllus* and *R. sceleratus* (1 week after germination) were used. For ethylene treatment, seedlings were transferred to half-strength MS medium with 50 μM 1-Aminocyclopropane-1-carboxylic acid (ACC), 1μM auxin, 10 μM GA, 1 μM epi-brassinolide (EBL), and 50 μM bikinin. In submerged condition experiments, 100 × 40 plant culture dish were used, then seedlings were transferred and filled with distilled water. After water filling, 1 μM ABA, 10 μM AgNO3, 1μM1-N-Naphthylphthalamic acid (NPA) and 10 μM paclobutrazol (PBZ) were added. After 1 week's growth in a growth chamber, the first true leaves of seedlings were used for next experiences.

For calculating leaf index and hypocotyl length, each seedling was placed on MS plate and snapped. Leaf lengths, widths, and hypocotyl lengths were measured by

comparing them to a ruler image. Leaf index was obtained by ratio of leaf length to leaf width.

For whole mount clearing, first true 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℃, epidermises and xylem elements were observed using an Axio Imager A1 microscope (Carl Zeiss) under DIC optics and were photographed using an AxioCam HRc camera (Carl Zeiss).

4. Expression studies by real-time quantitative polymerase chain reaction (qPCR)

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. *RtACTIN* (*RtACT*), *RsACT*, *AtPROTEIN PHOSPHATASE 2A* (*AtPP2A*) were used as reference genes for normalization. The relative transcript levels were calculated according to the ΔΔCtmethod (Livak and Schmittgen, 2001). The information of primer use is served in table 2.

5. Homolog genes sequencing for *R. trichophyllus* and *R. sceleratus*

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 know 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.

6. 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).

Table 2. List of primer sequences used in this study

Oligo name	Sequences (5’ to 3’)
For Gene cloning	
AqAAO F2	AAAAGGAATGGCAGAAGCTGA
AqAAO R2	ACCACAGGCATGGTCGC
AqAAO3 NF2	GATGCTTGCAAGAGTTTTGC
AqAAO3 NR	GCTCCTATTTCAATCCCT
AqACO F2	CAACCATGGAGATCATCAA
AqACO R2	AGCTTCATGTAGTCCTCAAA
AqACT F	TTTGCTGGTGATGATGCCC
AqACT R	TAGAGATCCTTCCTGATATC
AqAS1 F2	CCAGGGATCAAGAAAGG
AqAS1 R2	AACACTTCCCACCACTT
AqFAMA F2	GGAAGCAGATGAATGAACATCT
AqFAMA R2	ATGGTGGTAATATTAGTGTG
AqHD-ZIP III-b F	GACAATGGGAAGTATGTGAG
AqHD-ZIP III-b R	ACAGCTCTCTCATAGGATAC
AqHD-ZIP III-b F	CTAAACAGATCAAAGTCTGG
AqHD-ZIP III-b R	TTCATGAACATGAAAGCCAAG
AqHD-ZIP III-c F	AAGGCTACAGGAACTGCTG
AqHD-ZIP III-c R	GGAGCATACATCTGCGTG
AqKAN-a F	TTCCCAGCACAACCTGATC
AqKAN-a R	TCAATGTGGCCTGCCCAA
AqKAN-b F	CCTATCAAAGGGATTCC
AqKAN-b R	GGTGAATTCCAAGCTTGG
AqKAN-c F	AACTGCATCAACTTTCCCTG
AqKAN-c R	ACTTGAGAAGGGTTAGTTC
AqMUTE F	ATGTCTCACATTGCTGTAGA
AqMUTE R	ACAGTGTCTTCCATGCTACT
AqRAP2.2 F2	TGCTGATTACTTATGGCCTG
AqRAP2.2 R2	TCCCACCATCCTGAGTAG
AqXCP1 F	GCTTGGTTTGAATGAGTTTGC
AqXCP1 R	CCAGTGTTTCTCTTCATCC
AqYDA F	CCTAGAATGACAAGCCC
AqYDA R	AACAGTACATCCAAGACTCC
RtKAN-a 3' R	CACCACTGCTCCCTAGAG

RtKAN-a 5' F	AGGCACGAAACAACCATTTC
RtKAN-b 3' R	CCCAATTCCATTTGACTGC
RtKAN-b 5' F	CCATGCCTTTCTCTTGTC
RtKAN-c 5' F	ACTCATGATGGTATAATCACC
RtHD-ZIP III-a 3'	GGAACATTGGAAAGTACAC
RtHD-ZIP III-a 5'	TCCGTAAGATCTTTTAGTGC
RtHD-ZIP III-b 3'	ACACGAACATAGAGATGCC
RtHD-ZIP III-b 5'	ATCTTGCCTCGAGATGAG
RtHD-ZIP III-bseq F	GTCGCTTCTTGGTAGTGATGG
RtHD-ZIP III-c 3' R	AATGCGTCATTTTCACCAG
RtHD-ZIP III-c 5' F	TAGGATCGAAAATGGATGC
RtYAB 3' R	AGTGCAAGATGCAAAGTAAC
RtYAB 5' F	CCAGTCCATCAATCGAATC
RscHD-ZIP III- F	AGCCTTGAGGTTCTCCAAC
RscHD-ZIP III-R	AACCCTGCTGCATTATCTT
For RACE	
RkAAO3 RACE R1	ATTACGAGTATATGCGGGCAAC
RkAAO3 RACE R2	TCCTCCTTACTCCAAAATGAA
RkACO RACE F1	ACCCGCCATGCCCACGTC
RkACO RACE F2	CGACCAATGGATCGATGTACC
RtAS1 5'RACE R0	GTGACTTGTTACTCTCTTTCTGC
RtHD-ZIP III-a RACE R1	CCCAGCCAGTGACAGTCGAC
RtHD-ZIP III-a RACE R2	CGGACCACCTTGAGTACTACC
RtKAN-b RACE F1	TGAAGACAACTGACAAACCTGCA
RtKAN-b RACE F2	GAATGGTCTGCAAGGTCCG
RtKAN-c RACE F1	GGAAAGGGATGCTCGGAGCTC
RtKAN4-c RACE F2	CAGATAAACCGACTAAGGAAGC
RtHD-ZIP III-b RACE R1	GTTCGCAATAAAGCAGGTTGGCG
RtHD-ZIP III-b RACE R2	AGCTCTATGGTTCCGTCGTTC
RtHD-ZIP III-c RACE F	GGAGATCCTCAAAGACCGTC
RtHD-ZIP III-c RACE R	CACCTGTTTCTGGAGACGGT

RESULTS

1. Heterophylly of *R. trichophyllus*

R. trichophyllus is a herbaceous, amphibious, and annual plant. It showed aquatic leaf when it grew in submerged condition. It has complex leaves, and both terrestrial and aquatic leaves were dissected. The aquatic leaves were thin and thread-like with pale-green color, and theirleaflets were hardly distinguishable from its petiole, which are common properties among aquatic leaves (Jones, 1955; Schneider and Jeter, 1982; Givnish, 1987; Iida et al., 2007). It generated five to six rosette-leaves, and then stem elongation was started. Simultaneously, terrestrial shoot produced axillary shoot apical meristems, whereas in aquatic shoots, stem elongation was conspicuous growth (Figure 3Band 3C).

To compare the shape between terrestrial leaves and aquatic leaves, I measured leaflet length and width. Lengths of terrestrial leaves were shorter but widths were broader than those of aquatic leaves (Figure 4B). However, leaflet length or width only reflects leaf size. For example, under expanded aquatic leaflets represent shorter lengths similar to terrestrial leaflets. On the other hand, under expanded terrestrial leaflets show narrower widths similar to aquatic leaflets. Since I wanted to categorize leaflets through their shapes not their sizes, I adopted a parameter which was independent from leaflet expansion degree. The ratio of leaflet length to width fit to my purpose. If the leaflet is short and stumpy, the ratio would be small relatively. If it is

narrow and thin, the index would be larger. As expected, the index of aquatic leaves was greater than that of terrestrial leaves (Figure 4B).

At the microscopic level, I could find additional differences between two types of leaves. Terrestrial leaves had well developed stomata. Stomata were dominantly existed onadaxial surface; on abaxial surface, the stomata were rarely produced (Figure 5A and 5B). In *Arabidopsis* or maize, stomata are dominantly developed on abaxial epidermis (Geisler et al., 1998; Chen et al., 2003; Driscoll et al., 2006). This adaxial distribution of stomata on leaf surface were one of interesting characteristic of *R. trichophyllus* compared to model plants. Moreover, difference of stomatal densities between adaxial and abaxial side were more dramatic (Figure 5D). Aquatic leaves did not show any stoma structure (Figure 5C and 5D; Germ and Gaberščik, 2003).Terrestrial leaves had jigsaw puzzle-like epidermis, while aquatic leaves had rectangle-type epidermis (Figure 5A to 5C). Vascular structures were also affected by submergence. Vessel elements were developed well in terrestrial petioles, while in aquatic petioles, the number of vessel elements was reduced, and secondary cell wall structures were also under-developed (Figure 5E). *R. trichophyllus* lacked trichomes or any appendage structures on its epidermis, but aquatic leaves had some hair cells on its leaf tips (Figure 5F). Cross-sectioning revealed bifacial shape of the terrestrial leaf and cylindrical shape of the aquatic leaf (Figure 5G).

Figure 3

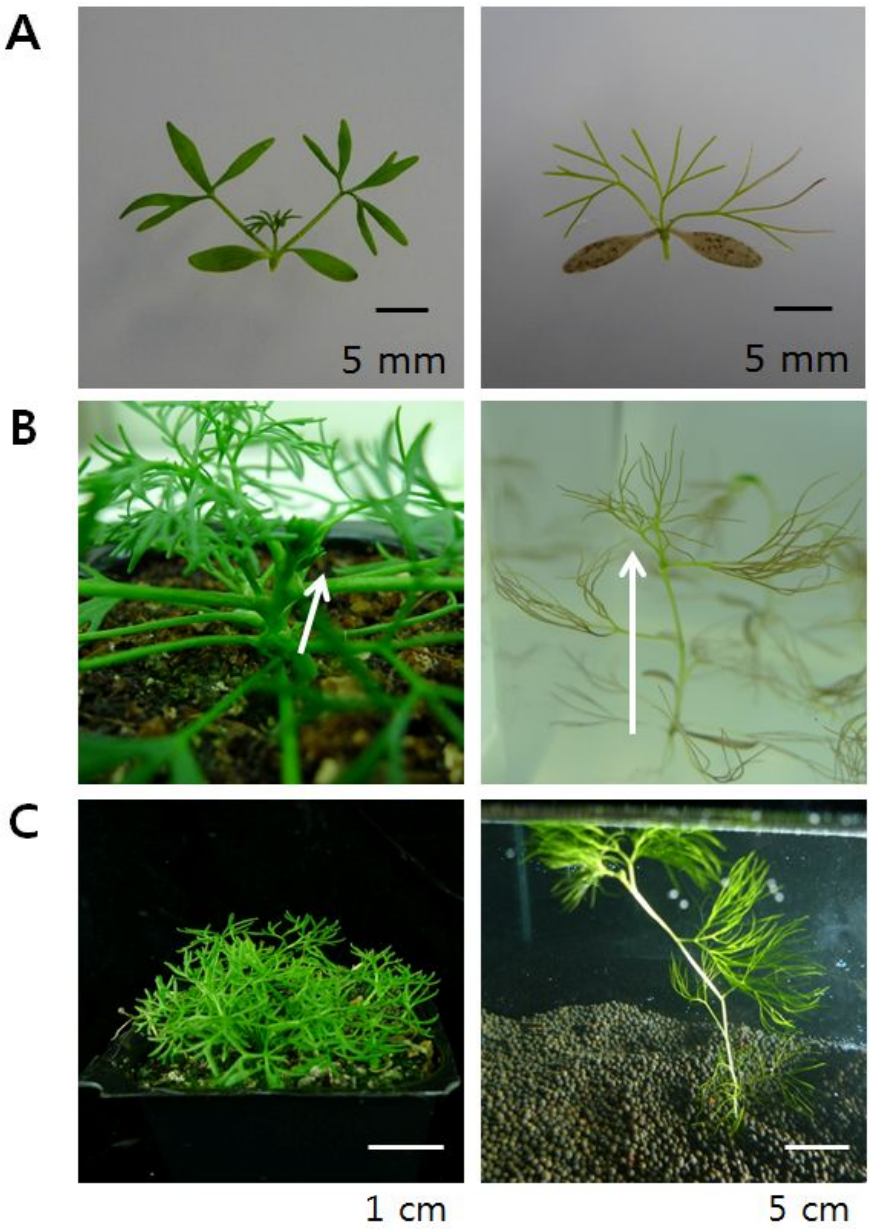


Figure 3. *R. trichophyllus* morphology according to developmental growth.

(A) 3-week-old seedling

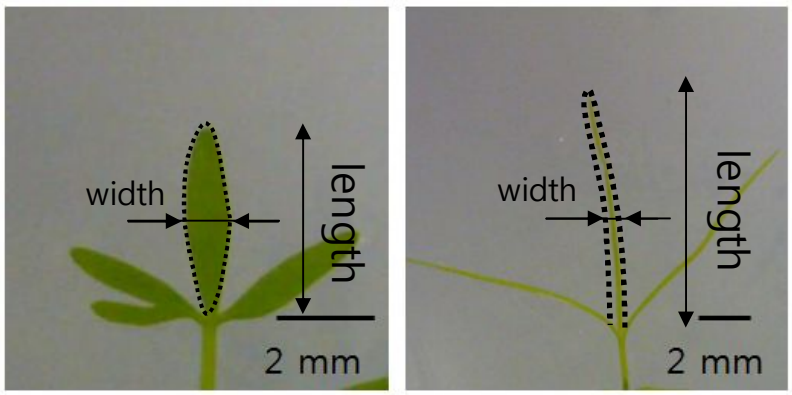
(B) 6-week-old seedling

(C) Adult plants just before flowering

Left panel shows terrestrial plants, and light panel shows aquatic plants.

Figure 4

A



B

	Terrestrial leaflet	Aquatic leaflet
Length (mm)	Short (3.24±0.49)	Long (9.28±0.74)
Width (mm)	Broad (0.78±0.11)	Thin (0.24±0.03)
Ratio (length/width)	Relative less (4.17±0.35)	Relative high (39.36±3.79)

Figure 4. *R. trichophyllus* heterophylly between terrestrial leaf and aquatic leaf.

(A) Leaf morphology of *R. trichophyllus* under terrestrial and aquatic environments. Left panel shows terrestrial leaf and right panel shows aquatic leaf. These are 2 week old seedlings. Dot lines represent leaflet margin.

(B) Table for brief comparison between terrestrial leaf and aquatic leaf. The index of ratio means the ratio of length to width. The data are presented as means ± SD from three biological replicates. (n=24)

Figure 5

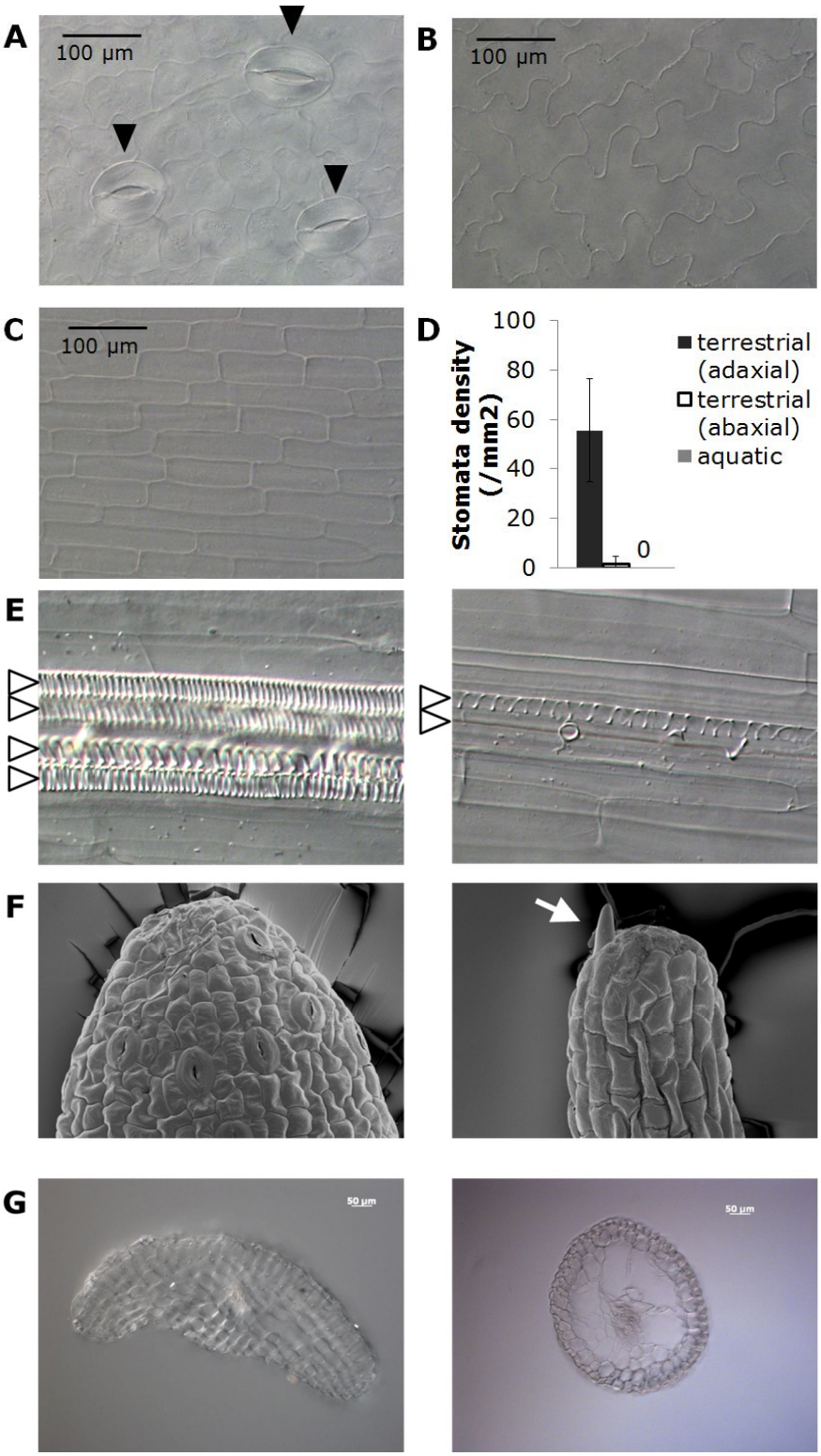

A
100 µm
B
100 µm
C
100 µm
D
Stomata density (/mm2)
100
80
60
40
20
0
0
terrestrial (adaxial)
terrestrial (abaxial)
aquatic
E
F
G
50 µm
50 µm

Figure 5. Microscopic analyses revealed differences between terrestrial leaf and aquatic leaf in *R. trichophyllus*.

(A to C) Epidermis cells of *R. trichophyllus*. (A) is adaxial side of terrestrial leaf, and (B) is abaxial side. (C) shows the epidermis of aquatic leaf.

(D) Statistical analyses of each stomatal density. I used eight leaflets for each leaf type.The data are presented as means ± SD from three biological replicates. (n=12)

(E to G) Left panel showed terrestrial leaf and right panel showed aquatic leaf.

(E) Vessel elements of each petiole.

(F) Leaf tips of each leaf.

(G) Cross-section of each leaf.

Black arrowhead indicates stoma. White arrowhead indicates each vessel element. White arrow points to a hair cell on a leaf tip.

2. Exogenous phytohormones affected to heterophylly of *R.trichophyllus*

Phytohormones are already known to provoke heterophyllous phenotypes in amphibious plants; especially, ABA induces terrestrial leaves on aquatic shoots in various species investigated so far (Anderson, 1978; Deschamp and Cooke, 1983; Yuong and Horton, 1985; Goliber and Feldman, 1989; Wells and Pigliucci, 2000). Thus, the present study investigated whether heterophylly in *R. trichophyllus* was also controlled by phytohormones.

Auxin, ethylene, and GA have been reported to induce aquatic responses in semi-aquatic and amphibious plants (McComb, 1965; Walters and Osborne, 1979; Deschamp and Cooke, 1984; Kuwabara et al., 2001; Kuwabara et al., 2003; Voesenek et al., 2006). To confirm whether these phytohormones also induce aquatic traits on terrestrial shoots, NAA and ACC, agonists for auxin and ethylene, and GA were treated to terrestrial seedlings. One of them, ethylene signal, induced narrow and pale-green leaf (Figure 5C), which were close traits of the aquatic leaves. GA also caused leaflet elongation, whereas leaf index was not significantly changed in the leaf treated with ACC (Figure 5D and 5E). Auxin did not induce any significant phenotypes.

The present study aimed to determine whether parameters such as stomata densities and the number of vessel elements were also affected by ethylene signaling. It was found that ethylene signaling reduced stomatal densities and the number of vessel elements in leaves on terrestrial shoots. Jigsaw-like shapes of epidermis cells were changed to tetragon-like shapes, which were also close to rectangle-like aquatic

epidermis (Figure 6B, 6D, and 6E). However, GA did not cause dramatic changes of structures—at least, not in microscopic levels (Figure 6C to 6E). Even though ACC did not generate complete aquatic traits on terrestrial shoot—for example, no hair cells on their leaf tips (Figure 6B)—these results suggested that ethylene signaling was linked to aquatic leaf development.

On the other hand, the terrestrial traits might be induced by exogenous molecules in aquatic shoots. In addition to ABA, $AgNO_3$ and PBZ, which are antagonists of ethylene and GA signaling, respectively, were chosen (Seyedmonir et al., 1990; Davidson et al., 2006). If ethylene and GA signaling are required for aquatic leaf growth, it is expected that these inhibitors produced the terrestrial traits on aquatic shoot. Aquatic shoots were treated with ABA, $AgNO_3$, and PBZ. Each of them affected normal aquatic leaf growths; they decreased leaf indices compared to untreated aquatic leaves (Figure 7C to 7E). Therefore, each of them interfered with normal growth of aquatic leaves.

Microscopic analyses indicated that ABA and $AgNO_3$ treatments provoked terrestrial traits on aquatic shoots. Stomatal densities and number of vessel elements were increased by ABA and $AgNO_3$ treatments (Figure 8B, 8C, 8E). Moreover, hairs of leaf tip also disappeared with ABA and $AgNO_3$treatments, which suggest that terrestrial traits are induced by these two molecules on aquatic shoots, at least at microscopic levels (Figure 8B and 8C). However, PBZ did not provoke terrestrial traits in aquatic shoots such as high stomatal densities and suppression of leaf hair development (Figure 8D to 8F). This result means that GA signaling is not required for aquatic leaf

development. Moreover, GA did not promote aquatic traits on terrestrial shoots, either. Taken together, GA signaling is independent from leaf plasticity of *R. trichophyllus*. Even though leaves treated with ABA and $AgNO_3$ were not identical to the terrestrial leaves (e.g., their vessel elements were not increased enough compared to terrestrial leaves), it was found that ABA and $AgNO_3$ elicited terrestrial traits in submerged condition. These results indicate that ethylene signaling is essential for aquatic leaf development and ABA signaling induces terrestrial leaf growth.

Figure 6

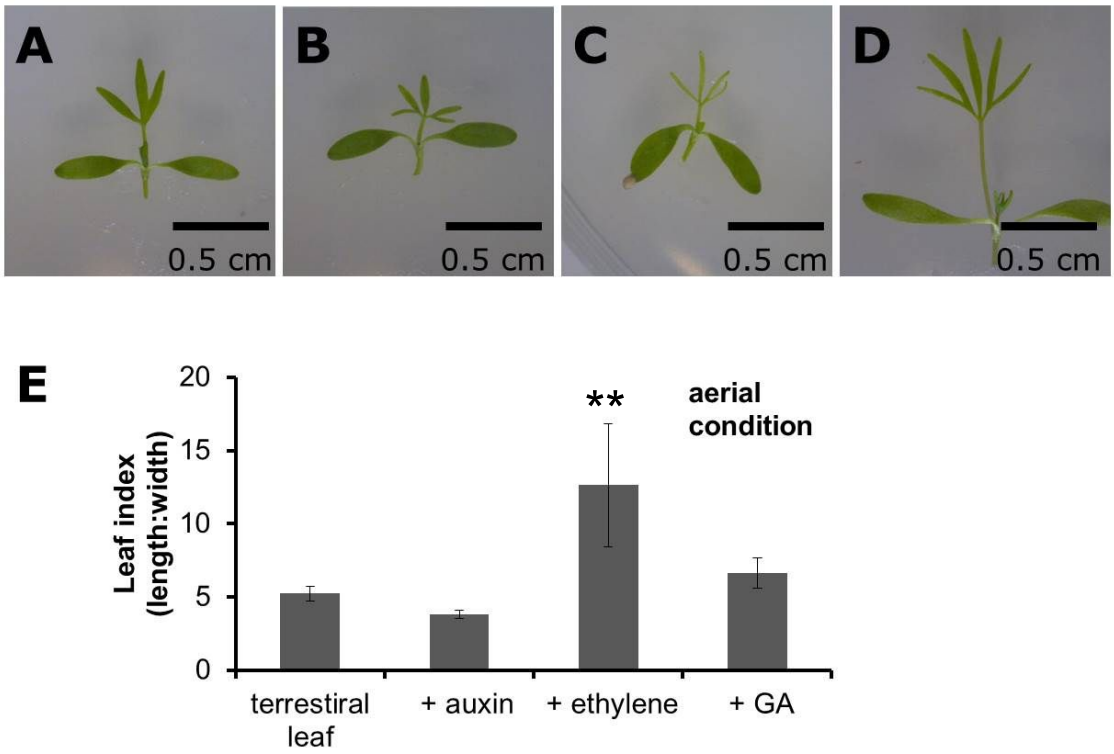


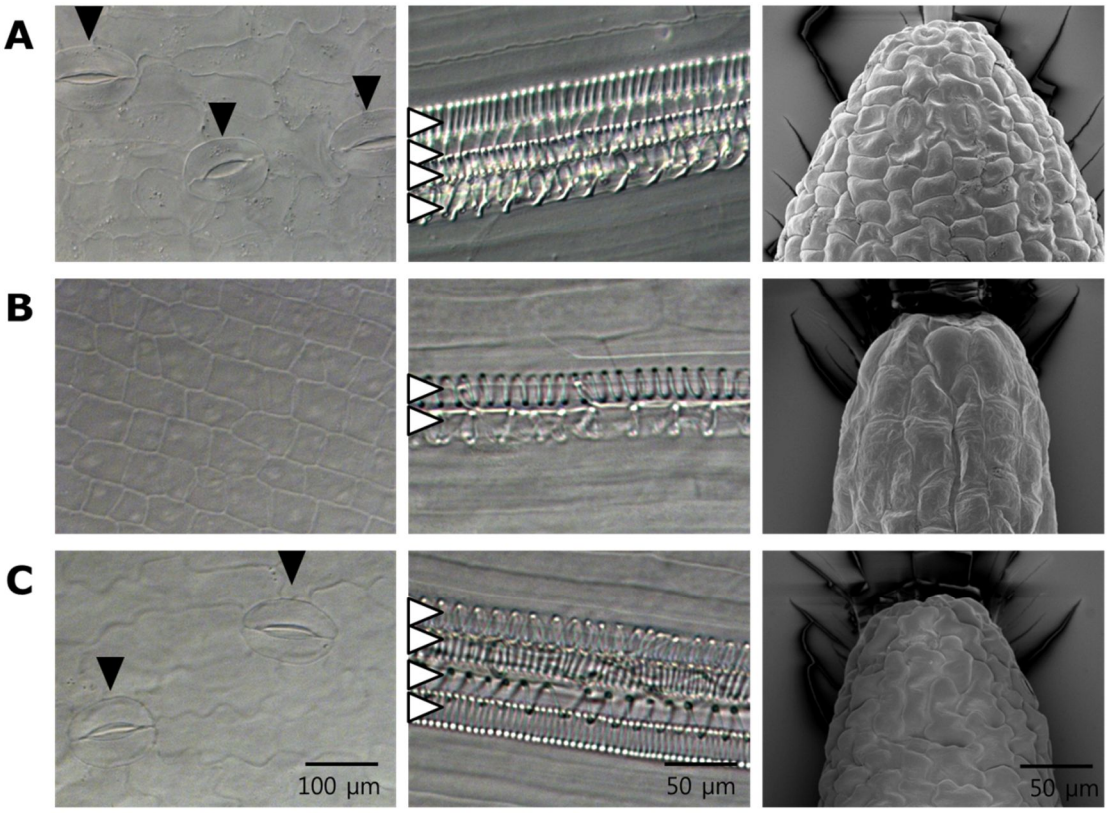
Figure 6. Effects of exogenous phytohormones in terrestrial shoots.

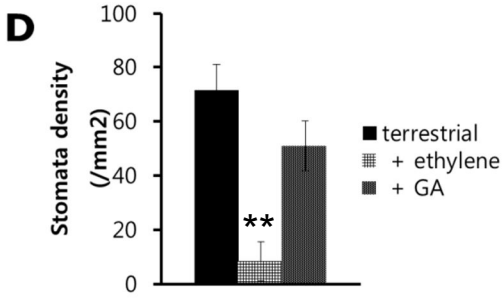
(A to D) Leaf morphologies by exogenous phytohormones, (A) is a picture of the control terrestrial shoot, and (B) to (D) are shoots treated with NAA, ACC, and GA, respectively.

(B) Leaf indices by exogenous phytohormones. Error bars indicate standard deviations.

The data are presented as means ± SD from three biological replicates. (n=24)

Figure 7


A
B
C
100 μm
50 μm
50 μm


D
Stomata density
(/mm2)
100
80
60
40
20
0
**
terrestrial
+ ethylene
+ GA

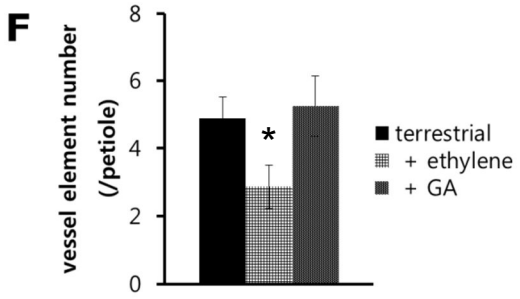

F
vessel element number
(/petiole)
8
6
4
2
0
*
terrestrial
+ ethylene
+ GA

Figure 7. Microscopic analyses of terrestrial leaf after phytohormone treatments.

(A to C) Microscopic images of terrestrial leaves treated with phytohormones. (A) is the control terrestrial leaf, and (B) and (C) were treated with ACC and GA, respectively. Left panels showed epidermis images. Middle panels show each vessel elements, and right panels are images of leaf tips.

(E and F) Stomatal density and number of vessel elements were plotted. Stomata were counted on adaxial sides. (n=12).

Black arrowhead indicates stoma and white arrowhead points to individual vessel element. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Figure 8

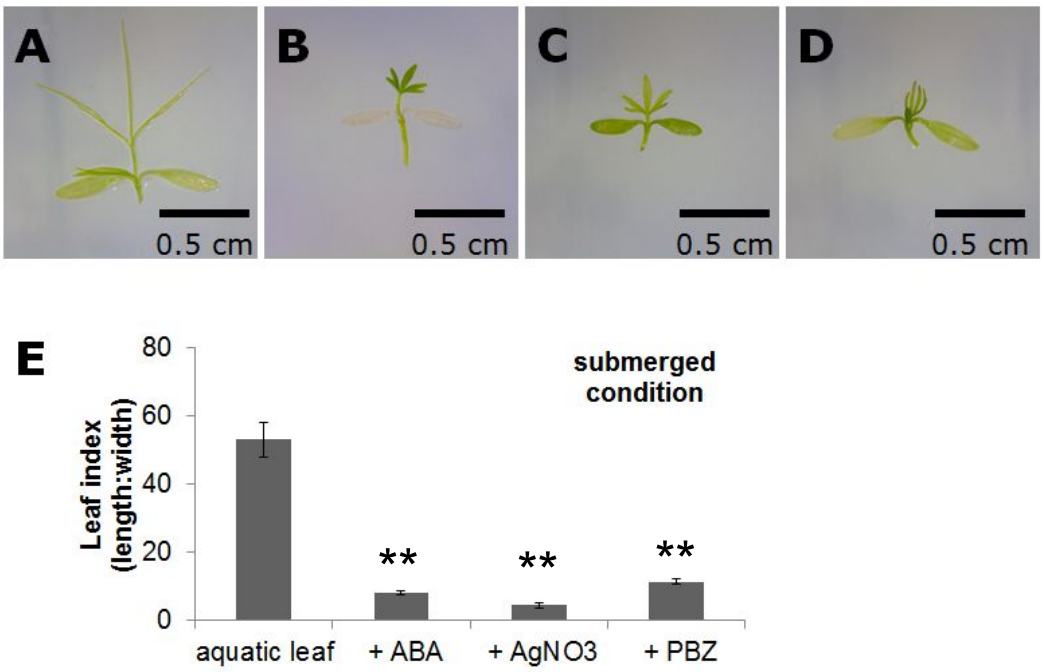


Figure 8.Effects of exogenous ABA and inhibitors of ethylene and GA in aquatic shoots.

(A to D) Leaf morphologies by exogenous ABA and hormone inhibitors, (A) is a picture of control aquatic shoot and (B) to (D) are pictures of aquatic shoot treated with ABA, AgNO3, and PBZ, respectively.

(B) Leaf indices for each aquatic shoots after treatments. Error bars indicate standard deviations.

The data are presented as means ± SD from three biological replicates. (n=24)

Figure 9

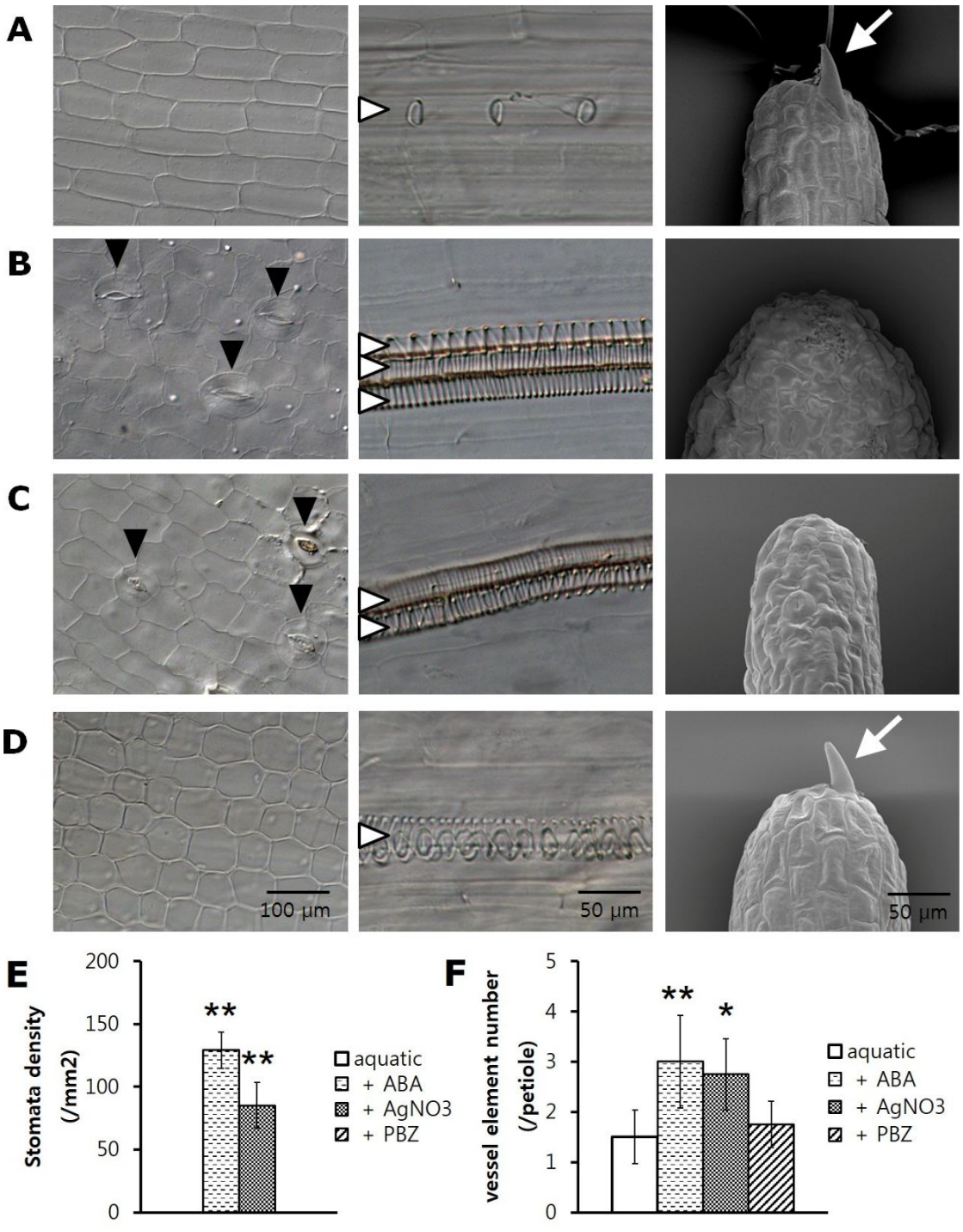

A
B
C
D
100 μm
50 μm
50 μm
E
Stomata density (/mm2)
200
150
100
50
0
**
**
aquatic
+ ABA
+ AgNO3
+ PBZ
F
vessel element number (/petiole)
5
4
3
2
1
0
**
*
aquatic
+ ABA
+ AgNO3
+ PBZ

Figure 9. Microscopic analyses for aquatic leaf after ABA and phytohormone inhibitors treatments.

(A to D) Microscopic images of aquatic leaves treated with ABA and phytohormone inhibitors. (A) is the control aquatic leaf, and (B) to (D) are images of aquatic leaf treated with ABA, $AgNO_3$, and PBZ, respectively. Left panels showed epidermis images. Middle panels show each vessel element, and right panels are images of leaf tips.

(E and F) Stomatal density and number of vessel elements, respectively. Stomata were counted on adaxial sides. (n=12)

Black arrowhead indicates stoma and white arrowhead points to individual vessel element. White arrow indicates hair cell on leaf tip. Error bars indicate standard deviations. The data are presented as means ± SD from three biological replicates. *P < 0.05; **P < 0.01

3. Brassinosteroid (BR) signaling did not affect stomatal density on terrestrial leaf of *R. trichophyllus*

In Arabidopsis, stomatal density is regulated by BR signaling. Stomatal density is decreased by exogenous BR treatment and increased by BR antagonists. Consistent with this, stomatal density is decreased in BR-deficient mutants (Kim et al., 2012). Moreover, BR-deficient mutants also show defects in xylem differentiation (Carlsbecker and Helariutta, 2005), which may be linked to the low number of vessel elements in aquatic leaves. I tested whether aquatic leaf traits such like absent of stomata and lower number of vessel elements were related to BR signaling—in other words, whether the heterophylly of *R. trichophyllus* is also regulated by BR and BR signaling compromised in aquatic leaves.

To know BR effects in heterophyllous phenomenome, I treated EBL (epi-brassinolide) and bikinin, BR agonists, to *R. trichophyllus*. Hypocotyl is a target of BR signaling; it is elongated by exogenous BR treatment (Mandava, 1988). I found that both EBL and bikini induced hypocotyl elongation in *R. trichopyllus* (Figure 10D). I concluded that response of hypocotyl by BR was conserved in *R. trichophyllus*. However, leaf indices were not changed by BR agonists. This result indicated that there are little relationship between heterophylly and BR signaling.

Further, I checked microscopic traits whether they were affected by BR agonists or not. EBL and bikinin did not suppress stoma development in *R. trichophyllus*. Stomatal densities of both adaxial and abaxial side were not decreased

by BR agonist treatments (Figure 11A to 11D). Moreover, the development of vessel elements was not changed by BR signaling induction, either (Figure 11E).These results indicate that BR signaling is a minor factor for leaf development of *R. trichophyllus*.

Figure 10

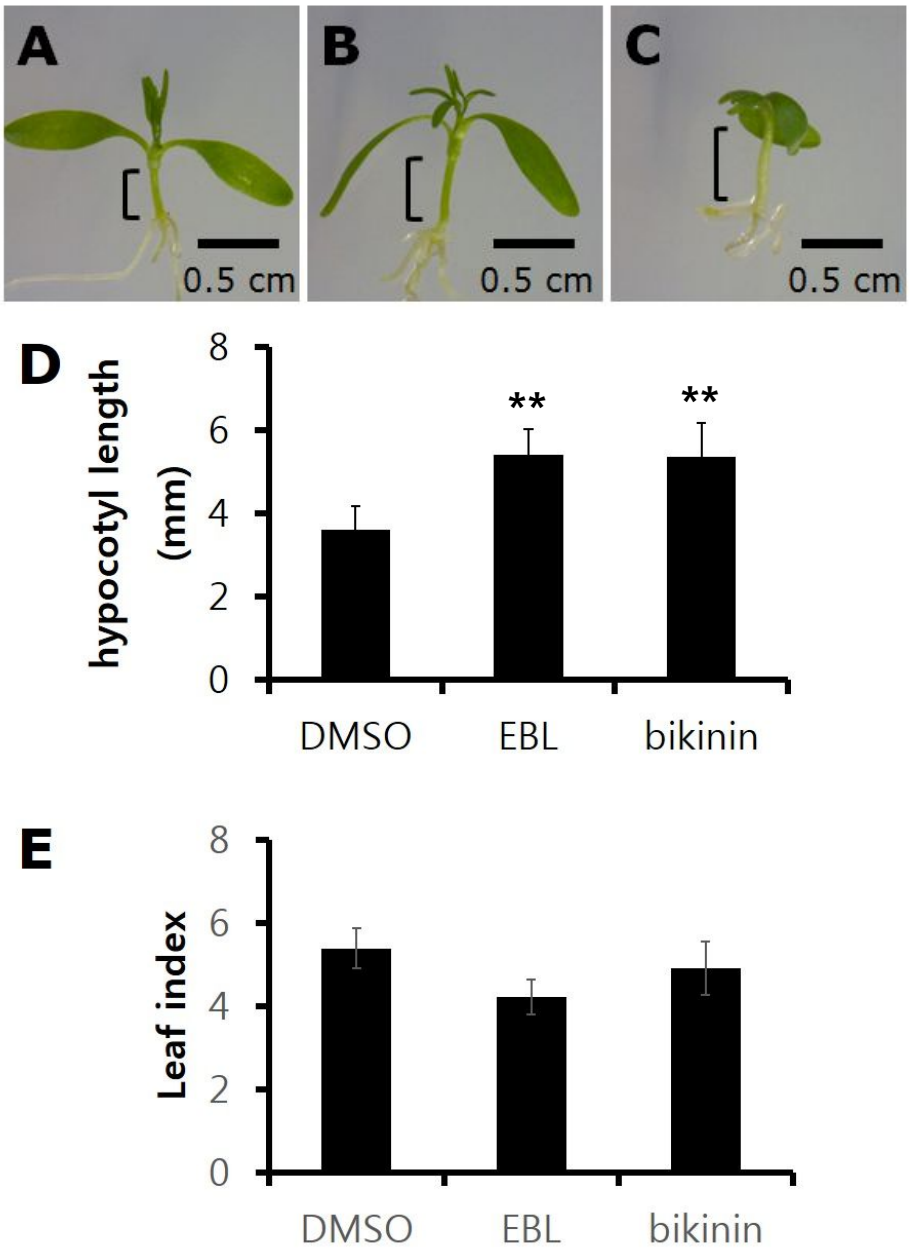

A
B
C
0.5 cm
0.5 cm
0.5 cm
D
hypocotyl length (mm)
8
6
4
2
0
**
**
DMSO
EBL
bikinin
E
Leaf index
8
6
4
2
0
DMSO
EBL
bikinin

Figure 10. BR signaling provokes hypocotyl length to elongation.

(A to C) Terrestrial shoots after treatments of BR agonists. (A) is a picture of the control terrestrial shoot on dimethyl sulfoxide (DMSO) and treated with EBL(B) and bikinin (C), respectively.

(D) Hypocotyl lengths according to BR signaling.

(E) Leaf indices by treatments of BR agonists.

The data are presented as means ± SD from two biological replicates. (n=16) **P < 0.01

Figure 11

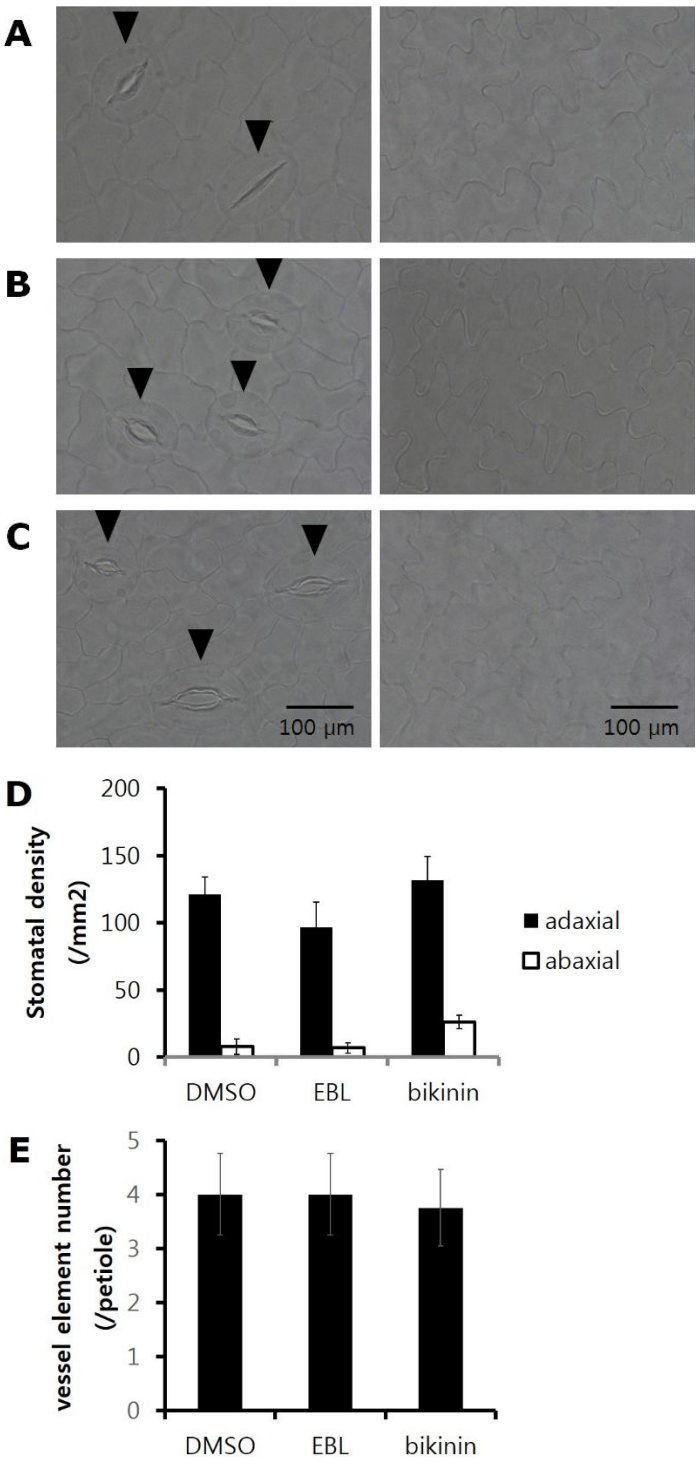

A
B
C
100 μm
100 μm
D
Stomatal density (/mm2)
200
150
100
50
0
adaxial
abaxial
DMSO
EBL
bikinin
E
vessel element number (/petiole)
5
4
3
2
1
0
DMSO
EBL
bikinin

Figure 11. Microscopic analyses of terrestrial leaf affected by BR agonists.

(A to C) Epidermis cells of terrestrial leaves treated with BR. (A) was control terrestrial leaf, and (B) and (C) were treated with EBL and bikini, respectively. Left panels showed adaxial sides and right panels showed abaxial sides.

(D and E) Statistical analyses of each stomatal density and vessel element number.

The data are presented as means ± SD from two biological replicates. (n=16)

4. Endogenous ethylene and ABA signaling were related to heterophylly of *R. trichophyllus*

The present findings showed that heterophylly was affected by exogenous ethylene and ABA. If ethylene and ABA are regulators for heterophylly, endogenous ethylene and ABA signaling should be also regulated properly. If ethylene signaling is not induced in submerged condition, terrestrial traits are generated on aquatic shoots. In the same case, if ABA signaling is not suppressed in submerged conditions, aquatic traits are not produced on aquatic shoots. Therefore, the present study aimed to check endogenous signaling between terrestrial leaves and aquatic leaves.

Ethylene is synthesized from methionine cycles. ACC synthase and ACC oxidase subsequently catalyze from S-adenosyl-L-methionine to ACC and from ACC to ethylene, respectively (Bleecker and Kende, 2000). ABA biosynthesis is started from zeaxanthin. ABA-aldehyde, a precursor of ABA, is produced by serial enzymatic steps. Finally, ABA-aldehyde oxidase3 (AAO3) converts ABA-aldehyde to active ABA (Barrero et al., 2006). ACC oxidase and AAO3 take last step of each phytohormone biosynthesis, and they receive positive feedbacks by ethylene and ABA, respectively (Bleecker and Kende, 2000; Barrero et al., 2006). Therefore, the present study focused on homologs of these two enzymes.

It was found here that *RtACO* was highly expressed in aquatic leaves, and *RtAAO* was mainly expressed in terrestrial leaves (Figure 12A). This is consistent with expected functions of each hormone in heterophylly regulation. Accumulated *RtACO*

would produce more ethylene in submerged condition; however, down-regulation of *RtAAO* would cause lower ABA status in aquatic leaves. Activated ethylene cascade and suppressed ABA signaling were necessary to produce aquatic leaves in *R. trichophyllus*. However, *AtAAO3* of Arabidopsis, a terrestrial and non-heterophyllous plant, was increased by submersion (Figure 12B). This is consistent with a previous investigation that ABA biosynthesis genes were accumulated in shoots under hypoxia environment (Hsu et al., 2011).

To confirm these signaling activities of ethylene and ABA in heterophylly, responsive genes that were positively regulated by ethylene and ABA, respectively, were sought. For ethylene, I found that homolog genes of *RELATED TO APETALA2.2* (*RAP2.2*) and *GLUTAMATE RECEPTOR* (*GLUR*) were accumulated by ethylene treatment (Figure 13A). *RAP2.2* was already known as ethylene responsive gene and was important to hypoxia resistance (Hinz et al., 2010). For ABA, the homolog genes of *RD22* and *CYTOCHROME P450, FAMILY 86* (*CYP86*) genes were selected. The expression levels of *RtRD22* and *RtCYP86* were increased by ABA treatment (Figure 13B). *RD22* was famous ABA responsive gene (Yamaguchi-Shinozaki and Shinozaki, 1993). Therefore, it was assumed that they reflected endogenous levels of ethylene and ABA, respectively, when checking their expression patterns between two types of leaves.

It was found that transcripts of both *RtRAP2.2* and *RtGLUR* were accumulated in aquatic leaves but *RtRD22* and *RtCYP86* were highly expressed in terrestrial leaves, relatively (Figure 14). This result suggested that ethylene signal was activated but ABA

signal was suppressed in aquatic leaves compared to terrestrial leaves. It is concomitant with the expression patterns of ethylene and ABA biosynthesis genes between terrestrial leaves and aquatic leaves. Considered with the ethylene roles of ethylene and ABA in heterophylly formation of *R. trichophyllus*, it was reasonable that ethylene signal was upregulated but ABA signal was downregulated in aquatic leaves. Therefore, I concluded that ethylene signal is activated but ABA signal is compromised in aquatic leaves.

Figure 12

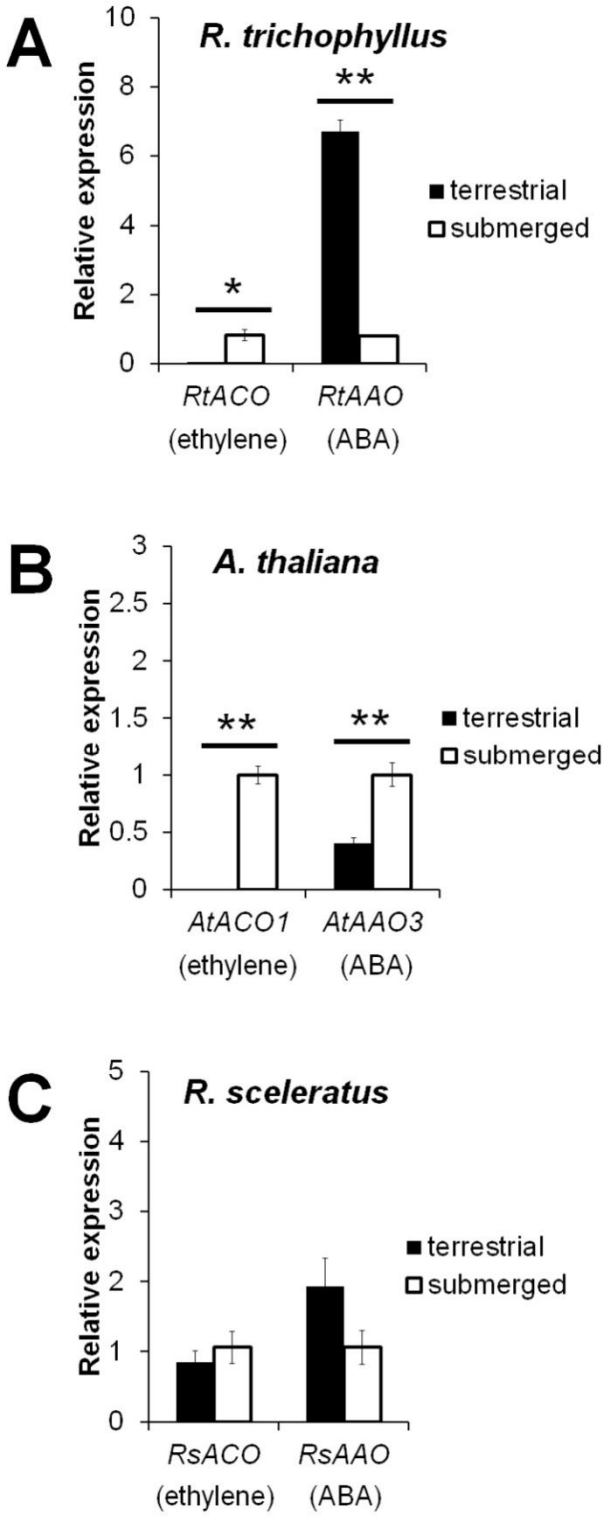

A
R. trichophyllus
Relative expression
10
8
6
4
2
0
**
*
terrestrial
submerged
RtACO
(ethylene)
RtAAO
(ABA)
B
A. thaliana
Relative expression
3
2.5
2
1.5
1
0.5
0
**
**
terrestrial
submerged
AtACO1
(ethylene)
AtAAO3
(ABA)
C
R. sceleratus
Relative expression
5
4
3
2
1
0
terrestrial
submerged
RsACO
(ethylene)
RsAAO
(ABA)

Figure 12. The expression levels of ethylene and ABA biosynthesis genes in *R. trichophyllus* and Arabidopsis according to aerial condition and submerged condition.

Effects of submergence on the expression levels of *ACO*, an ethylene biosynthesis geneand*AAO*, an ABA biosynthesis genes *in R. trichophyllus* (A), *Arabidopsis* (B), and *R. sceleratus* (C). *RtACT*, *AtPP2A,* and *RsACT* gene were used as references for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Figure 13

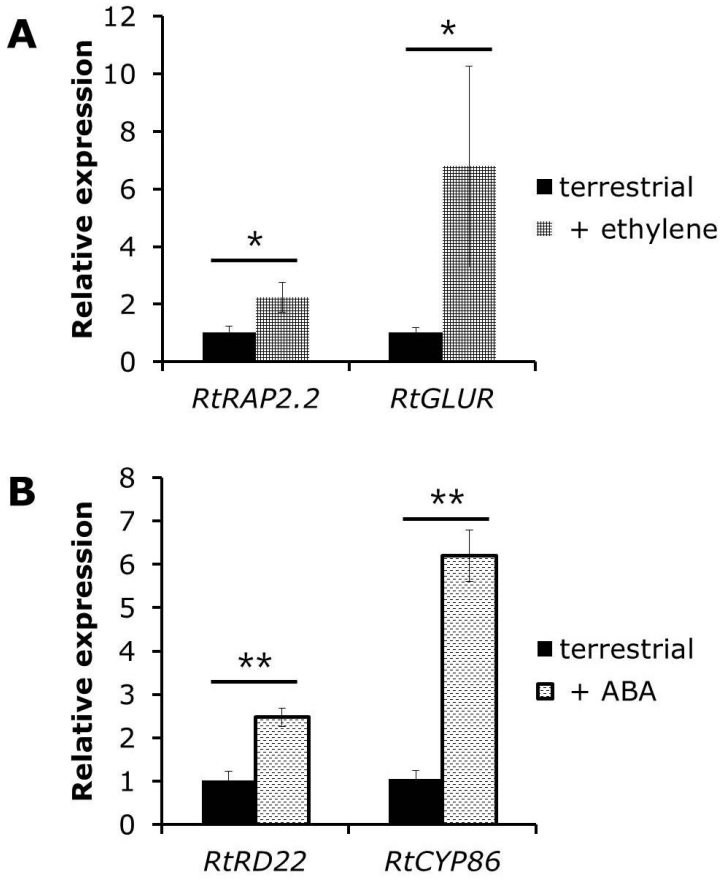


Figure 13. The expression levels of candidate genes for ethylene and ABA signaling.

(A) Real-time qPCR of *RtRAP2.2* and *RtGLUR* according to ethylene treatment.

(B) Real-time qPCR of *RtRD22* and *RtCYP86* according to ABA treatment.

RtACT gene was used as a reference for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Figure 14

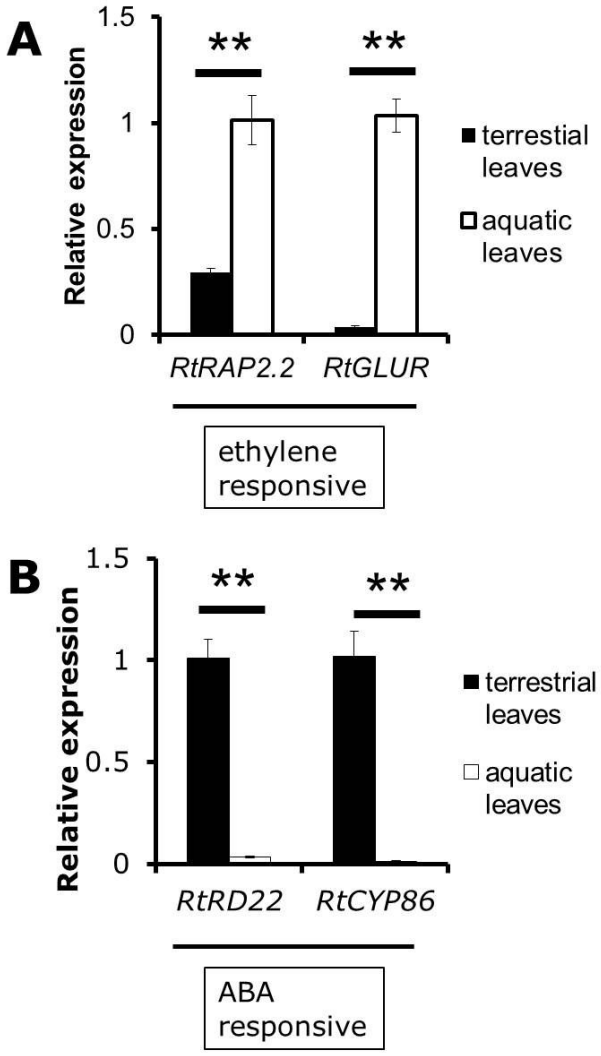


Figure 14. The expression levels of ethylene and ABA responsive genes in *R. trichophyllus* according to aerial condition and submerged condition.

(A) Real-time qPCR of ethylene responsive genes, *RtRAP2.2*, and *RtGLUR*.

(B) Real-time qPCR of ABA responsive genes, *RtRD22*, and *RtCYP86*.

RtACT gene was used as a reference for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05.

5. Leaf polarity genes were related to heterophylly in *R. trichophyllus*

A differential stomatal density on adaxial-abaxial side of the terrestrial leaves means that stomata distributions are controlled by leaf polarity in *R. trichophyllus*. Lack of any stomata in aquatic epidermal cells might correspond to the abaxial epidermis of terrestrial leaves. Moreover, leaf shapes and vascular development are regulated by leaf polarity genes (Carlsbecker and Helariutta, 2005; Kidner and Timmermans, 2010; Choudhary et al., 2012). Therefore, we investigated possible relationships between heterophylly and leaf polarity in *R. trichophyllus*. Three conserved pathways have already been investigated for leaf polarity: *TRANS-ACTING SIRNA3-AUXIN RESPONSE FACTOR* (*TAS3–ARF*), *ASYMMETRY1*(*AS1*)-*AS2*, and *KANADI-Class III Homeodomain-Leucine Zipper*(*KAN-HD-ZIP III*)pathways. Among the three pathways, the antagonistic relationship between KAN and HD-ZIP III has been investigated well so far (Kidner and Timmermans, 2007; Husbands et al., 2009; Ilegems et al., 2010; Reinhart et al., 2013). The present study also focused on these gene clades.

KAN genes are GARP-type transcription factor, and *HD-ZIP III* genes encode homeodomain proteins, which have putative lipid binding motifs. *KAN* genes are expressed and control the abaxial side of leaf; however*, HD-ZIP III* genes determine adaxial cell fates (Carlsbecker and Helariutta, 2005). *R. trichophyllus* has three *RtKAN* genes and three *RtHD-ZIP III* genes. Phylogenetic analysis revealed that three *RtKAN* genes corresponded to Arabidopsis *AtKAN1*, *AtKAN2*, and *AtKAN4* (Figure 15). Three genes of *HD-ZIP III* clade were matched to Arabidopsis *AtCNA*, *AtPHB*, and *AtREV* (Figure 15). Leaf polarity-regulated genes of *R. trichophyllus* were named following the

phylogenetic tree which is generated by amino acids similarities. Because duplication events of HD-ZIP III genes were occurred in eudicot lineage (Prigge and Clark, 2006), it is correct that *R. trichophyllus* had three *RtHD-ZIP III* genes.

All *RtKAN* genes were accumulated in aquatic leaves, while *RtHD-ZIP III* genes were suppressed in aquatic leaves than those in terrestrial leaves (Figure 16). Since *KAN* genes and *HD-ZIP III* genes are originated from ancient basal plants and their leaf polarity functions are also well conserved in land plants (Townsley and Sinha, 2012; Yamaguchi et al., 2012), these results indicate that leaf polarity pattern of terrestrial leaves is different from that of aquatic leaves. This may suggest that abaxial identity was reinforced in aquatic leaves. Thus, that these characteristic expression patterns of *RtKAN* and *RtHD-ZIP III* genes may be responsible for producing the cylindrical shape of aquatic leaves in *R. trichophyllus*.

I found that the expression patterns of *RtKAN* genes and *RtHD-ZIP III* genes were different according to leaf shapes. However, I had a question if these results might be a trivial consequence because leaf shapes were tied with leaf polarity genes. In other words, I wondered whether these transcriptional changes of leaf polarity genes were caused by submerged environment. If these genes mediated cascades from submergence to heterophyllous development, they should be controlled by submergence directly. If so, their expression patterns of mature leaves also showed similar to those of newly developed aquatic leaves. I checked transcription levels of *RtKAN* and *RtHD-ZIP III* genes in mature leaves before and after submergence.

In mature leaves, transcription levels of *RtKAN-b* and *RtKAN-c* were also increased by submergence except *RtKAN-a* as aquatic leaves (Figure 17A). Meantime, *RtHD-ZIP III* genes were down-regulated within 2 days of submergence (Figure 17B). This result suggested that these leaf polarity genes were downstream of submerged signaling perception. Since leaf polarity genes controls leaf shape and leaf axis patterning, it proposes a possibility that heterophylly of *R. trichophyllus* is controlled by leaf polarity genes.

Figure 15

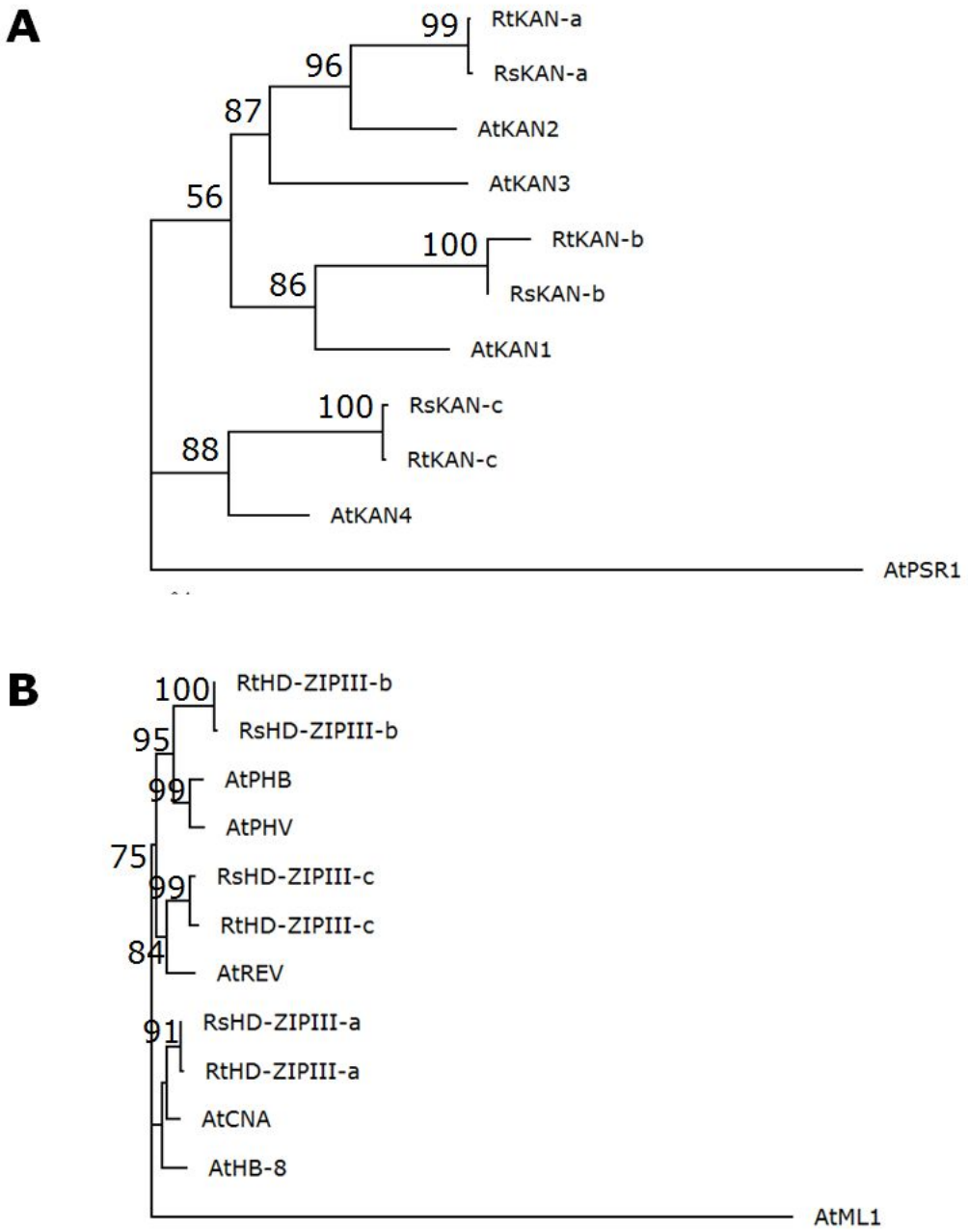

A
99
RtKAN-a
96
RsKAN-a
87
AtKAN2
AtKAN3
56
100
RtKAN-b
86
RsKAN-b
AtKAN1
100
RsKAN-c
88
RtKAN-c
AtKAN4
AtPSR1
B
100
RtHD-ZIPIII-b
95
RsHD-ZIPIII-b
99
AtPHB
AtPHV
75
RsHD-ZIPIII-c
99
RtHD-ZIPIII-c
84
AtREV
91
RsHD-ZIPIII-a
RtHD-ZIPIII-a
AtCNA
AtHB-8
AtML1

Figure 15. Phylogenetic trees of KAN genes and HD-ZIP III genes of *R. trichophyllus* and *Ranunculus sceleratus* with Arabidopsis.

The families of *KAN* (a) and *HD-ZIPIII* (b) genes in *R. trichophyllus* and *R. sceleratus* were aligned with those of A. thaliana based on amino acid sequences. AtPSR1 and AtML1 were used as out-groups. Bootstrap values are denoted beside branch nodes. Only values greater than 75 are presented.

Figure 16

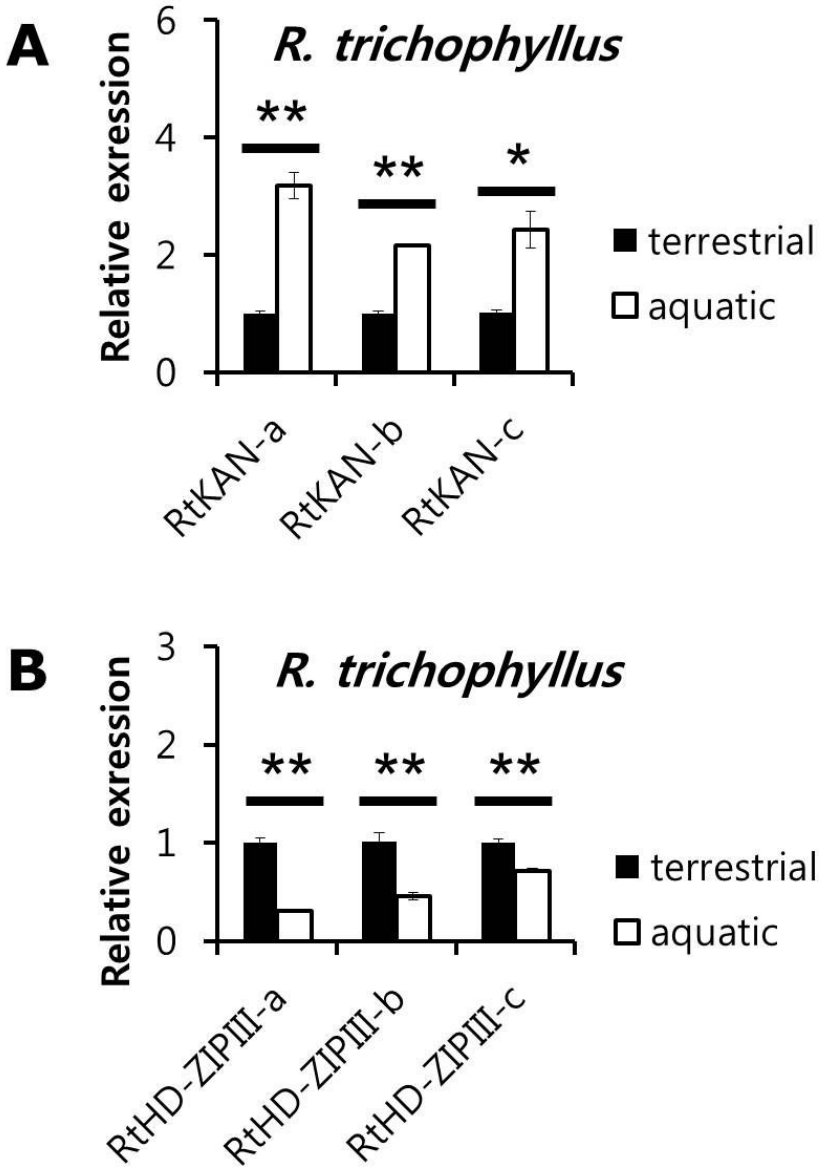


Figure 16. *RtKANs* and *RtHD-ZIP IIIs* were changed according to leaf types.

Real-time qPCR of *RtKAN* gene clade (A) and *RtHD-ZIP III* gene clade (B), respectively. *RtACT* gene was used as a reference for normalizing. Error bars indicate standard deviations.The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Figure 17

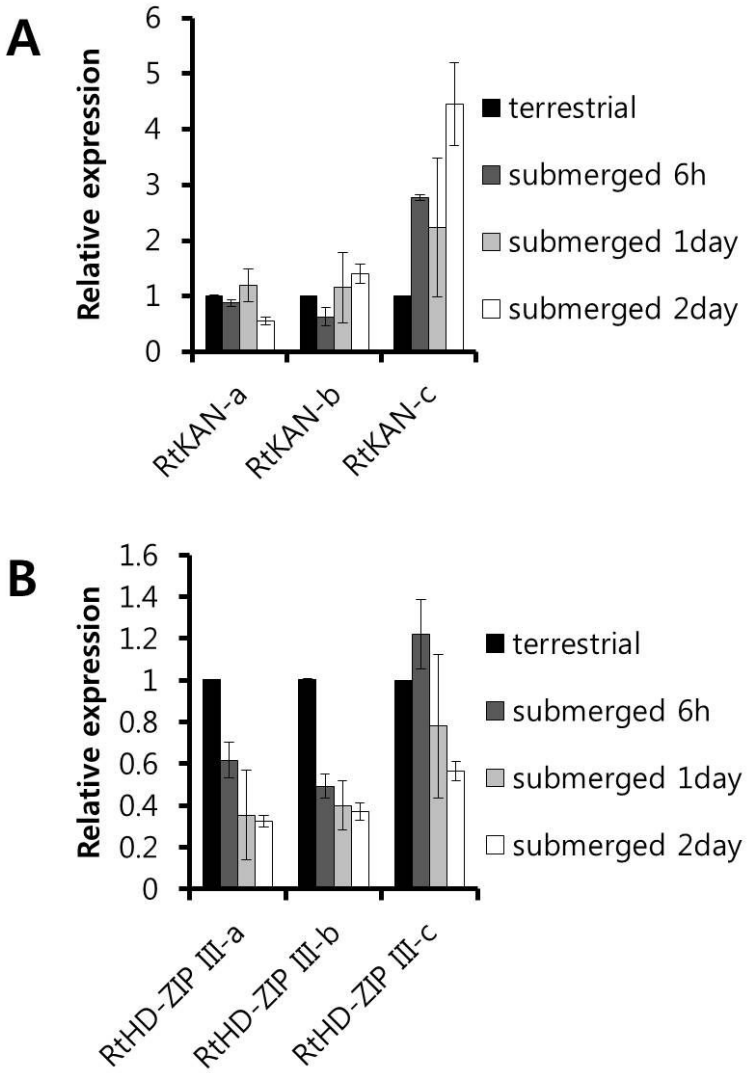


Figure 17. Flooding regulates leaf polarity genes of *R. trichophyllus*.

Transcript levels of leaf polarity genes after flooding treatment. Real-time qPCR of *RtKAN* genes (A) and *RtHD-ZIP III* genes (B) in matured terrestrial leaves after submergence.

RtACT gene was used as a reference for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from two biological and two technical replicates.

6. Leaf polarity genes of *R. sceleratus* were not affected by submerged environment

If there is relationship between heterophylly and *RtKAN–RtHD-ZIP III* pathways, it will be questionable whether non-heterophyllous plants show any changes in *KAN–HD-ZIP III* ratio. To test this hypothesis, I selected another plant species, which named *Ranunculus sceleratus*. This plant is relative species of *R. trichophyllus* and classified as a semi-aquatic plant (Hörandl and Emadzade, 2012). *R. sceleratus* did not show strong morphological heterophylly by submergence. On the other hand, *R. sceleratus* had an escape mechanism that provoked petiole elongations when submerged (Figure 18; Park et al., 2011). If expression patterns of *RsKANs–RsHD-ZIP IIIs* are similar to those of *R. trichophyllus*, it will support that the transcriptional changes of *RtKANs–RtHD-ZIP IIIs* play a major role in heterophylly of *R. trichophyllus*.

There also existed three *KAN* genes and *HD-ZIP III* genes in *R. sceleratus* (Figure 15). As *R. sceleratus* does not generate specialized leaves for underwater adaptation, the leaf generated under the water is defined as submerged leaf in the present study. It was found that expression patterns of *RsKANs–RsHD-ZIP IIIs* were not altered by submergence (Figure 19B and 19C). These results confirm the relationship between leaf shape and leaf polarity genes. Thus, because *RsKANs–RsHD-ZIP IIIs* were not altered by submergence, *R. sceleratus* did not show heterophylly according to terrestrial and aquatic environment. I also tested two *KAN* genes and two *HD-ZIP III* genes of Arabidopsis. Arabidopsis *KANs–HD-ZIP IIIs* were not changed according to submergence, either (Figure 19A). These results emphasize that heterophylly needs to be accompanied with transcriptional changing of leaf polarity

genes, *KANs* and *HD-ZIP IIIs.* In conclusion, *R. trichophyllus* could have noble mechanisms in KANs-HD ZIP IIIs regulation to generate heterophyllous leaves.

To know the relationship between water adaptation and leaf polarity, I tested viability of *pAS1-AS2* transgenic line under submerged condition. This transgenic line showed up-curled leaf phenotype which means the leaf is adaxialized. I thought that if leaf polarity was linked to water adaptation, the transgenic line would show altered viability compared to control. Consistent with hypothesis, the radicalized transgenic line was more resistant to submerged than control Col-0 (Figure 20).

Figure 18

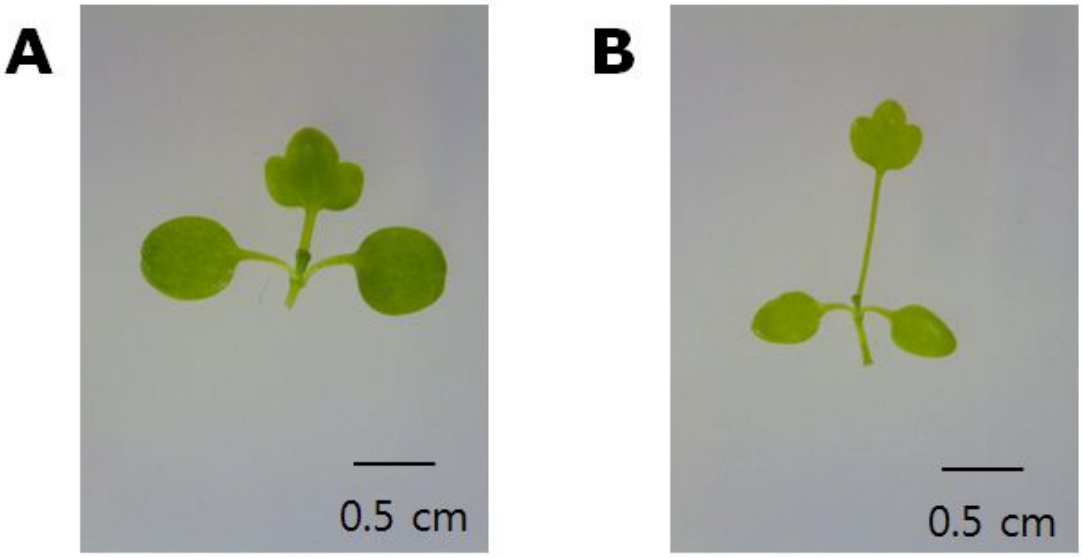


Figure 18. *R. sceleratus* seedling morphology with or without submersion.

(A) Seedling of *R. sceleratus* without submersion treatment.

(B) Seedling of *R. sceleratus* with submersion during 1 week.

Figure 19

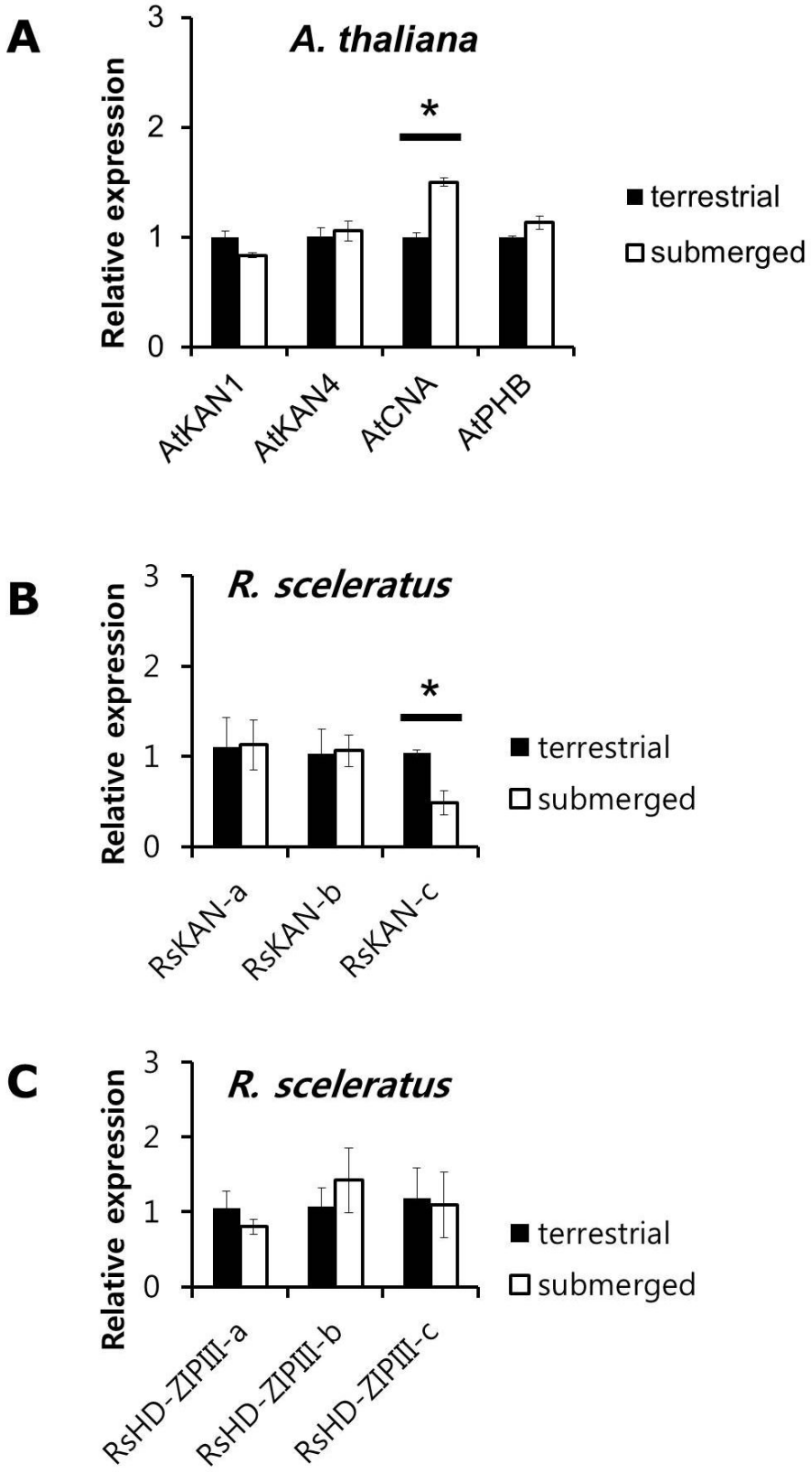

A
A. thaliana
Relative expression
3
2
1
0
*
terrestrial
submerged
AtKAN1
AtKAN4
AtCNA
AtPHB
B
R. sceleratus
Relative expression
3
2
1
0
*
terrestrial
submerged
RsKAN-a
RsKAN-b
RsKAN-c
C
R. sceleratus
Relative expression
3
2
1
0
terrestrial
submerged
RsHD-ZIPIII-a
RsHD-ZIPIII-b
RsHD-ZIPIII-c

Figure 19. *KANs* and *HD-ZIP IIIs* were not altered by submersion in non-heterophyllous plants.

(A) Transcript levels of *KANs* and *HD-ZIP IIIs* in aerial and submerged leaves of *A. thaliana*.

(B and C) Transcript levels of *KANs* and *HD-ZIP IIIs* in aerial (B) and submerged leaves (C) of *R. sceleratus*.

*AtPP2A*gene and *RsACT* were used as references for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05

Figure 20

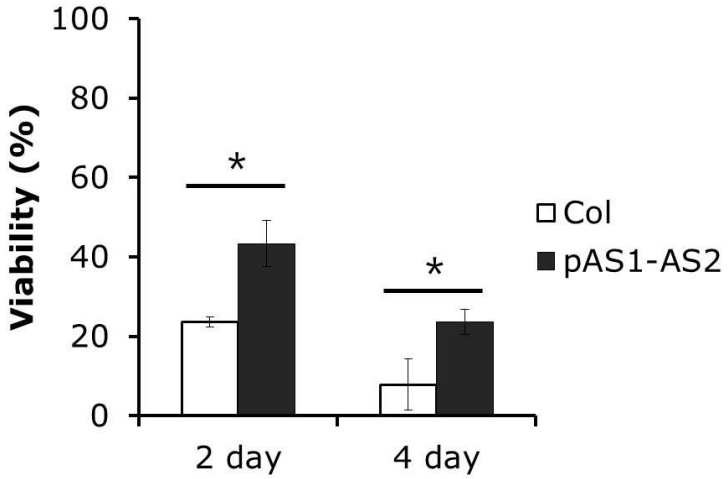


Figure 20. Cylindrical leaves showed resistance to submergence stress.

Increased viability of cylindrical Arabidopsis leaves under submergence stress. The transgenic line, *pAS1-AS*, shows narrow and up-curled leaf phenotypes. Ten-day-old seedlings were submerged in water for 2 and 4 days, and were then allowed to recover for 10 days under normal growth conditions. The data are presented as means ± SD from three biological repeats. *P < 0.05 (n=50)

7. Ethylene induced *RtKAN* genes in terrestrial leaves and ABA induced *RtHD-ZIP III* genes in aquatic leaves

If the transcript patterns of *RtKANs–RtHD-ZIP IIIs* are related to heterophylly, their expressions are also changed by exogenous ACC and ABA because these two molecules regulated heterophylly of *R. trichophyllus*. It was found here that the transcriptional levels of *RtKAN* genes were increased by ethylene signaling, which indicated that *RtKAN* genes were targets of ethylene signaling cascades. Since ethylene signaling would be activated in submerged condition, up-regulations of *RtKANs* in aquatic leaf are results of this strengthened ethylene signaling (Figure 21A).

In ABA treatment experiment, terrestrial leaves were generated but increased transcription levels of *RtKAN* genes were not reduced to terrestrial levels (Figure 21C). These results might suggest that ABA signaling did not control *RtKANs* gene expressions directly. If ethylene signaling was blocked by $AgNO_3$, the transcription levels of *RtKANs* were not induced. Thus, ethylene signaling is required for the transcriptional activation of *RtKAN* genes in water

On the other hand, the transcription levels of *RtHD-ZIP III-a* and *-c* genes were slightly decreased by exogenous ACC treatments (Figure 21B). By submergence, the expressions of *RtHD-ZIP IIIs* were reduced but the levels were recovered by ABA treatments (Figure 21D). This suggested that *RtHD-ZIP III* genes were targets of ABA signaling. By blocking ethylene signaling, the expression levels of them were also returned. Ethylene signaling is not enough to suppress *RtHD-ZIP IIIs* in aerial condition

but it is necessary in submerged signaling perception to reduce expression levels of them.

Therefore, in aerial condition, ABA signals were activated and leaf polarity can be established. In submerged condition, ethylene signaling was induced but ABA signaling was attenuated. This hormonal status caused upregulation of *RtKAN* genes and downregulation of *RtHD-ZIP III* genes (Figure 22).

Figure 21

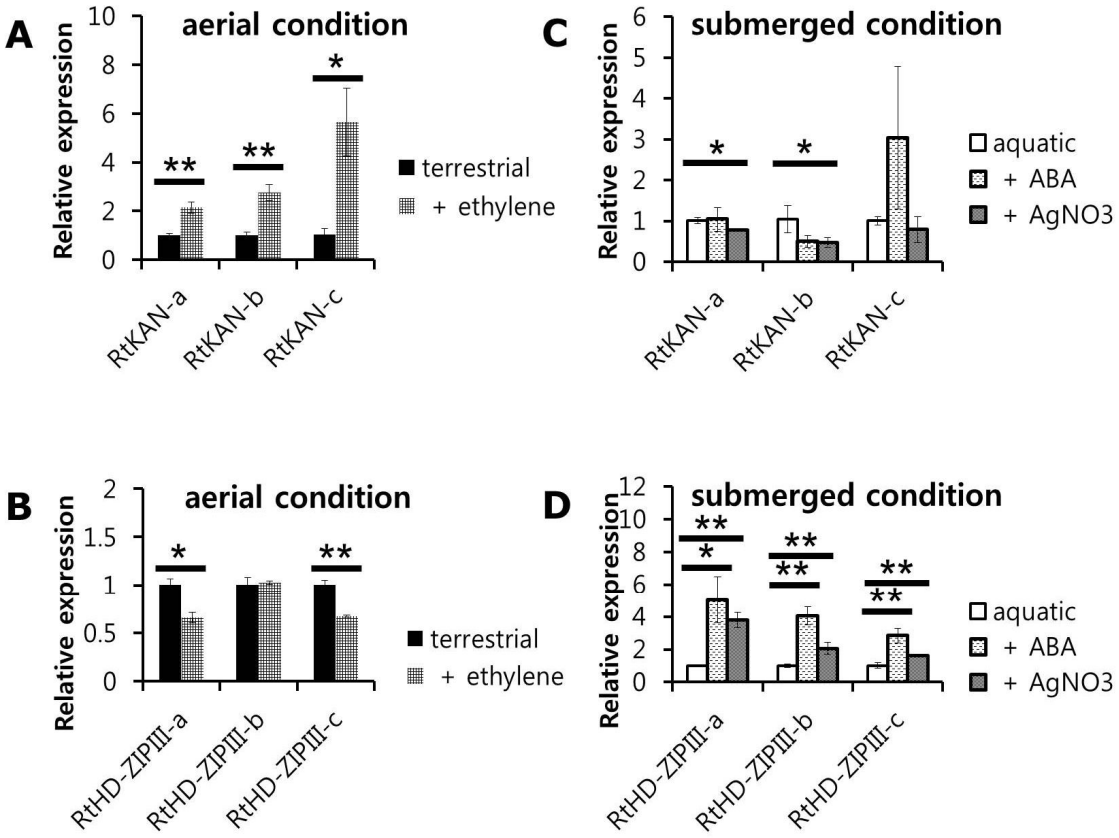


Figure 21. Transcript levels of *RtKAN* genes are increased by ethylene treatment and suppression of *RtHD-ZIP III* gene expressions in submerged condition are recovered by ABA and AgNO3 treatment, respectively.

Transcript levels of leaf polarity genes after chemical treatments. Real-time qPCR of *RtKAN* genes and *RtHD-ZIP III* genes treated with in aerial condition (A and B) and treated with ABA and $AgNO_3$ in submerged condition (C and D).

RtACT gene was used as a reference for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Figure 22

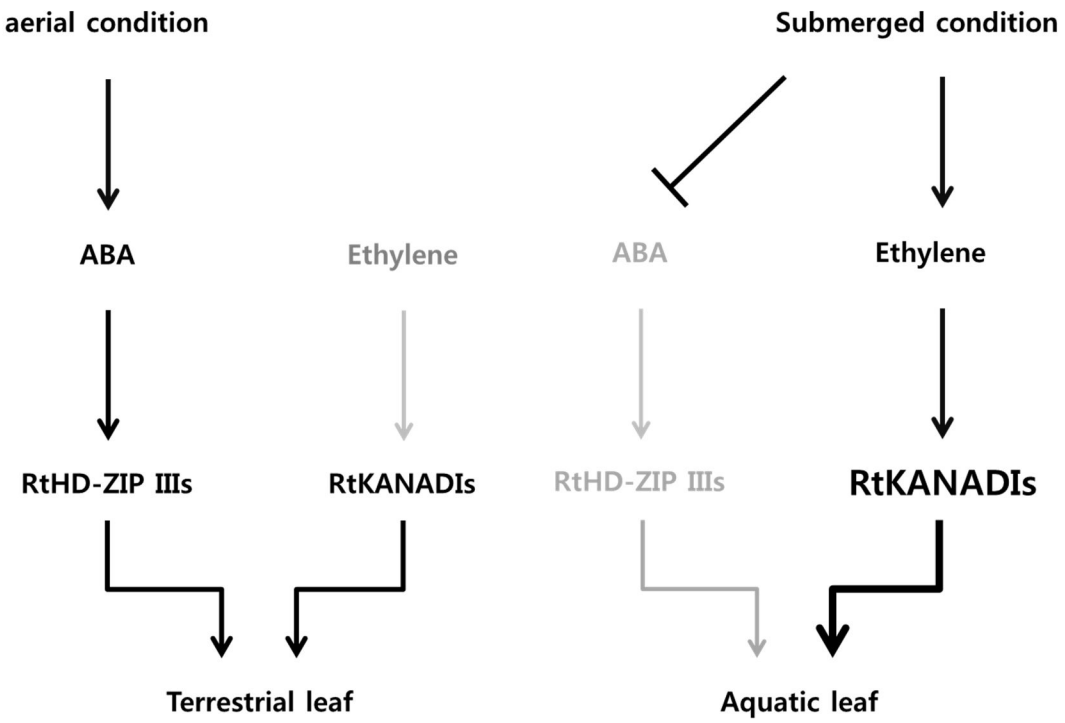


Figure 22. Brief model between submerged signaling and *KAN-HD-ZIP III* genes in *R.trichophyllus.*

In aerial condition, ABA signaling is superior to ethylene signaling. In this status, both *RtHD-ZIP IIIs* and *RtKANs*are expressed with balancing and generate terrestrial leaf. In submerged condition, RtKANs are activated but RtHD-ZIP IIIs are suppressed. Reinforced RtKANs produce aquatic leaves when RtHD-ZIP IIIs are decreased.

8. Induction of aquatic leaves by high humidity.

As I mentioned, submergence are accompanied with various changes of physio-chemical property.I wanted to know what factors of them could cause heterophyllous phenomenom in *R. trichophyllus*. In other words, I hoped to find a mechanism how *R. trichophyllus* could know that it was drowning. Several environmental cues were already known as an inducer for heterophylly in various heterophyllous plants (Wells and Pigliucci, 2000). I tested several environmental cues whether some of them could mimic submergence signaling.

I found that water-saturated air produced aquatic leaves on terrestrial shoots (Table 3 and 4). 95% humidity condition could not even induce aquatic leaves, which suggests that blocking of water evaporation might be a signal for drowning perception to *R. trichophyllus*. This result showed that *R. trichophyllus* can prepare aquatic growth before submergence.

Table 3.Tests for candidate environmental cues of eliciting heterophylly in *R. trichophyllus*.

	terrestrial leaf	aquatic leaf
Blue light	—	—
Red light	—	—
Dim light *	—	—
NaCl *		—
Ambient temperature (12℃)*	—	—
High humidity	Aquatic leaf	

—: not changed

These data were collected from two biological replicates. (n≧50) (* means that the data did not confirmed by replicate experiment)

Table 4 . High huminity induced aquatic leaves on terrestrial shoots.

Humidity	Ratio (seedling with aquatic leaves/total seedlings)
55 %	0 (0/50)
75 %	0 (0/25) *
95 %	2% (1/50)
100 %	48% (24/50)

These data were collected from two biological replicates. (n≧50) (* means that the data did not confirmed by replicate experiment)

9. Transcripts of stomatal and vascular developmental gene in aquatic leaves

Stomata development is controlled by phosphorylation signaling cascades and serial basic-helix-loop-helix (bHLH) transcription factors (Lampard et al., 2008; Bringmann and Bergmann, 2013). Since stomatal densities differed between terrestrial leaves and aquatic leaves, there are possibilities that development of stomata was also altered according to leaf types, so these were checked. Some homolog genes were obtained for stomatal development, such as *RtYODA*, etc. *YODA* is a mitogen activated protein kinase kinase kinase (MAPKKK) that mediates phosphorylation relay from receptor kinase to downstream kinases (Bergmann et al., 2004) *SPEACHLESS* (*SPCH*),*ICE1*, *MUTE* and *FAMA* belong to bHLH protein and control asymmetric division process for stomata production (MacAlister et al., 2006; Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007; Kanaoka et al., 2008). Their expression patterns were checked in both types of leaves in the present study. I found that the expression level of *RtICE1* and *RtFAMA* were greatly decreased in aquatic leaves compared to terrestrial leaves but other genes were not affected or slightly up-regulated, respectively (Figure 23A). This down-regulation of*RtICE1* and *RtFAMA* might be linked with lacking of stomata on aquatic leaf surface in *R. trichophyllus* (Figure 4A). In other words, submerged signaling interfered with expression of *RtICE1*and *RtFAMA*. ICE1 can form heterodimer with *SPCH*, *MUTE*, and *FAMA* and promote stomatal development (Kanaoka et al., 2008). *FAMA* is expressed in guard mother cells and controls transition from guard mother cells to guard cells and prevents over-proliferation of guard mother cells (Ohashi-Ito and Bergmann, 2006). Therefore,

this transcriptional suppression of *RtICE1*and *RtFAMA* suggests that the flow of stomatal developmental cascades or the generation of guard mother cells are blocked in aquatic leaves.

Xylem development is controlled by NAC and MYB transcription factors and structural genes related to cell wall deposition and program cell death (Pimrote et al., 2012). Since vessel numbers of aquatic leaves were decreased that terrestrial leaves, I checked transcription levels of xylem developmental genes. *RtSND* is NAC transcription factor and its homolog of *A. thaliana* is expressed in xylem and interfascicular structure and controls xylem differentiation (Zhong et al., 2006; McCarthy et al., 2009). XYLEM-SPECIFIC PAPAIN-LIKE CYSTEIN PEPTIDASE1 (XCP1) is xylem specific protease, which is expressed in xylem of both *Arabidopsis* and *Brachypodium* (Funk et al., 2002; Valdivia et al., 2013). *XCP1* controls autolysis of vacuoles and tracheary element differentiation (Bollhöner et al., 2012). Even though *RtSND* was not significantly changed between leaf shapes, *RtXCP* was suppressed in aquatic leaves (Figure 23B).This result was consistent with a smaller number of vessel elements in aquatic petioles (Figure 5E). This result corresponds with xylem differentiation being delayed or xylem tissue being reduced in aquatic leaves.

Figure 23

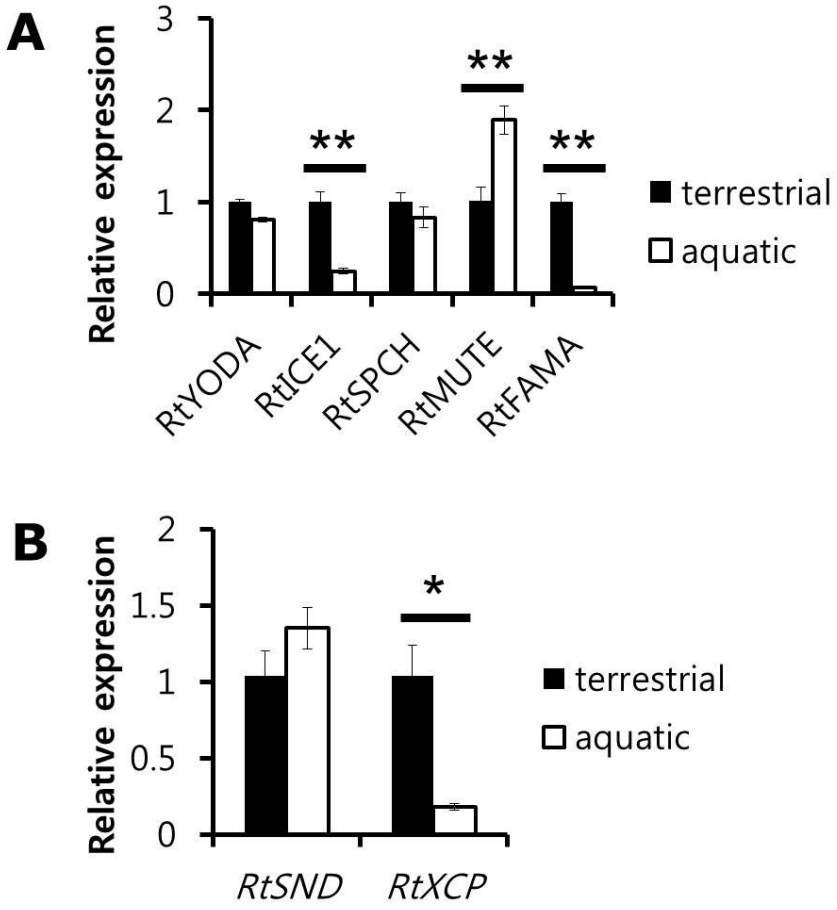


Figure 23. The expression levels of molecular markers corresponded to specific tissues.

(A) Transcript levels of stomatal developmental genes between terrestrial leaves and aquatic leaves.

(B) Transcript levels of vascular genes.

RtACT gene was used as a reference for normalizing. Error bars indicate standard deviations. The data are presented as means ± SD from three biological and two technical replicates. *P < 0.05; **P < 0.01

Discussion

This study aimed to reveal which factors cause heterophyllous development in amphibious plants to know evolutionary history of aquatic plants. *R. trichophyllus*, an amphibious plant, was a good material for this purpose. Several parameters were confirmed to be different between the terrestrial leaf and the aquatic leaf. These phenotypes must be induced by submersion, which means that amphibious plants have unique cross-talks from submerged perception to developmental processes. In spite of their convergent evolution, there are two common points among amphibious plants at least. One is that leaf polarity genes should be changed at the transition time. This is because that leaf shape is determined by boundary outgrowth and that outgrowth region is determined by leaf polarity genes (Yamaguchi et al., 2012; Fukushima and Hasebe, 2014). The other one is that ABA signaling should be controlled at the transition time because high ABA concentration can block aquatic leaf development (Deschamp and Cooke, 1985; Kuwabara et al., 2003; Lin et al., 2005). Thus, any steps of ABA signaling perceived submerged alarms, and then its expression patter is changed to compromise of ABA. Based on these concepts, the present study focused on the differential expression of hormone signal cascades and leaf polarity genes according to submersion.

The present findings indicate that *R. trichophyllus* was using ethylene and ABA as signal mediators from submerged cues to leaf development. Final steps of ethylene

and ABA biosynthesis were sensitively regulated in transcriptional levels (Figure 11A). These mechanisms may give some advantages against dynamic environmental changes because fast transition of hormone signaling would lead to fast shift of developmental and physiological responses. Thus, these checkpoint-like mechanisms immediately turn on or off the switch for end-product. With submergence, blackout of *RtAAO* transcription stops ABA supply and accumulation of *RtACO* oversupply ethylene. Moreover, ethylene biosynthesis requires oxygen (Jackson, 1985); therefore, the fast turn-on ensures promotion of ethylene accumulation or compensates biosynthesis rates even in hypoxia condition (Vriezen et al., 1999).

These processes may offer another advantage. If *RtAAO* is suppressed by submergence, active ABA contents are decrease, while the precursors of ABA are waiting until escaping time of shoots from the water. After that time, the level of *RtAAO* is increased to that of terrestrial leaves, and then the accumulated *RtAAO* catalyzes precursor to ABA immediately. On the other hand, if the upstream-most component is a single target of submergence, down-stream enzymes exhaust precursors of ABA during submersion time. After draining, precursors should be synthesized to generate ABA. This *de novo* biosynthesis process takes more time than the checkpoint process, which also causes delay of the signaling cascades.

Regulation of stomatal development process by submergence looks similar to regulation mechanisms of hormone biosynthesis. Upstream genes of stomatal development, such as *RtYODA*, *RtSPCH*, and *RtMUTE*, were not suppressed in aquatic leaves; however, *RtFAMA* was significantly decreased (Figure 23A). In stomata

production, several asymmetric cell divisions occurred, and these steps change cell identities from meristemoid cells to guard mother cells to guard cells(Bringmann and Bergmann, 2013).*FAMA* participates in differentiation of guard mother cells to guard cell (Ohashi-Ito and Bergmann, 2006).Therefore, shutdown of *RtFAMA* means ceasing of stomata production. This mechanism may guarantee the absence of stoma phenotype in aquatic leaves.

This *RtFAMA* regulation also suggests that submerged signaling connect to the promoter of *RtFAMA*. This cross-talk would not exist in Arabidopsis because its stomatal index is increased by ethylene and decreased by ABA (Saibo et al., 2003; Tanaka et al., 2013) while that of *R. trichophyllus* is decreased by ethylene and restored by ABA (Figure 6D and Figure 8E). On the other hand, the cross-talks between BR and stomata in *R. trichophyllus* remain obscure (Figure 11). In Arabidopsis, BR signaling activate stomata development through inhibition of mitogen activated protein kinase cascades (Kim et al., 2012; Khan et al., 2013) but also suppress it through inhibition of bHLH cascades (Carlsbecker and Helariutta, 2005). Hence, delicate approaches of BR signaling may reveal their functions in heterophyll of *R. trichophyllus*. However, there are also possibilities that those BR signaling is not conserved in *R. trichophyllus*. At any rate, by species-specific manners, new cross-talks have been obtained or useless cross-talks have been abandoned, which are results of adaptation and products of evolution.

R. trichophyllus showed heterophylly according to terrestrial and aquatic condition (Figure 3 and 4). Since the phylogenetic tree indicated that the ancestor of *R.*

trichophyllus was terrestrial plant (Hörandl and Emadzade, 2012), it is obvious that this plant invented the connection between submerged perception and aquatic leaf production. It was found in this study that ethylene and ABA signaling controlled heterophylly of *R. trichophyllus* (Figures 5 to 8) and related gene expressions for ethylene and ABA were consistent to physiological data (Figures 12 and 13). Thus, ABA signaling is not blocked or compromised in aerial condition, while the signaling is stopped in submerged condition. On the other hand, ethylene signaling is increased in submerged condition. Leaf polarity genes, *RtKANs* and *RtHD-ZIP IIIs*, were correlated with heterophylly (Figures 16 and 19), and their transcription levels were affected by ethylene and ABA (Figure 21). Since leaf polarity genes are well-known controllers for leaf shape, it is reasonable that ethylene and ABA regulates leaf polarity genes to produce specialized aquatic leaves.

Submergence is accompanied with various environmental changes. Among them, *R. trichophyllus* use high humidity as an indicator for aquatic environment (Table 3). In water-saturated aerial condition, *R. trichophyllus* could produce aquatic leaves, which suggests a possibility that *R. trichophyllus* monitors water-demand or evaporation rate. Taken together, I could draw a brief model for heterophylly determination of *R. trichophyllus* (Figure 24). Submergence and/or high humidity are start points for aquatic leaf development. If *R. trichophyllus* perceives submergence signal, ethylene biosynthesis is increased but ABA biosynthesis is decreased. These changes of hormones destroy the balance between *RtKAN* and *RtHD-ZIP III* genes. By these transcriptional changes, leaf blade formation would be affected then thin and

cylindrical aquatic leaves are produced. Meantime, stomata development and xylem formation are also regulated by submergence. Finally, aquatic leaves of R. trichophyllus show narrow and cylindrical shape with lack of stomata and lower number of xylem elements.

Several traits are lost or compromised in the aquatic leaf such like stoma, vessel elements of xylem, bifacial expanded blades (Raven, 2002; Sperry, 2003; Beerling, 2005). Leaf polarity-regulated genes, *KANs* and *HD-ZIP IIIs*, can be ancient genes that originated from early plant species. Even though their ancestral roles are questioned, they also are conserved in moss, the successors of first land plant, and have similar roles in leaf development (Prigge and Clark, 2006; Floyd and Bowman, 2007; Tomescu, 2009). *R. trichophyllus* might invent new mechanisms to return to the water through abolishing or compromising of leaf polarity. There are two possible explanation of why *R. trichophyllus* have to abandon the leaf pattering. Leaf polarity genes might be the main-regulators that control various water adaptive traits. Developmental genes for stomata and vascular structure, etc. may be targets of leaf polarity genes. Alternatively, leaf polarity genes are just one of numerous targets of submerged perception signaling. In this case, developmental genes of them are not downstream genes of leaf polarity genes. At any rate, leaf polarity genes are changed according to environmental cues to produce aquatic leaves. This is because the phenotypes of aquatic leaves such as thin and thread-like structures are beneficial in the water. As a result, observed phenotypes are close to the ancient prototype by losing organs and traits to change hormone signaling and leaf polarities. If leaf polarity

is connected to land–water transition at the molecular level, it suggests that the evolution of leaf patterning would co-opt with land adaptation (Sanders et al., 2007).

Figure 24

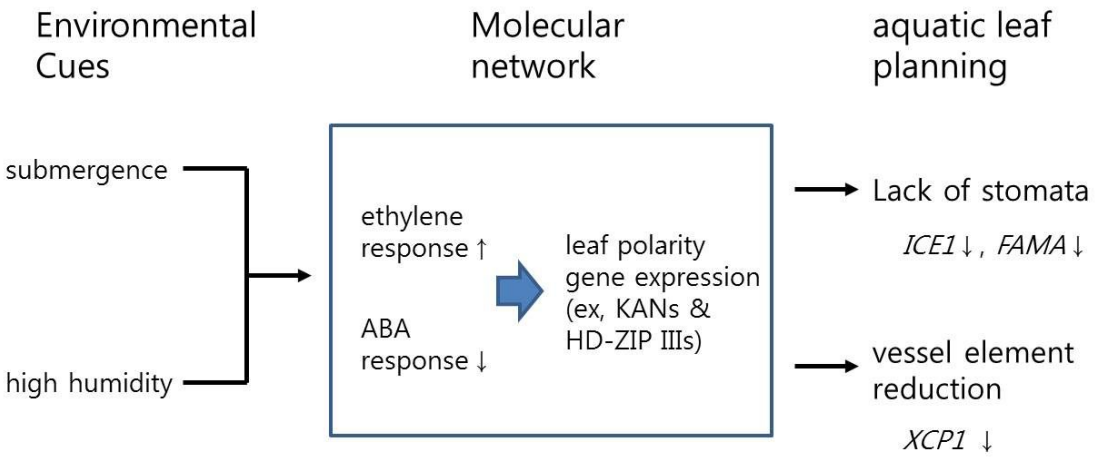


Figure 24.Brief mechanism for aquatic leaf production in *R. trichophyllus*.

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ABSTRACT IN KOREAN

수생 식물은 다양한 분류군에 속하는 육상 식물로부터 독립적으로 진화하였다. 그럼에도 불구하고 수생식물은 수렴진화를 통해 수중 환경에 적응하는 유사한 형질을 공유하고 있다. 그 중 하나로 진화과정에서 수생식물은 수생잎이라고 불릴 수 있는 특수한 잎을 만들게 되었다. 여러 선행연구가 이 공통 형질을 연구하였으나 수생잎이 어떠한 분자 기작을 통해 만들어지는지에 대한 연구는 아직 미흡한 부분이 있다. 매화마름(*Ranunculus trichophyllus*)은 육상과 수중에서 동시에 살 수 있는 양서식물로 수중에서는 가늘고 둥근 단면의 잎을, 육상에서는 상대적으로 넓적한 잎을 만들어낸다. 생리적인 연구를 통해 이 매화마름의 잎 이형성이 두 식물 호르몬 에틸렌과 ABA에 의해 조절된다는 것을 알 수 있었다. ABA는 육상잎을 만드는 것에, 에틸렌은 수생잎을 만드는 것에 관계되어 있었다. ABA 생합성 유전자와 ABA 반응 유전자는 수생잎에서 그 발현이 감소되어 있었으나 에틸렌 생합성 유전자와 그 반응 유전자는 수생잎에서 발현이 증가되어 있었다.

수중 환경에서 증가하는 에틸렌은 잎 아랫면을 결정하는 유전자 *RtKAN*의 발현을 증가시켰으며, 반대로 ABA의 감소는 잎 윗면을 결정하는 유전자 *RtHD-ZIP III*의 발현 감소와 연관되어 있었다. 이 잎 축 형성 유전자는 잎의 형태를 조절하므로 수중에서는 잎 아랫면의 성질이 강해져 둥근 단면의 수생잎이 만들어질 수 있다는 것을 유추할 수 있었다. 이러한 잎 축 발달 유전자의 발현 변화는 수생잎의 다른 형질인 기공의 소멸, 물관 조직의 감소도 잘 설명해 주고 있다. 이를

통해 매화마름의 잎 이형성 발달 과정에 대한 모델을 제안할 수 있었다. 침수 시에 식물 호르몬인 에틸렌과 ABA를 각각 증가-감소되고 이로 인해 잎 축 형성 유전자 *RtKAN*와 *RtHD-ZIP III*의 발현이 각각 증가-감소되어 잎 축 발달이 망가지는 기작이 매화마름이 수생 식물 진화로 진화하면서 획득한 주요 형질로 생각된다.

APPENDIX : The FRIGIDA Complex Activates Transcription of *FLC*, a Strong Flowering Repressor in Arabidopsis, by Recruiting Chromatin Modification Factors

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The FRIGIDA Complex Activates Transcription of *FLC*, a Strong Flowering Repressor in *Arabidopsis*, by Recruiting Chromatin Modification Factors [C] [W]

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The flowering of *Arabidopsis thaliana* winter annuals is delayed until the subsequent spring by the strong floral repressor *FLOWERING LOCUS C* (*FLC*). *FRIGIDA* (*FRI*) activates the transcription of *FLC*, but the molecular mechanism remains elusive. The *fri* mutation causes early flowering with reduced *FLC* expression similar to *frl1*, *fes1*, *suf4*, and *flx*, which are mutants of *FLC*-specific regulators. Here, we report that FRI acts as a scaffold protein interacting with FRL1, FES1, SUF4, and FLX to form a transcription activator complex (FRI-C). Each component of FRI-C has a specialized function. SUF4 binds to a *cis*-element of the *FLC* promoter, FLX and FES1 have transcriptional activation potential, and FRL1 and FES1 stabilize the complex. FRI-C recruits a general transcription factor, a TAF14 homolog, and chromatin modification factors, the SWR1 complex and SET2 homolog. Complex formation was confirmed by the immunoprecipitation of FRI-associated proteins followed by mass spectrometric analysis. Our results provide insight into how a specific transcription activator recruits chromatin modifiers to regulate a key flowering gene.

INTRODUCTION

Seasonal flowering is fundamental to the reproductive success and survival of higher plants. Plants have evolved a complex genetic network to control flowering time in response to endogenous cues and environmental factors such as daylength and temperature (Simpson and Dean, 2002). *Arabidopsis thaliana* is distributed in a wide range of environments, and its flowering behavior has adapted to these local environments (Napp-Zinn, 1979; Gazzani et al., 2003). Most of the *Arabidopsis* accessions collected from northern areas display winter annual flowering characteristics: late flowering that is accelerated by a long-term winter cold period, called vernalization (Gazzani et al., 2003; Michaels et al., 2003). The genetic analysis of natural variation in the flowering time of *Arabidopsis* has revealed two major genes conferring such characteristics, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Napp-Zinn, 1979; Clarke and Dean, 1994; Lee et al., 1994; Gazzani et al., 2003). Winter annuals have functional genes of both *FRI* and *FLC*, whereas summer annuals (rapid cycling accessions) have mutations in either *FRI* or *FLC* or both (Johanson et al., 2000; Gazzani et al., 2003; Michaels et al., 2003). *FRI* encodes a protein with two coiled-coil motifs and is required to increase the *FLC* transcript level (Johanson et al., 2000). *FLC* encodes a MADS box transcription factor that blocks flowering quantitatively by repressing the transcription of downstream floral pathway integrator genes (Michaels and Amasino, 1999; Sheldon et al., 1999). Although the *FRI* gene was cloned a decade ago (Johanson et al., 2000), its biochemical function remains unknown.

The expression of *FLC* is regulated by diverse and sophisticated mechanisms. Whereas *FRI* is required for an increase of the *FLC* transcript, vernalization completely suppresses *FLC* expression (Michaels and Amasino, 2001). Such suppression is accomplished through a series of histone modifications by the plant polycomb repressive complex, including *VERNALIZATION INSENSITIVE3*, *VERNALIZATION2* (*VRN2*), and the subsequent maintenance of the heterochromatin state by *VRN1* and *LIKE HETEROCHROMATIN PROTEIN1* (Kim et al., 2009). In summer annuals that have a nonfunctional *FRI*, *FLC* expression is repressed by the cooperation of the so-called autonomous pathway genes, which are mostly involved in chromatin modifications and RNA processing (Simpson, 2004). The late flowering caused by the mutations in autonomous pathway genes is also suppressed by vernalization (Michaels and Amasino, 2001). Thus, vernalization overrides the function of *FRI*, and *FRI* overrides the function of all autonomous pathway genes.

In elucidating the FRI-mediated *FLC* regulation mechanism, two classes of *FLC* regulators were revealed: *FLC*-specific regulators and *FLC* nonspecific regulators. When mutated, *FLC*-specific

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regulators show an early flowering phenotype without any other obvious phenotype, whereas nonspecific regulators show pleiotropic phenotypes such as small size and abnormal morphology (He et al., 2004; Kim et al., 2009). To date, six *FLC*-specific regulators, FRI, FRIGIDA LIKE1 (FRL1), FRL2, FRIGIDA ESSENTIAL1 (FES1), SUF4, and FLC EXPRESSOR (FLX), have been reported (Michaels et al., 2004; Schmitz et al., 2005; Kim et al., 2006; Andersson et al., 2008). All the mutants for the *FLC*-specific regulators show reduced *FLC* expression, and the overexpression of one gene does not induce late flowering in the other mutant backgrounds. These findings suggest that the proteins encoded by the *FLC*-specific regulators act as the components of the same signaling pathway or the same protein complex rather than acting independently in a dosage-dependent manner. However, protein-to-protein interactions among the *FLC* specific regulators were not detected by a yeast two-hybrid analysis in previous reports (Michaels et al., 2004; Schmitz et al., 2005; Andersson et al., 2008). Although we have reported that SUF4, which contains two C2H2-type zinc finger motifs and a Pro-rich region, physically interacts with FRI, the existence of a FRI-containing complex (FRI-C) is controversial, and the molecular function remains to be elucidated (Kim et al., 2006).

FLC nonspecific regulators are involved in either chromatin modification or RNA processing. The full activation of *FLC* by *FRI* requires chromatin modifiers, including proteins catalyzing histone H2B ubiquitination/deubiquitination, histone H3 lysine 4 (H3K4) methylation, H3K36 methylation, and the PAF1 (RNA polymerase associated factor 1) complex (Kim et al., 2009). In addition, the orthologous components of the yeast ATP-dependent SWR1 chromatin remodeling complex (SWR1-C), such as PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1), ACTIN-RELATED PROTEIN6 (ARP6)/SUPPRESSOR OF FRIGIDA3 (SUF3), and SWC6, are also required for incorporation of H2A.Z into *FLC* chromatin (Noh and Amasino, 2003; Choi et al., 2007; Deal et al., 2007). These chromatin-modifying proteins are well conserved among eukaryotes and are required for the epigenetic marks that are used for active transcription (Li et al., 2007). How these proteins, including SWR1-C, are recruited to the *FLC* gene remains unclear. Additionally, the RNA-processing factors, HUA2 and SERRATE, and an mRNA cap binding protein, CBP80/ABH1, are also involved in *FLC* activation (Bezerra et al., 2004; Doyle et al., 2005).

Here, we show that FRI acts as a scaffold protein interacting with known *FLC* specific regulators and that the resulting protein complex, FRI-C, acts as a specific transcription factor of *FLC* by recruiting the general transcription machinery and chromatin modification factors.

RESULTS

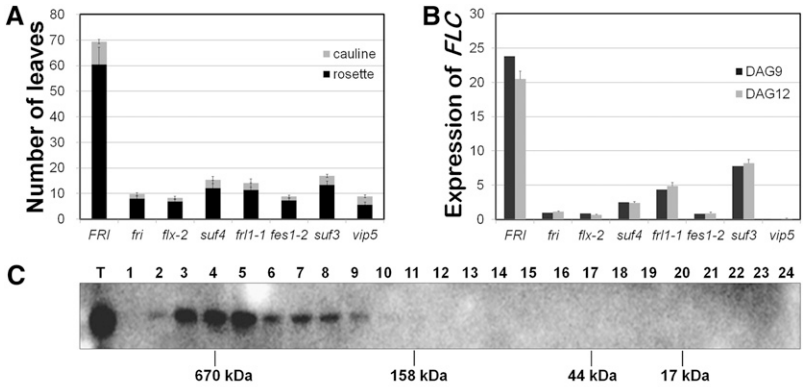
FRI Forms a Large Protein Complex

To elucidate the FRI-mediated *FLC* activation mechanism, we characterized early flowering *suf* mutants that were generated by fast-neutron mutagenesis; the *suf* mutations suppress the late flowering phenotype in the *FRI*-containing line. We isolated a total of 33 early flowering mutants, which were grouped into 12 independent loci (*suf1-suf12*) based on a complementation analysis (see Supplemental Figure 1A online). All of the *suf* mutants exhibited reductions in the expression of *FLC*; some showed only a flowering phenotype, similar to *fri*, but others showed a pleiotropic phenotype. Thus, we tentatively classified the *suf* mutants into two groups, one for *FLC*-specific regulators and the other for *FLC* nonspecific regulators. The *FLC*-specific regulators we analyzed were *SUF4*, *SUF5*/*FLX*, and *SUF8*/*FRL1*. Among these, the *suf4* and *frl1* mutations caused slightly later flowering and a higher expression of *FLC* than did the *fri* mutation (Figures 1A and 1B).

suf5 was allelic to *flx*, which was identified in the C24 background (Andersson et al., 2008), and was labeled as *flx-2*. It showed only early flowering, which was similar to the *fri*, *frl1*, *fes1*, and *suf4* mutants (Figures 1A and 1B; see Supplemental Figure 2 online). *FLX* was highly expressed in the shoot and root apical meristems and vascular tissues (see Supplemental Figure 2F online), where *FLC* and the *FLC* positive regulators, *FES1*, *SUF3*, *SUF4*, and *SWC6*, are expressed (Michaels and Amasino, 2001; Choi et al., 2005, 2007; Schmitz et al., 2005; Kim et al., 2006). In addition, the overexpression of *FLX* rescued *flx-2* but failed to induce late flowering in the Columbia (Col) (*fri*) background (see Supplemental Figure 2E online). These results suggest that FLX acts in a protein complex with FRI.

To reveal the size of the protein complex that includes FRI, we performed gel filtration analyses using a myc-tagged *FRI* transgenic line, namely, *35S-myc:FRI fri* (Figure 1C). The *35S-myc:FRI fri* plants flowered (68 $\pm$ 7.6 rosette leaves) as late as the wild type, indicating that the introduced transgene was functional. The size of the protein complex detected by the myc antibody was $\sim$670 kD, ranging from 400 to 1000 kD. This size is much higher than the expected size for a FRI monomer (68.4 kD), indicating that FRI exists mostly as a component of a large protein complex. To determine whether the integrity of the protein complex was affected by the mutations in the *FLC*-specific regulators, we determined the size of this protein complex in each mutant. The size was not affected by the individual mutation (see Supplemental Figure 3 online). However, *fes1* and *frl1* consistently showed a decreased steady state level of FRI and the FRI complex (FRI-C) (Figure 1D; see Supplemental Figure 3 online). Taken together, the results suggested that FES1 and FRL1 are required for the stabilization of FRI-C.

To identify the FRI-C components and any additional interacting proteins in vivo, we performed biochemical purifications using *35S-myc:FRI fri* seedlings. After isolating a nuclear protein extract from *35S-myc:FRI fri* seedlings, the FRI-associated proteins were obtained by immunoprecipitation using c-myc antibody–conjugated agarose beads and then analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). As a negative control, the same procedure was performed using *fri* seedlings. FRI, FES1, FRL1, FLX, and SUF4 were identified as FRI-associated proteins that were not detected in the *fri* negative control (Figure 1E). This result indicates that these proteins comprise FRI-C in vivo. In addition, we detected EFS, TAF14, and YAF9 as FRI-associated proteins (Figure 1E; see Supplemental Table 1 online for a full list of the proteins detected), which may explain why the size of the FRI-C was nearly 1 MD. EFS, YAF9, and TAF14 are components of chromatin modification



E

FRI-C component proteins			
Gene Name	AGI Number	Mass(KDa)	Matched peptides
FRI	At4g00650	68.4	12
FES1	At2g33835	64.4	6
YAF9	At5g45600	30.2	4
FRL1	At5g16320	52.8	4
EFS	At1g77300	198.8	4
FLX	At2g30120	30.1	2
TAF14	At2g18000	22.9	1
SUF4	At1g30970	39.8	1

Figure 1. The Flowering Phenotype Caused by Mutations in *FLC* Regulators and the Presence of the FRI Complex.

(A) Flowering time was measured by counting total rosette and cauline leaves at the bolting stage. A higher number of leaves at bolting represents later flowering. Twenty plants per genotype were used for the flowering time measurement. Error bars indicate the SD.

(B) Real-time RT-PCR analysis of the *FLC* transcript level in the wild type and mutants grown for 9 and 12 d after germination (DAG) under long days. Levels of *FLC* transcripts were normalized to the levels of *ACTIN* and presented as a relative value to that of *fri.* Error bars indicate the SD of triplicate experiments. **(C)** An immunoblot of the gel filtration fractions from a Superdex 200 column and protein extracts of the *35S-myc:FRI fri* transgenic plant. T indicates the total protein as input, and the numbers above the blot represent the fraction order. Calibrated molecular masses are shown below the blot. The myc:FRI protein was detected with a monoclonal myc antibody.

(D) An immunoblot showing the level of myc:FRI protein in *fri*, *fes1*, *frl1*, *suf4*, and *flx* backgrounds. Ponceau staining is shown as a loading control.

(E) The myc-FRI–associated proteins. Plants carrying fully functional myc-FRI protein expressed under the control of the *35S* promoter were harvested after 2 weeks of growth under long days and used in the immunoprecipitation purification. The proteins were identified by LC-MS/MS. The *fri* mutant plants were used as a negative control to determine whether a protein was associated with the myc-FRI protein. The immunoprecipitation and ensuing LC-MS/MS analysis was repeated three times independently, and the representative data are presented. Full details on the peptides and scores from the myc purification and control sample are available in Supplemental Table 1 online.

factors and general transcription factors, respectively (see below).

FRI Is a Scaffold Protein Interacting with *FLC*-Specific Regulators

Because the genetic and biochemical data showed that the *FLC* specific regulators were components of the FRI-C, we carefully performed a yeast two-hybrid (Y2H) interaction analysis with FRI, FRL1, FES1, SUF4, and FLX using activation domain (AD) and binding domain (BD) constructs that contained both the full-length coding sequences and a series of deletions (Figure 2A; see Supplemental Figure 4 online). As expected, FRI interacted with FLX, SUF4, and FES1. The interaction between AD-FRL1 and BD-FRI was not detected when the full coding sequence was used, but we found that the C-terminal deleted FRL1 could interact with FRI (see Supplemental Figure 4 online), indicating either that the AD-fused FRL1 may not be well translated or that the protein structure may impede the interaction in yeast. The interaction between FRI and FRL1 has also been reported in Y2H assays using different yeast strains (Geraldo et al., 2009). Such a result suggests that FRI may act as a scaffold protein to assemble a protein complex that activates *FLC*. However, any combinations between BD-FES1 and AD-other proteins could not be considered as interaction because the combination of BD-FES1 and AD alone showed good growth even in –LTHA medium due

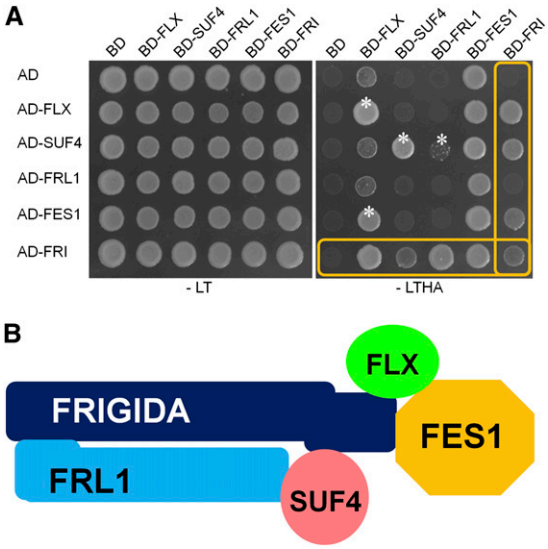


Figure 2. Y2H Interaction Analyses among FRI, FRL1, FLX, SUF4, and FES1.

(A) Interactions among full-length FRI, FRL1, FLX, SUF4, and FES1. The -LT indicates the synthetic drop (SD) medium lacking Leu and Trp, whereas -LTHA indicates the SD medium without Leu, Trp, His, and Ade. Boxes show the FRI interactions with FLX, SUF4, FRL1, and FES1. White stars mark the interactions with other proteins except FRI.

(B) A schematic drawing showing the interactions among the components of the FRI complex. FRI functions as a scaffold protein interacting with all of the *FLC*-specific regulators analyzed, FRL1, FES1, SUF4, and FLX. The Y2H analysis using a series of deletion constructs showed that the N terminus of FRI binds to the N terminus of FRL1 and that FRI-CTR interacts with the N terminus of FLX and with the C termini of both SUF4 and FES1. SUF4 interacts with FRL1, and FLX interacts with FES1 (see Supplemental Figure 4 online). All components also form homodimers, but these are not shown.

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to strong transcriptional activity of FES1 itself. Thus, interactions between BD-FES1 and AD-other proteins require independent confirmation by bimolecular fluorescence complementation (BiFC) as below.

The Y2H analysis performed using a deletion series of FRI and FRL1 showed that the N termini, including the coiled-coil domains, were required for the interaction between the two, and that the C terminus of FRI (FRI-D4, amino acids 459 to 609) was required for the interaction with FLX, SUF4, and FES1 (see Supplemental Figure 4 online). The C-terminal sequence of FRI (FRI-D4, amino acids 459 to 609) is unique and is extended compared with its paralogs, FRL1 and FRL2 (Michaels et al., 2004); this region is Pro rich and has 17 predicted phosphorylation sites (see Supplemental Figure 5 online), the characteristics of which likely provide the scaffolding activity to interact with various proteins, including CBP20 (Geraldo et al., 2009). The deletion series also showed that the N terminus of FLX (amino acids 35 to 78), the C terminus region that includes the Pro-rich region of SUF4, and the C terminus (at least amino acids 459 to 587) of FES1 were required for the interaction with the C terminus of FRI (see Supplemental Figure 4D online). By contrast, two C2H2 zinc finger motifs in SUF4 and the CCCH-type zinc finger motif in FES1 were dispensable for the interaction with FRI. In addition, it was shown that FRI could homodimerize through the C-terminal region, the FRIGIDA domain, and the coiled-coil motif and that FLX, SUF4, FES1, and FRL1 could also homodimerize (Figure 2A; see Supplemental Figure 4D online).

We confirmed the interactions observed in the Y2H assays by BiFC analysis using *Arabidopsis* protoplasts (Figure 3). The BiFC analysis using YN (amino acids 1 to 173 of yellow fluorescent protein [YFP])-FRI and YC (residues 174 through the end of the YFP sequence)-FRI fusion constructs showed a florescence pattern of nuclear speckles (Figure 3A), which was similar to the previously reported nuclear localization pattern of YFP-FRI (Kim et al., 2006). We observed similar nuclear speckle patterns from the interactions of FRI and FLX, SUF4, FRL1, and FES1 (Figures 3B to 3E). The nuclear fluorescence signals for the combination of FES1 and FRI were relatively weak and disappeared during the period of signal detection using confocal microscopy (Figure 3E). We also confirmed the interactions of SUF4/FRL1 and FLX/FES1 by BiFC because the Y2H data were ambiguous (Figures 3F and 3G). Indeed, the results showed nuclear speckles similar to those of the FRI homodimer, which provided further evidence supporting their interaction. When transfected alone, FLX localized to both the cytosol and the nucleus, as was previously reported (Andersson et al., 2008). However, the BiFC analysis showed that most of the FLX homodimers were present in the cytosol, but the signals of the FLX/FRI and FLX/FES1 heterodimers were strongly detected only in the nucleus (Figures 3B and 3F; see Supplemental Figure 6 online), suggesting that FRI-C recruits FLX to the nucleus. Their physical interactions and similar nuclear speckle patterns supported to the notion that FRI-C is composed (at least partially) of the *FLC*-specific regulators that we have analyzed.

Each Component of the FRI Complex Has a Specialized Function

An obvious function of FRI-C is the transcriptional activation of *FLC*, such that it overrides the coordinated repression of *FLC* by autonomous pathway genes. However, it is not known how FRI-C can activate *FLC* transcription. Thus, we hypothesized that each component of the protein complex has a specialized function and designed experiments to test this hypothesis as below.

FLX and FES1 Have Transcriptional Activation Potential

Initial evidence supporting the idea of a specialized function was obtained from the Y2H assay of FLX and FES1. Both FLX and FES1 showed intrinsic transcriptional activity when fused with the DNA binding domain of GAL4 (Figure 2A). To confirm this finding, a yeast one-hybrid (Y1H) assay, using a deletion series of FLX and FES1, was performed (Figure 4). The results showed that a specific region was required for the intrinsic transcription activation of each protein in yeast: for FLX, a domain similar to SMC (structural maintenance of chromosomes) was required, whereas the CCCH-type zinc finger motif in FES1 was essential for transcription activation (Figures 4C and 4D). These observations indicated that FLX and FES1 have two separable domains:

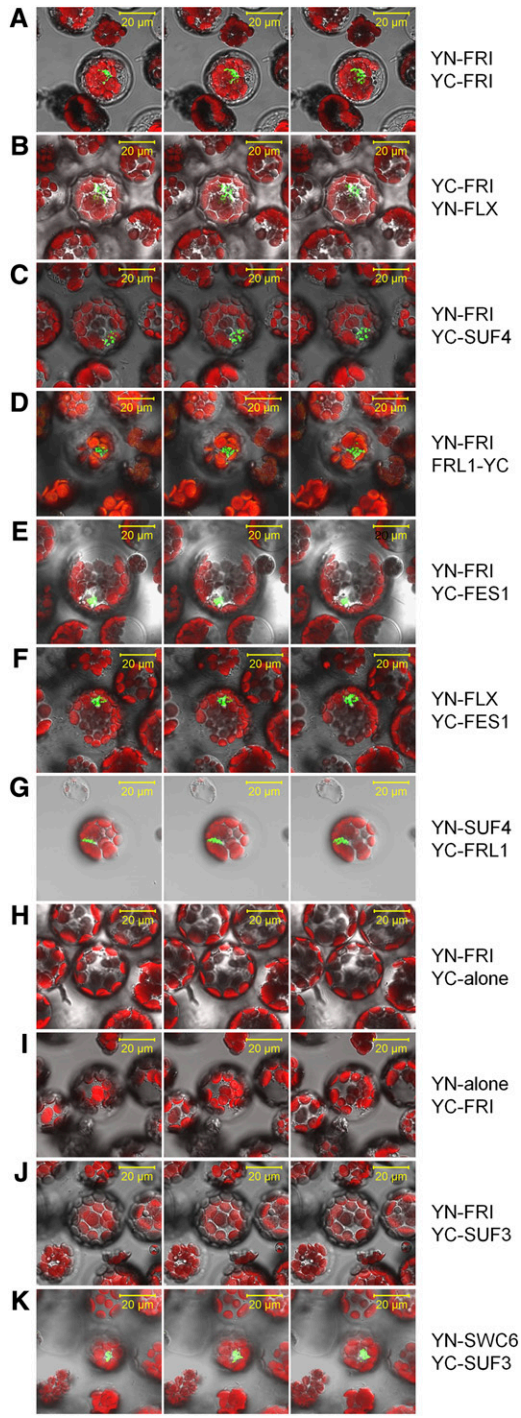

Figure 3. In Vivo Interactions among FRI, FRL1, FLX, SUF4, and FES1.

BiFC analyses were performed to confirm the interactions among FRI, FRL1, FLX, SUF4, and FES1. In the YN-/YC-interest or interest-YN/-YC fusion proteins, YN indicates the N-terminal region (amino acids 1 to 173), whereas YC indicates the C-terminal region (from residue 174 to the end of the polypeptide) of YFP. Each image was serially sectioned at intervals of 1 or 1.5 μm and is shown as merged panels of YFP, autofluorescence, and bright-field detections. Shown are the confocal images of BiFC to display the formation of FRI homodimers **(A)** and the interactions between FRI and FLX **(B)**, FRI and SUF4 **(C)**, FRI and FRL1 **(D)**, FRI and FES1 **(E)**, FLX and FES1 **(F)**, and SUF4 and FRL1 **(G)**. YN-FRI/YC-alone **(H)**, YN-alone/YC-FRI **(I)**, and YN-FRI/YN-SUF3 **(J)** are shown as negative controls. The previously shown physical interaction between SWC6 and SUF3 is shown as a positive control **(K)**. Bars = 20 μm.

one for the interaction with FRI and the other for transcription activation. In addition, the observations suggested that FLX and FES1 play roles as transactivators or coactivators for *FLC* transcription, likely through a conserved mechanism in yeast. It is likely that the specific activation domains of FLX and FES1 may bind to components of general transcription factors and chromatin modifiers in yeast.

FES1 is a unique protein, but there are four additional FLX-like proteins in *Arabidopsis* (see Supplemental Figure 7 and Supplemental Data Set 1 online). The four proteins were also capable of binding to FRI in the Y2H assay, but they had very weak or no intrinsic transcription activity in yeast (see Supplemental Figures 7C and 7D online). Consistently, the T-DNA insertion mutants in three genes (*FLX-LIKE1*, *2*, and *3*) did not suppress *FRI* activity. (The segregation ratios of F2 populations from crosses between *FRI*-Col and *flx like-1*, *2*, and *3* were approached to three late flowering to one early flowering [61:23, 64:20, 59:25; χ^2 = 16.4, 21.7, and 13.4 respectively], indicating that *FLX-LIKE* genes do not affect flowering time.) Thus, it is very likely that only FLX, which demonstrated strong transcription activity, functions in the FRI-C to activate the transcription of *FLC*.

Direct Binding of SUF4 to the *FLC* Promoter

Next, we hypothesized that at least one of the FRI-C components has DNA binding specificity for the *FLC* promoter. Previously, chromatin immunoprecipitation (ChIP) analysis showed that SUF4 is enriched at the *FLC* promoter region; however, direct binding of SUF4 was not demonstrated (Kim et al., 2006). To address this, we performed a Y1H assay using the *FLC* promoter fused with the *lacZ* reporter gene (Figure 5). Among the *FLC*-specific regulators, only SUF4 showed binding to the *FLC* promoter region but not to the first exon and intron, which is the location of the *cis*-element for the vernalization response (Figure 5B; Sheldon et al., 2002). To determine the exact binding sequence for SUF4, we analyzed a deletion series of the *FLC* promoter using the Y1H assay. The results showed that SUF4 binds to a 33-bp sequence in the proximal region (−363 to ~−331 from the transcription start site) of the *FLC* promoter (Figures 5C; see Supplemental Figure 8A online). We then generated a series of point mutations within the sequence to determine the essential nucleotides for SUF4 binding (Figure 5D), which resulted in a minimal 15-bp sequence (5′-CCAAATTT-TAAGTTT-3′). This result was confirmed by an electrophoretic mobility shift assay using a recombinant MBP-SUF4 fusion protein (Figure 5E). The MBP-SUF4 protein bound to the 15-bp sequence, and the wild-type competitor DNA eliminated the binding, whereas the mutant competitor DNA did not.

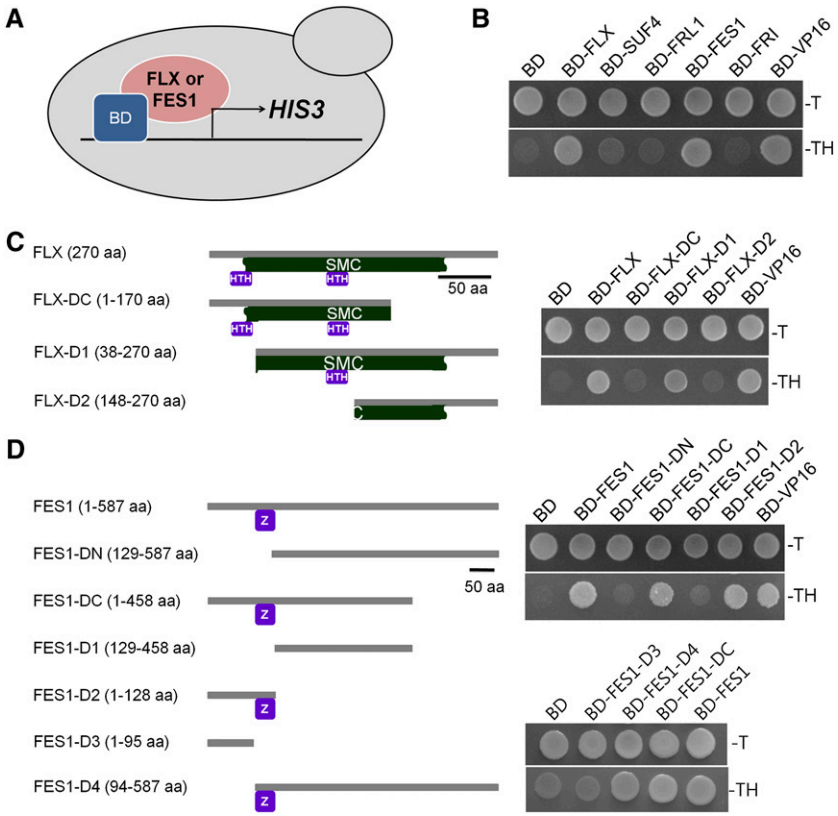

Figure 4. Intrinsic Transcription Activity of FLX and FES1 in Yeast.

(A) A diagram of the Y1H system used to monitor the intrinsic transcription activity. Each protein was fused to the Gal4-DNA BD. When a yeast cell expresses proteins with intrinsic transcription activity, the yeast cell can grow in a medium without TH (Trp and His) by expressing the *HIS3* reporter gene.

(B) Intrinsic transcription activity among *FLC*-specific regulators in yeast. Six independent colonies from each construct were tested, and representative samples are shown. The AD of VP16 (VP16) was fused to BD and used as a positive control. –T and –TH indicate the media lacking Trp only or both Trp and His, respectively.

(C) and **(D)** Y1H analysis showing that FLX and FES1 contain domains with intrinsic transcription activity. The deletion constructs of FLX **(C)** and FES1 **(D)** are shown in the middle of each panel, and the results of the one-hybrid analysis are shown to the right. The SMC region in FLX and the CCCH-type zinc finger region in FES1 were necessary for the intrinsic transcription activity. Boxes under the gray line representing the sequences show the following: SMC, SMC motif in FLX; HTH, HTH domain in FLX; and Z, CCCH-type zinc finger motif in FES1.

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We confirmed the binding of SUF4 to the *FLC* promoter by an *Arabidopsis* protoplast transient assay (Figure 5F). For this assay, we introduced either the *proFLC-LUC* (*FLC* promoter fused to *luciferase*) or the *proFLCm-LUC* (using an *FLC* promoter with a mutation in the SUF4 binding sequence) construct into wild-type and *fri* mutant protoplasts. When the wild-type *FLC* promoter was used, the luciferase activity was approximately 3 times higher than that of the mutant promoter, indicating that this *cis*-element is necessary for full *FLC* activation. Furthermore, when the *fri* mutant plant was used for this assay, the luciferase activity was reduced dramatically, irrespective of the promoter, indicating both that SUF4 bound to the *FLC* promoter and that the intact FRI-C was required for the activation of *FLC*.

Because SUF4 is a component of the FRI-C and a DNA binding factor bound to the *cis*-element in the *FLC* promoter, we reasoned that all of the components of the FRI-C should bind to this same region of the promoter, either directly or indirectly. Indeed, the ChIP analysis using transgenic plants expressing epitope-tagged proteins driven by a 35S promoter showed that the FRI, FRL1, and FLX proteins were highly enriched near the SUF4 binding region (Figure 5G). When the endogenous promoter of *SUF4* was used for the generation of the myc-tagged *SUF4* transgenic lines, it also showed the enrichment at the same binding region at FLC-5Q (see Supplemental Figure 8B online). Therefore, our results clearly demonstrated that SUF4 is a sequence-specific DNA binding factor that recruited FRI-C to the *FLC* promoter.

The FRI Complex Recruits Chromatin Modification Factors

In general, high gene expression depends on physical interactions between sequence-specific activators and general

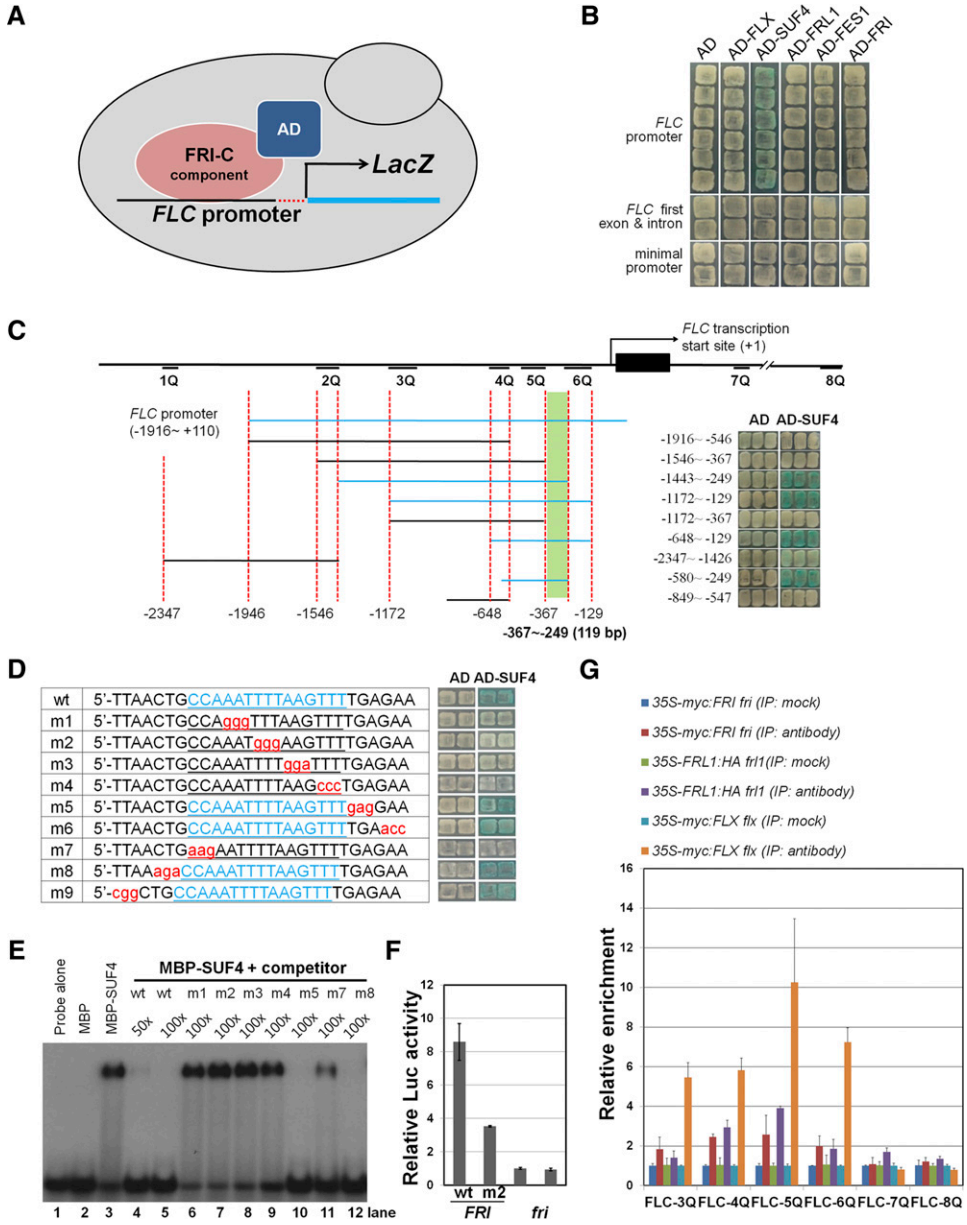

Figure 5. SUF4 Binds to the *FLC* Promoter and the Elucidation of the *cis*-Element.

(A) The Y1H system used to determine DNA binding protein at the *FLC* promoter. Each protein was fused to the AD of B42 and used as the effector protein. When a protein with *FLC* promoter binding activity is expressed, the reporter gene, bacterial *lacZ*, is induced, and as a consequence, a blue color appears on the X-gal plate. The red dots just 5′ to the *LacZ* coding region indicate the minimal promoter from the cytochrome C1 gene fused with the reporter gene.

(B) Y1H analysis of FLX, SUF4, FRL1, FES1, and FRI using the *FLC* promoter. The *FLC* promoter (*proFLC*) construct contains the upstream promoter of *FLC* from −1916 to +110, before the ATG. Six independent colonies were tested for each *FLC*-specific regulator. The *FLC* first exon and intron have a DNA fragment from the translational start ATG (+111) to the first three base pairs of the second exon (+3794). Two independent colonies were tested for each *FLC*-specific regulator. Vectors containing a minimal promoter (two bottom rows) and the AD (effector) alone were used as negative controls,.

(C) Diagrams of the *FLC* promoter deletion series and Y1H analysis of SUF4. Blue lines indicate the promoter regions that provided blue colonies on Y1H plates. The green box indicates the minimal region required for SUF4 binding to the *FLC* promoter. The numbers are the distance from the transcription start site.

(D) A diagram of Y1H analysis to delineate the SUF4 binding sequences. The 28 bp of the DNA fragment containing wild-type or mutated sequences of the *FLC* promoter, as shown on the left, were fused with the minimal promoter and the *lacZ* reporter gene and tested for the AD-SUF4 protein activation of the reporters. The 15-bp sequence, 5′-CCAAATTTTAAGTTT-3′, was essential for SUF4 binding.

(E) Electrophoretic mobility shift assay using the MBP-SUF4 recombinant protein and the SUF4 binding elements. Oligoduplex sequences of wild-type

transcription factors or chromatin modification factors. Therefore, we examined whether FRI-C interacts with the previously identified chromatin modification factors regulating *FLC* expression, such as the SWR1 complex that replaces the histone variant H2A.Z and the PAF1 complex that affects transcriptional elongation and histone methylation (He et al., 2004; Choi et al., 2007). None of the components of the PAF1 complex showed any interaction with FRI-C (data not shown). However, three components (SWC6, YAF9, and RVB1) of SWR1-C bound to components of the FRI-C (Figure 6A). SWC6 bound to both FLX and SUF4, YAF9 interacted with FLX alone, and RVB1 interacted with FLX, SUF4, FES1, and FRI in Y2H analysis (Figure 6A). The interactions of SWC6 and YAF9 with the FRI-C components were confirmed by BiFC analysis (Figure 6B), suggesting a direct link between FRI-C and SWR1-C.

If FRI-C interacts with SWR1-C, the recruitment of SWR1-C to the *FLC* promoter may be reduced in the absence of FRI. To address this question, we performed a ChIP assay using the transgenic plants of *suf3 fri* and *suf3 FRI* expressing myc-tagged SUF3, which is a subunit of SWR1-C (Figure 6C). As expected, SUF3 was enriched approximately twofold at the proximal region of the *FLC* promoter when *FRI* was present, demonstrating that FRI-C enhanced the recruitment of SWR1-C to the *FLC* promoter. By contrast, SUF3 was not enriched at the promoter of *HSP70* by the presence of *FRI* (Figure 6C), the transcription of which is induced by heat and affected by the activity of the SWR1-C (Kumar and Wigge, 2010). These data support the proposed specificity of FRI-C onto the *FLC* promoter.

Interestingly, the BiFC analysis showed that SWC6, a subunit of SWR1-C, also binds to EFS-N and TAF14, as well as to FRI-C (Figures 7B). Recently it was reported that EFS interacts with FRI and catalyzes the trimethylation of both H3K4 and H3K36 of *FLC* chromatin, thus activating transcription (Ko et al., 2010). Therefore, our results suggest that SWC6 plays a critical role in coordinating SWR1-C, FRI-C, EFS, and general transcription factors (Figure 7C).

The FRI Complex Recruits General Transcription Factors

YAF9, a component of SWR1-C, contains the YEATS (Yaf9/GAS41-ENL-AF9-Taf14-Sas5) domain that has been linked to chromatin modification and transcription (Schulze et al., 2009). The *Arabidopsis* genome has only two YEATS domain proteins, YAF9 and TAF14 (AT2G18000). TAF14 is a shared component of general transcription factors (TFIID and TFIIF), chromatin remodeling factors (INO80, SWI/SNF, and RSC), and histone H3 acetylation (NuA3) complexes in yeast and humans (Kabani et al., 2005). To determine whether TAF14 also interacts with FRI-C, both Y2H and BiFC analyses were performed (Figure 7; see Supplemental Figure 9 online). In the Y2H assay, interactions with TAF14 were detected with the FRI-C components except for FRL1; however, an interaction with FRL1 was detected in the BiFC analysis. The Y2H analysis, using a truncated version of TAF14 sequence revealed that the FRI-C components bind specifically to the C-terminal half of TAF14, whereas FRI requires the entire TAF14 sequence for interaction (see Supplemental Figure 9C online). It is noteworthy that the sequence of the C terminus of TAF14 is variable among species, in contrast with the conserved YEATS domain at the N terminus (see Supplemental Figures 9A and 9B and Supplemental Data Set 1 online). When single mutants of *TAF14* and *YAF9* were examined, they showed the same phenotype as the wild type. However, the double mutant showed a pleiotropic phenotype that included small size and abnormal leaf morphology, suggesting that the two genes have functional redundancy (see Supplemental Figure 10 online).

Last, we determined whether FRI-C recruits RNA polymerase II (polII), together with SWR1C and EFS, to the *FLC* transcription initiation site (Figure 7B). Indeed, RNA polII was enriched approximately twofold higher than the control at the first exon of the *FLC* when FRI was present. As expected, a mutation in *SUF3* or *EFS* reduced the RNA polII enrichment at the first exon. Moreover, the double mutants, *suf3 fri* and *efs fri*, showed an additive effect for RNA polII enrichment compared with the single mutants. These data suggest that SWR1-C, FRI-C, and EFS have partially independent functions in recruiting RNA polII onto the *FLC* promoter. Taken together, our results indicate that FRI-C mediates *FLC* transcription through interactions with chromatin modification factors, SWR1-C and EFS, as well as the general transcription factor TAF14, at the proximal region of the *FLC* promoter (Figure 7D).

The FRI Complex Leads to the Active Chromatin State of the *FLC* Gene

To define further the function of FRI-C at the molecular level, we monitored the chromatin state of the *FLC* gene in the mutants for FRI-C components. The epigenetic modifications of H3K4 trimethylation (H3K4me3), H3K36me2, H3 acetylation (H3ac), and

Figure 5. (continued).

probes, the wild type, or cold mutant competitors are shown. Recombinant proteins were mixed with labeled probes and competitors. The 50-fold (lane 4) and 100-fold (lanes 5 to 12) unlabeled wild type or mutant (m1, m2, m3, m4, m5, m7, and m8) oligoduplexes were used as competitors to confirm the specific interaction between SUF4 and the *cis*-element identified by Y1H analysis.

(F) The FRI complex activates the *Luc* reporter gene under the control of the wild-type *FLC* promoter with the first intron (wild type, −1916 to +3794) but not under the mutant promoter (m2) in *Arabidopsis* protoplasts. The plasmid DNA of two reporters, wild type and m2, was transformed into *FRI*-Col and Col (*fri*) protoplasts. Error bars indicate the SD of triplicate experiments. Luc, luciferase. The *35S-GUS* gene was cotransformed and used as an internal control.

(G) ChIP–quantitative PCR analysis showing FRI, FRL1, and FLX enriched on the *FLC* promoter. Epitope-tagged transgenic plants of *35S-myc:FRI fri*, *35S-myc:FLX flx*, and *35S-FRL1:HA frl1* grown for 12 d under long-day conditions used for ChIP analysis. Mock indicates immunoprecipitation without the specific antibody for MYC or HA. FLC-3Q~8Q indicates primers occupying the region of the *FLC* promoter, as shown in **(C)**. The *TUB2* promoter region was used for the normalization of the qPCR. Error bars indicate the SD of triplicate experiments.

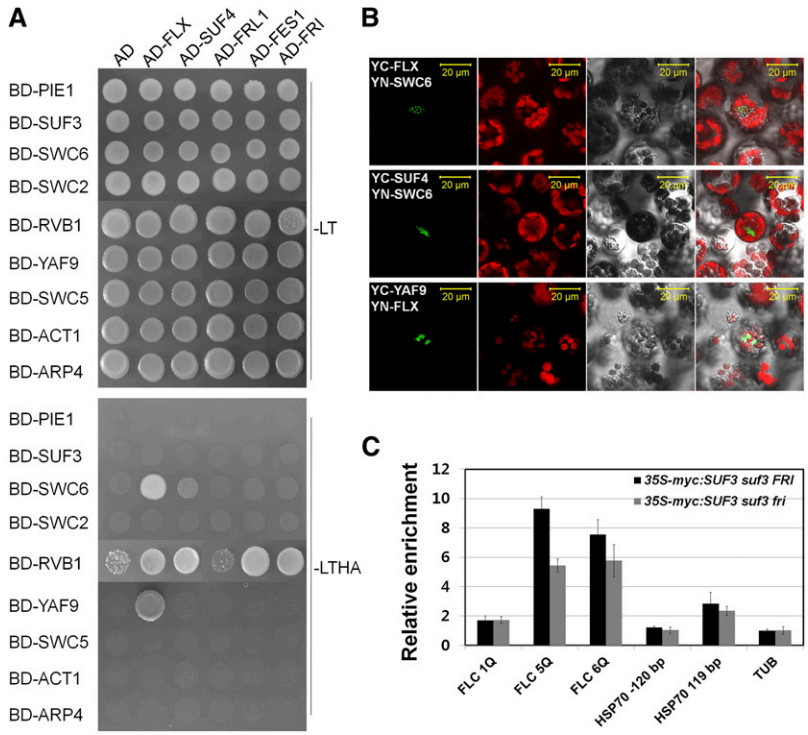

Figure 6. FRI-C Recruits SWR1-C to the *FLC* Promoter.

(A) Y2H analysis between components of FRI-C and SWR1-C.
(B) BiFC analysis used to confirm the interactions between FLX and SUF4, and SWC6 and YAF9. From left, fluorescence from YFP, autofluorescence, bright-field, and merged panel, respectively. Bars = 20 μm.
(C) ChIP–quantitative PCR analysis used to show that FRI-C recruits SWR1-C to the *FLC* promoter. The transgenic plants, *35S-myc:SUF3 suf3 fri* and *35S-myc:SUF3 suf3 FRI*, were used for ChIP analysis. Enrichment of the ChIP products was normalized based on results using *TUB2* promoter primers. The location of 4Q∼7Q primers is depicted in Figure 5C. The information of *HSP70* primer sets is obtained from previous report (Kumar and Wigge, 2010). Error bars indicate the SD of triplicate experiments.

H4ac were assessed across the promoter and the first exon and intron of *FLC.* Typical landscapes of eukaryotic epigenetic marks for active transcription were observed in the wild type: that is, the active epigenetic marks of H3K4me3, H3ac, and H4ac were enriched highly at the 5′ region (the FLC-2 region), and the H3K36me2 level was highest at the first intron region (Figure 7E). By contrast, when mutated in the components of FRI-C, the active epigenetic marks were severely reduced at the 5′ region or the first intron, a result that may reflect the decreased transcription state of *FLC* in the mutants (Figure 7E). A similar reduction was observed in *efs* and *vip5*, a mutant of a PAF1-C component, indicating that the functions of chromatin modification factors and FRI-C are tightly correlated. Taken together, our results support the hypothesis that FRI-C recruits chromatin modification factors to the *FLC* promoter, resulting in an active chromatin state.

DISCUSSION

FRI is a major determinant of the natural variations in flowering time and vernalization responses observed among *Arabidopsis* ecotypes. In this report, we show that FRI forms a large protein complex that we call FRI-C, the components of which have specialized functions, such as DNA binding, transcription activation, and maintenance of the complex. In addition, we show the interactions of FRI-C with a diverse range of chromatin modification factors and general transcription factors, which appear to result in the active chromatin state of the *FLC* gene. Therefore, our results clearly demonstrate that FRI-C acts as a transcription activator complex for *FLC* expression.

FRI Activates *FLC* Transcription by Forming a Large Protein Complex

The mutants of the five *FLC*-specific regulators, *fri*, *frl1*, *fes1*, *suf4*, and *flx*, all showed the same phenotype, indicating that they may be components of the same protein complex. As expected, FRI was shown to act as a scaffold protein that assembles a large protein complex with these five regulators. Furthermore, the biochemical and functional analyses of the FRI-C components allowed us to propose a model for the FRI-C–mediated transcriptional activation of *FLC*, as summarized in Figure 7C. As depicted, SUF4 directly binds to the *cis*-element

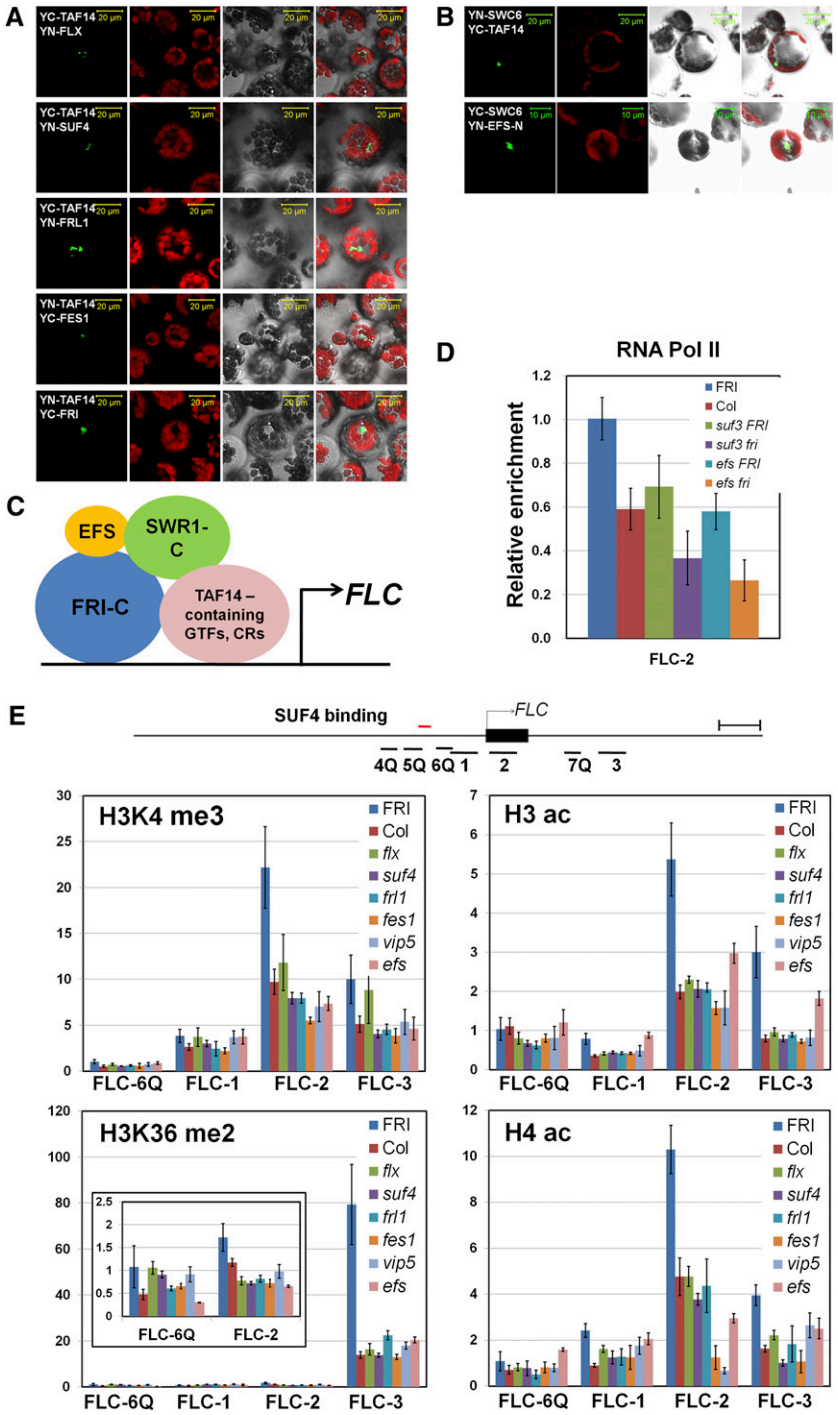

Figure 7. Physical Interactions of FRI-C with TAF14 and EFS.

(A) and **(B)** BiFC analyses used to show that TAF14 interacts with all of the FRI-C components **(A)** and that SWC6 interacts with both TAF14 and the N terminus of EFS **(B)**. From left, fluorescence from YFP, autofluorescence, bright-field, and merged panel, respectively. Bars = 20 μm, except bottom panels of **(B)**, where they = 10 μm.

(C) A model of how FRI-C functions for the transcriptional activation of *FLC* through physical interactions with chromatin modification factors and general transcription factors, such as SWR1-C, EFS, and TAF14.

(D) The relative enrichment of RNA polymerase II at the first exon of *FLC* in *FRI*, *fri*, *suf3*, *efs*, *suf3 fri*, and *efs fri*. Ten-day-old plants were used for ChIP analysis using the anti-polII antibody. FLC-2 primers for amplifying the region of the first exon of *FLC* were used. *ACTIN* was used for the normalization

(5′-CCAAATTTTAAGTTT-3′) located on the proximal promoter of *FLC* and forms a transcription complex (FRI-C) with the other *FLC*-specific regulators. The FRI-C then recruits chromatin modification factors and general transcription factors, such as SWR1-C, EFS, and TAF14. This recruitment promotes the transcription from the basal to the active state and subsequently mediates transcription elongation via the interaction with EFS at the transcribed region. There are many examples of specific transcription factors working in a protein complex with separate DNA binding and transcription activation components (Kidd et al., 2005; Nam et al., 2006; Wilson and Kovall, 2006). However, how such complexes activate transcription is not well known. Our study showing that a plant-specific transcription complex (FRI-C) recruits chromatin modification factors and general transcription factors provides unique insight into the mechanism of this complicated eukaryotic transcription process.

Recruitment of Chromatin Modification Factors and General Transcription Factors

FRI-C interacts with the eukaryotically conserved chromatin modification factors SWR1-C and EFS (Ko et al., 2010). The function of the incorporation of H2A.Z at transcriptional start sites, which is catalyzed by SWR1-C, is highly controversial: it is involved in both gene activation and inactivation (Zlatanova and Thakar, 2008). In *Arabidopsis*, it has also been implicated in both positive and negative transcriptional regulation. Mutations in the components of SWR1-C cause the derepression of biotic and abiotic plant response genes, such as plant disease resistance genes, heat shock–induced genes, and phosphate starvation response genes, supporting the negative role of H2A.Z (March-Díaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2010). By contrast, the mutations cause the suppression of the developmentally regulated *FLC* gene, supporting the positive role of H2A.Z (Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007; March-Díaz et al., 2007).

The contradictory role of SWR1-C/H2A.Z may be due to changes in the stability of H2A.Z-containing nucleosome particles by the acetylation of H2A.Z and the coincorporation of another histone variant, H3.3, as has been reported (Millar et al., 2006; Jin and Felsenfeld, 2007; Jin et al., 2009). The H2A.Z-containing nucleosomes exhibit a tighter wrapping of their DNA and thus are likely to maintain the inactive state of the genes by preventing the access of RNA polII or specific transcription factors (Thambirajah et al., 2006; Kumar and Wigge, 2010). However, when acetylated or coincorporated with H3.3, the H2A.Z-containing nucleosomes become labile and are evacuated from the promoter region for the onset of active transcription (Millar et al., 2006; Jin et al., 2009). The specific loading of FRI-C onto the proximal promoter of *FLC* may enhance the replacement of H2A.Z at the transcription start site by a direct interaction with SWR1-C. Thereafter, H2A.Z acetylation and the incorporation of H3.3 may occur at the *FLC* promoter, resulting in the release of the histone octamer and activation of transcription.

Such a model can explain why the H2A.Z level at the *FLC* chromatin is negatively correlated with the transcript level of *FLC* such that the steady state level of H2A.Z is less in *FRI* than in Col (a *fri* mutant) (Deal et al., 2007). Recruitment of SWR1-C seems to be specific to the *FLC* promoter because FRI-C does not affect the enrichment of ARP6 on the *HSP70* promoter (Figure 6C), which is one of the targets of H2A.Z incorporation (Kumar and Wigge, 2010). In addition, FRI-C interacts with AtYAF9 and AtTAF14, which are also a shared component of the NuA4 and NuA3 complexes involved in histone acetylation (Lu et al., 2009; Schulze et al., 2009). Thus, FRI-C likely orchestrates the dynamic process in which H2A.Z is reassembled and evacuated for the active transcription of the *FLC* gene. A similar mechanism has been observed in estrogen receptor signaling in humans, in which H2A.Z incorporation is enhanced by the direct interaction between p400, a component of human SWR1-C, and the estrogen receptor for targeted gene induction (Gévry et al., 2009). Thus, the recruitment of SWR1-C by specific transcription factors seems to be common. It is noteworthy that SWR1-C can also be recruited to the *FLC* gene independent of FRI because *FLC* transcription in the *fri* background is suppressed by the *suf3* mutation (Choi et al., 2005). Therefore, FRI-C is not absolutely required, yet it enhances the recruitment of SWR1-C to the *FLC* promoter.

The interaction of FRI-C with EFS has recently been reported, and EFS has a dual catalytic activity for the trimethylation of both H3K4 and H3K36, which are active epigenetic marks for transcription initiation and elongation (He et al., 2004; Sims et al., 2004; Ko et al., 2010). In agreement, there is a report showing that Set2, an EFS homolog, and H3K36 methylation affect the recruitment of RNA polII at the promoter as well (Krogan et al., 2003b). Therefore, likely through EFS, FRI-C may enhance both the initiation and the elongation steps of transcription. Prerecruitment of EFS to the promoter of *FLC* by FRI-C may help the dynamic transcription progress from preinitiation to initiation and to productive elongation for *FLC* transcription. Together with the fact that FRI-C also interacts with general transcription factors and the mRNA cap binding protein, CBP20 (Geraldo et al., 2009), our results support the concept of a unified model of gene expression in which each step regulating gene expression is physically and functionally connected to the next (Orphanides and Reinberg, 2002).

Conservation and Specification of the FRI Complex

Molecular genetic analyses in *Arabidopsis* have revealed two flowering pathways that respond to environmental signals: the

Figure 7. (continued).

of ChIP-qPCR. Error bars indicate the SD of triplicate experiments.

(E) ChIP and quantitative PCR analyses used to detect the active epigenetic marks of H3K4me3, H3ac, H4ac, and H3K36me2 in FRI-Col, *fri*, *suf4*, *flx*, *frl1*, *fes1*, *vip5*, and *efs.* The top part of the panel represents the *FLC* gene structure and the region used for the primers in the ChIP–quantitative PCR analyses. Ten-day-old seedlings were used. *Ta3* was used for the normalization of the quantitative PCR analysis. Error bars indicate the SD of triplicate experiments.

photoperiod and vernalization pathways. Although the CO-FT regulatory module that perceives daylength in the photoperiod pathway seems to be well conserved among both monocots and dicots (Izawa et al., 2003; Hayama and Coupland, 2004; Böhlenius et al., 2006), the FRI-FLC regulatory module in the vernalization pathway seems not to be conserved in monocots; *FLC*-like genes have not been identified in monocots, and vernalization in wheat (*Triticum aestivum*) suppresses the expression of a flowering repressor that is distinct from *FLC*, Ta *VRN2*, which encodes a B box zinc finger protein (Goff et al., 2002; Yan et al., 2004). In addition, Ta *VRN2* expression is not suppressed by vernalization under short days, a normal photoperiod during the winter season, suggesting that the molecular mechanism of vernalization in monocots is quite dissimilar with that in *Arabidopsis* (Trevaskis et al., 2007). Interestingly, we could detect the homologs of the components of FRI-C by database searching in both monocots and dicots (see Supplemental Figure 11 and Supplemental Data Set 3 online). However, the amino acid sequence identities, compared with rice (*Oryza sativa*) homologs, were as low as 25% with the exception of SUF4. In addition, the C terminus (amino acids 459 to 609) of FRI, where the majority of the FRI-C components interact, is very unique: no homologous sequence was detected outside the Brassicaceae family. Therefore, it is likely that the C terminus of FRI has evolved recently and provides a function as a scaffold for FRI-C in this Brassicaceae family. It is tempting to propose that *FRI* underwent neofunctionalization for generating FRI-C to regulate the flowering repressor *FLC*. Recently, the *FLC* homolog in sugar beet (*Beta vulgaris*), which is outside the Brassicaceae family, has been reported to play a critical role in the vernalization pathway (Reeves et al., 2007). It may be interesting to ascertain whether the *FRI-FLC* regulatory module is also conserved in the plant family that includes the sugar beet.

By contrast, SUF4 is relatively highly conserved in both sequence (60% identity) and biological function: the overexpression of the rice *SUF4* homolog (EEE70223) successfully rescued the *suf4* mutant (see Supplemental Figures 12 and 13 online). Although the obvious function of all of the components of FRI-C is to regulate *FLC*, the higher conservation of SUF4 may indicate that it has an additional function regulating the transcription of other genes in a subtle way. Indeed, when double mutants for the components of FRI-C were generated in *Arabidopsis*, the *suf4* mutation caused an increase in the floral organ number (in *suf4 flx* and *suf4 fes1*) and the retardation of root growth (in *suf4 fes1*). Such an additional function may constrain the evolutionary change of *SUF4*.

The genetic variation in flowering time among *Arabidopsis* ecotypes is mainly caused by aberrations in *FRI* and *FLC*, although a mutation in any of the components of FRI-C causes a similar flowering phenotype (Gazzani et al., 2003; Michaels et al., 2003; Lempe et al., 2005; Werner et al., 2005). This suggests that evolutionary constraints may exist in the other components, such that they have other functions in addition to the regulation of *FLC*. The phenotype regulated by these additional functions may be expressed in natural environments, which then leads to different outcomes of genotypes compared with standard laboratory conditions (Wilczek et al., 2009).

METHODS

Plant Materials and Growth Conditions

The wild type, Col:*FRI*^{SF2} (*FRI*-Col), and all the flowering time mutants used in this study have been previously described (Michaels et al., 2004; Choi et al., 2005; Schmitz et al., 2005; Kim et al., 2006). Seed for SALK insertion lines (see accession numbers section) were obtained from the ABRC. Plants were grown and the flowering time was measured by counting the number of rosette leaves from at least 10 plants.

Plasmid Construction

The sequence information of the primer pairs used for PCR amplification is presented in Supplemental Table 2 online. To generate the constructs for the plant transformation, the full open reading frames (ORFs) of *FLX*, *FRI*, and *FRL1* were cloned into pCGN18, myc-pBA, or the pGWB14 binary gateway vector.

For the Y2H analysis, the ORFs of FLX, FES1, and other *FLC* regulators were amplified, cloned into the pGEM-T Easy vector obtained from Promega, sequenced, and finally subcloned into the proper restriction sites of the pGBKT7 and pGADT7 vectors obtained from Clontech (Matchmaker GAL4 Yeast Two-Hybrid System 3). To generate the truncated constructs, proper restriction sites within the genes and vectors were used, or amplified PCR fragments including the truncated region were cloned into the Y2H vectors.

For the reporter constructions used in the Y1H analysis, various *FLC* promoter fragments, the first intron of *FLC*, and 28-bp oligomer duplexes were cloned into the *Sma*I site of the pLacZi2μ vector (kindly provided by H. Wang). For the effector constructions, the ORFs of FRI, FRL1 (*Sma*I and *Bam*HI), FES1 (*Bgl*II), SUF4, and FLX (*BamH*I), within the pGBKT7 vectors, were removed by restriction enzyme digestion, and the overhanging ends were filled in using DNA polymerase Klenow fragment. The inserts were cloned into the *Eco*RI site of the pJG4-5 vector, whose ends had been blunted using Klenow.

To produce the constructs for the BiFC analysis or the cyan fluorescent protein (CFP) and YFP localization analyses using the protoplast transient assay, the ORFs or the ORFs without the stop codon of FRI, FRL1, FES1, SUF4, and FLX were cloned into the *Bam*HI site of E3081/pSAT4-nEYFP-C1, E3082/pSAT4-cEYFP-C1, E3083/pSAT4-nEYFP-N1, E3084/pSAT4-cEYFP-N1 (http://www.bio.purdue.edu/people/faculty/gelvim/nsf/index.htm), the YFP vector (Kim et al., 2006), or the CFP vector, which was modified from the YFP vector by exchanging the ORF of YFP (*Nco*I-*Bam*HI) with the ORF of CFP.

Y2H Analysis

The vectors and yeast strains (Matchmaker GAL4 Two-Hybrid System 3) were obtained from Clontech. The Y2H assay was performed according to the manufacturer's instructions. After 3 d of incubation at 30°C, yeast cells were spotted on the selection plates containing SD medium lacking Leu, Trp, Ade, and His. These plates were further incubated at 22 or 30°C until the yeast cells formed colonies.

Y1H Assay

To detect intrinsic transcriptional activity, plasmids with GAL4 DNA binding domain fusions were transformed into the yeast strain AH109 (Matchmaker GAL4 Yeast Two-Hybrid System 3 from Clontech) using standard transformation techniques. To find the DNA binding proteins, plasmids for the AD fusions were cotransformed into the yeast strain EGY48, with the *LacZ* reporter gene driven by various *FLC* promoter fragments.

Protoplast Transient Assay

Rosette leaves of Col plants that had been grown for 4 weeks on short days (8 h light/16 h dark) were sampled for the isolation and transformation of protoplasts as described (Yoo et al., 2007). All of the plasmid DNA for the protoplast transformation was prepared by the CsCl gradient method. After ~12 h of transformation, the protoplasts were observed as previously described (Choi et al., 2005) or used for luciferase activity assays and ChIP analysis.

Gel Filtration Assays

Two hundred micrograms of total protein extracts from *35S-myc:FRI fri* transgenic plants grown for 10 d was injected on a Superdex 200 10/300 GL column (Amersham Biosciences). After fractionation by the AKTA fast protein liquid chromatography system (Amersham Biosciences), 24 tubes containing 0.5 mL were collected. Proteins in each fraction were concentrated using 10 μL of Strataresin (Stratagene), electrophoresed, blotted, and identified using monoclonal anti-c-myc antibodies (Sigma-Aldrich M5546).

Purification of the Components of FRI-C

Purification of the FRI complexes was performed following a protocol previously described (Cho et al., 2006). Two-week-old plants of *35S:myc-FRI* (50 g fresh weight), which fully rescues the *fri* mutant, were used. As a negative control, the *fri* mutant was used while following the same procedure. Nuclear extracts were obtained as previously described (Lee et al., 2007), incubated with 300 μL of anti-c-myc affinity agarose in TBS (50 mM Tri-HCl, pH 7.4, and 150 mM NaCl) overnight, and then washed and eluted with 100 μL sample buffer. The eluates were analyzed by LC-MS/MS (ProteomeX LTQ 2D/MS/MS mass spectrometer). The analysis was processed according to the manufacturer's procedures. Full details of the data on the matching peptides and scores from the purification of the protein complexes and control samples are included in Supplemental Table 1 online.

Electrophoretic Mobility Shift Assays

The MBP and MBP-SUF4 recombinant fusion proteins were expressed in *Escherichia coli* BL21 strain and purified using amylose resin. The single-stranded oligonucleotides were labeled with [α-^{32}P]ddATP using terminal transferase (Roche 03333566001) and then annealed. One hundred nanograms of protein and ~40,000 cpm of ^{32}P-labeled probe were incubated in 20 μL of binding mixtures, including 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 μg/μL BSA, 100 μM $ZnCl_2$, 6% glycerol, and 1 mM DTT at 4°C for 1 h and separated on a 6% polyacrylamide gel in Tris-acetate-EDTA buffer (40 mM Tris and 2.5 mM EDTA, pH 7.8) at 10 V/m for 2 h. For the competition assay, excess unlabeled probes (50× and 100×) were added to the binding mixture.

ChIP Analysis

All ChIP procedures were followed according to a previous report (Choi et al., 2007). Whole plants grown for 10 d under long-day conditions and antibodies against acetyl H4K5/8/12/16 (Upstate 06-866), acetyl H3K9/14 (Upstate 06-599), trimethyl H3K4 (Upstate 17-614), dimethyl H3K36 (Upstate 07-274), and the RNA polymerase II C-terminal domain (Abcam ab817) were used for immunoprecipitation. To determine the enrichment in the epitope-tagged transgenic plants, monoclonal anti-c-myc (Sigma-Aldrich M5546) and monoclonal anti-HA (Sigma-Aldrich H9658) were used. Information on the primer pairs for the ChIP–quantitative PCR is presented in Supplemental Table 2 online.

Phylogenetic Analysis

Multiple alignments of amino acid sequences were performed using the ClustalX2.1 program (http://www.clustal.org/download/current/). For phylogenetic analysis, the PHYLIP program (version 3.69) was used (http://evolution.genetics.washington.edu/phylip.html). In the PHYLIP software, SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE programs were used to draw phylogenetic trees (unrooted) and obtain bootstrap values. For the phylogenetic tree, JTT model, neighbor joining, and bootstraps with 1000 trial options were used. The phylogenetic tree was drawn with the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Quantitative PCR

For real-time quantitative PCR, total RNA was isolated using the RNeasy plant mini kit (Sigma-Aldrich 74904). Four micrograms of total RNA was treated with recombinant DNaseI (TaKaRa 2270A) to eliminate genomic DNA. cDNA was generated using RNA with reverse transcriptase (Fermentas EP0441) and oligo(dT). Quantitative PCR was performed using the 2× SYBR Green SuperMix (Bio-Rad 170-8882) and monitored by the CFX96 real-time PCR detection system (Kim et al., 2006). The relative transcript level and the ChIP enrichment level were normalized with *actin7* and *tubulin2* genes according to the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database or the Arabidopsis Genome Initiative database under the following accession numbers: *ACT1* (*AT2G37620*), *ARP4* (*AT1G18450*), *ARP6* (*AT3G33520*), *EFS* (*AT1G77300*), *FES1* (*AT2G33835*), *FLX* (*AT2G30120*), *FRI* (*AT4G00650*), *FRL1* (*AT5G16320*), *HSP70* (*AT3G12580*), *PIE1* (*AT3G12810*), *SUF4* (*AT1G30970*), *SWC2* (*AT2G36740*), *SWC4* (*AT2G47210*), *SWC5* (*AT5G30490*), *SWC6* (*AT5G37055*), *RVB1* (*AT5G22330*), *TAF14* (*AT2G18000*), *TUB* (*AT5G62690*), *VP16* (*U8963*), and *YAF9* (*AT5G45600*). SALK insertion lines from ABRC are as follows: SALK_002678 (*flx like 1*), SAIL_535_H09 (*flx like 2*), SAIL_156_B10 (*flx like 3*), and SALK_075203 (*yaf9*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Grouping of *suf* Mutants and *FLC* Expression.

Supplemental Figure 2. Characterization of *flx-2* and the expression patterns of *FLX*.

Supplemental Figure 3. Gel Filtration Analysis of myc:FRI Proteins Expressed in the *fes1*, *frl1*, *suf4*, and *flx* Mutant Backgrounds.

Supplemental Figure 4. Yeast Two-Hybrid Interaction Analyses among the Deletion Forms of FRI, FRL1, FLX, SUF4, and FES1.

Supplemental Figure 5. Characteristics of the C-Terminal 150 Amino Acids of FRI.

Supplemental Figure 6. Localization of FLX, FRL1, and FES1.

Supplemental Figure 7. *FLX*-Like Genes in *Arabidopsis thaliana*.

Supplemental Figure 8. SUF4 Binding to the *FLC* Promoter in Vitro and in Vivo.

Supplemental Figure 9. YEATS-Containing Proteins in Different Organisms.

Supplemental Figure 10. Phenotype of *atyaf9* and *attaf14-kd* Plants.

Supplemental Figure 11. Phylogenetic Trees of FRI-C Components.

Supplemental Figure 12. Alignment of SUF4 Homologs from Different Plant Species.

Supplemental Figure 13. The Overexpression of *OsSUF4* Rescued the *suf4* Mutant Phenotype.

Supplemental Table 1. LC-MS/MS Results for the myc-FRI IP and the Control Sample.

Supplemental Table 2. Primers for Plasmid Construction and PCR.

Supplemental Data Set 1. Text File of Sequences Used for the Phylogenetic Analysis in Supplemental Figure 7A.

Supplemental Data Set 2. Text File of Sequences Used for the Phylogenetic Analysis in Supplemental Figure 9A.

Supplemental Data Set 3. Text File of Sequences Used for the Phylogenetic Analysis in Supplemental Figure 11.

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