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ORIGINAL ARTICLE

Brassinosteroid Signaling Modulates Submergence-induced Hyponastic Growth in *Arabidopsis thaliana*

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Abstract The role of brassinosteroids (BRs) in hyponastic growth induced by submergence was investigated in *Arabidopsis thaliana*. Under flooding conditions, exogenously applied BRs increased hyponastic growth of rosette leaves. This hyponastic growth was reduced in a BR insensitive mutant (*bri1-5*), while it was increased in a BR dominant mutant (*bes1-D*). Further, expression of hypoxia marker genes, *HRE1* and *HRE2,* was elevated in submerged *bes1-D.* These results indicate that BRs exert a positive action on hyponastic growth of submerged Arabidopsis leaves. Expression of ethylene biosynthetic genes, such as *ACS6, ACS8* and *ACO1,* which are up-regulated by submergence, was also activated by application of BRs and in *bes1-D*. The enhanced hyponastic growth in submerged *bes1-D* was significantly reduced by application of cobalt ion, suggesting that BRs control hyponastic growth via ethylene, which seems to be synthesized by *ACO6* and *ACO8* followed by *ACO1* in submerged leaves. A double mutant, *bes1-Dxaco1-1,* showed hyponastic growth activity similar to that seen in *aco1-1*, demonstrating that the BR signaling for regulation of hyponastic growth seems to be an upstream event in ethylene-induced hyponastic growth under submergence in Arabidopsis.

Keywords: Brassinosteroids, Ethylene, Hyponastic growth, Submergence

Introduction

The life cycle of plants consists of germination, vegetative and reproductive growth and development, and senescence. Each stage of the plant life cycle is regulated by various factors which can be divided into biotic and abiotic factors. Among abiotic factors, water is considered to be one of the most important factors which needs to be properly maintained for the normal growth and development of plants. If water levels are improperly maintained, plants recognize it as a stress and activate a defense mechanism to overcome this water stress. The stresses related to water are divided into two categories - drought and submergence. Due to global warming and water shortages, studies on drought stress have been actively pursued. However, research on flooding stress in plants has been relatively scarce.

The exchange of gases such as oxygen, carbon dioxide and ethylene is necessary for normal growth of plants. Under flooding conditions, a plant is completely isolated from its external atmosphere. The diffusion rate of gases in liquid is dramatically reduced relative to the rate in the atmosphere, which results in a decreased gas exchange ratio between plants and the atmosphere (Gibbs and Greenway 2003; Voesenek et al. 2006; Jackson 2008). Aquatic plants, such as deep-water rice and *Rumex palustris*, are known to respond to submergence by sensing the altered composition of internal gases (Van der Straeten et al. 2001; Voesenek et al. 2004). Lowered oxygen inhibits photosynthesis, causes a transition of glycolysis and anaerobic respiration and promotes both the synthesis and accumulation of ethylene (Setter et al. 1996, 2003; Cox et al. 2003; Millenaar et al. 2005; Pierik et al. 2006; Voesenek et al. 2006; Jackson, 2008). To overcome this situation, plants are known to use two strategies, quiescence and escape, which are collectively referred to as 'Low oxygen escape syndrome (LOES)' (Bailey-Serres and Voesenek, 2008). In the quiescence strategy, the growth of a plant is inhibited until the plant is no longer exposed to flooding conditions (Fukao and Bailey-Serres 2004; Perata and Voesenek 2007). In contrast, in the escape strategy, the growth of the shoot portion of plants, such as the stem and petioles of leaves, is promoted to achieve an exit out of the water (Setter and Laureles 1996;

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Visser et al. 1996; Colmer et al. 1998; Sauter 2000; Colmer 2003; Evans 2004; Mommer et al. 2005; Mommer and Visser 2005).

Under submergence, petioles grow differentially on the adaxial and abaxial sides, which causes upward movement due to the increased angle between the petiole and the ground (Cox et al. 2004; Polko et al. 2012; Rauf et al. 2013). This is referred to hyponastic growth and is one of the representative phenotypes observed in submerged plants. Ethylene is regarded as a key factor regulating hyponastic growth in submerged plants (Millenaar et al. 2005, 2009; Van Zanten et al. 2010; Rauf et al. 2013). As it is a gas, ethylene accumulates inside plants under flooding stress and activates the ethylene signaling pathway (Visser et al. 2000; Pierik et al. 2006; Jackson 2008). As a result, ethylene regulates the re-orientation of microtubules in the abaxial side of leaf petioles and the expression of cell wall loosening proteins, which lead to the uneven growth of petioles for hyponastic growth under flooding stress conditions (Vriezen et al. 2000; Polko et al. 2012).

Brassinosteroids (BRs) are steroidal plant hormones which promote plant growth and development through the regulation of cell elongation and cell division. (Mitchell et al. 1970; Clouse and Sasse 1998; Sasse 2003). BRs interact with other plant hormones, indicating that cross-talk between BRs and other plant hormones is necessary to control the growth and development of plants. In particular, the activities for BRs frequently overlap with those induced by auxin and ethylene, suggesting that interactions between these three hormones are important for their physiological activities (Arteca et al. 1983; Goda et al. 2002; Nakamura et al. 2003; Goda et al. 2004; Halliday 2004; Nemhauser, et al. 2004; Gendron et al., 2008; Vert et al. 2008). BRs are known to increase ethylene production in plants (Arteca et al. 1983; Lim et al. 2002). Recently, the Arabidopsis *decreased low light angle1 (ddd1)* mutant, which exhibits reduced hyponastic growth under submergence, was shown to inhibit the expression of a BR biosynthetic gene, *ROT3*/CYP90C1 (Polko et al. 2012). They also reported that ethylene promotes hyponastic growth through interaction with ROT3/CYP90C1 in the plant (Polko et al. 2013). In addition, an ethylene response factor related to flooding stress, SUBMERGENCE 1A (SUB1A) was shown to regulate BRs biosynthesis in rice (Schmitz et al. 2013). These findings indicate that BRs can mediate hyponastic growth in submerged plants. However, little is known about the mechanisms of action of BRs in hyponastic growth in submerged plants.

In this study, we tested the effect of BRs on hyponastic growth under flooding stress in Arabidopsis. In addition, the effects of interactions between BRs and ethylene action on hyponastic growth were examined in submerged Arabidopsis. Finally, we investigated the connection between BRs- and ethylene-induced hyponastic growth in the submerged Arabidopsis plants. Our results provide a clue as to how BRs control the hyponastic growth of plants under flooding stress.

Results and Discussion

Rosette plants from wild type (Col-0) Arabidopsis were submerged in distilled water for 24 h and the lengths of petioles and the angle between the ground and petioles were measured. Compared to non-submerged plants, submerged plants had increases in both the lengths of petioles and the angle between the ground and the petioles (Fig. 1A, B). Under flooding conditions, exogenously applied brassinolide (BL, 10^{-8} M) increased the flooding-induced petiole length and angle (Fig. 1A, B). In a BR-insensitive mutant (*bri1-301*), both the petiole length and angle were reduced compared to wild type (Col-0) Arabidopsis plants. In contrast, both the petiole length and angle were significantly increased in a BR-dominant mutant (*bes1-D*) relative to in wild type (En-2) (Fig. 1C-E). Two ethylene response factors (ERFs), hypoxia–responsive ethylene response factor 1 (*HRE1*) and 2 (*HRE2*), are known to be marker genes which are up-regulated by submergence in Arabidopsis (Licausi et al, 2010). In *bes1-D*, the expression of *HRE1* and *HRE2* was approximately 3.3 and 1.7 times higher than in wild type plants under submergence (Fig. 1F). These findings demonstrate that BR signaling via BES1 enhances hyponastic growth, and that this BRs-induced hyponastic growth is likely mediated via the ethylene response in submerged Arabidopsis plants.

Application of an ethylene biosynthetic inhibitor, cobalt ion, slightly decreased hyponastic growth induced by submergence, suggesting that the endogenous ethylene level is important for hyponastic growth in submerged Arabidopsis (Fig. 2A, B). To examine how ethylene production is controlled, the expression of ethylene biosynthetic genes was examined by semi-quantitative RT-PCR (semi q-PCR) in submerged Arabidopsis. As shown in Fig. 2C, expression of *ACS8* was significantly increased for 12 h and then decreased. Similarly, expression of *ACS6* was activated for 12 h and then subsequently decreased. As for the *ACO* genes, *ACO1* exhibited continuous increases in gene expression up to 24 h when strong hyponastic growth was also observed. In contrast, *ACO4* did not show any significant alterations in gene expression over 24 h (Fig. 2D). Collectively, submerged-Arabidopsis increases the expression of ethylene biosynthetic genes, such as *ACS6, ACS8* and *ACO1,* which implies that the endogenous ethylene which induces hyponastic growth under submergence is likely to be produced by *ACO1* via two *ACS* genes*, ACS6* and *ACS8,* in Arabidopsis.

The BR biosynthetic gene *CPD* is known to be down-regulated by BL in *A. thaliana.* The application of 10^{-8}M BL

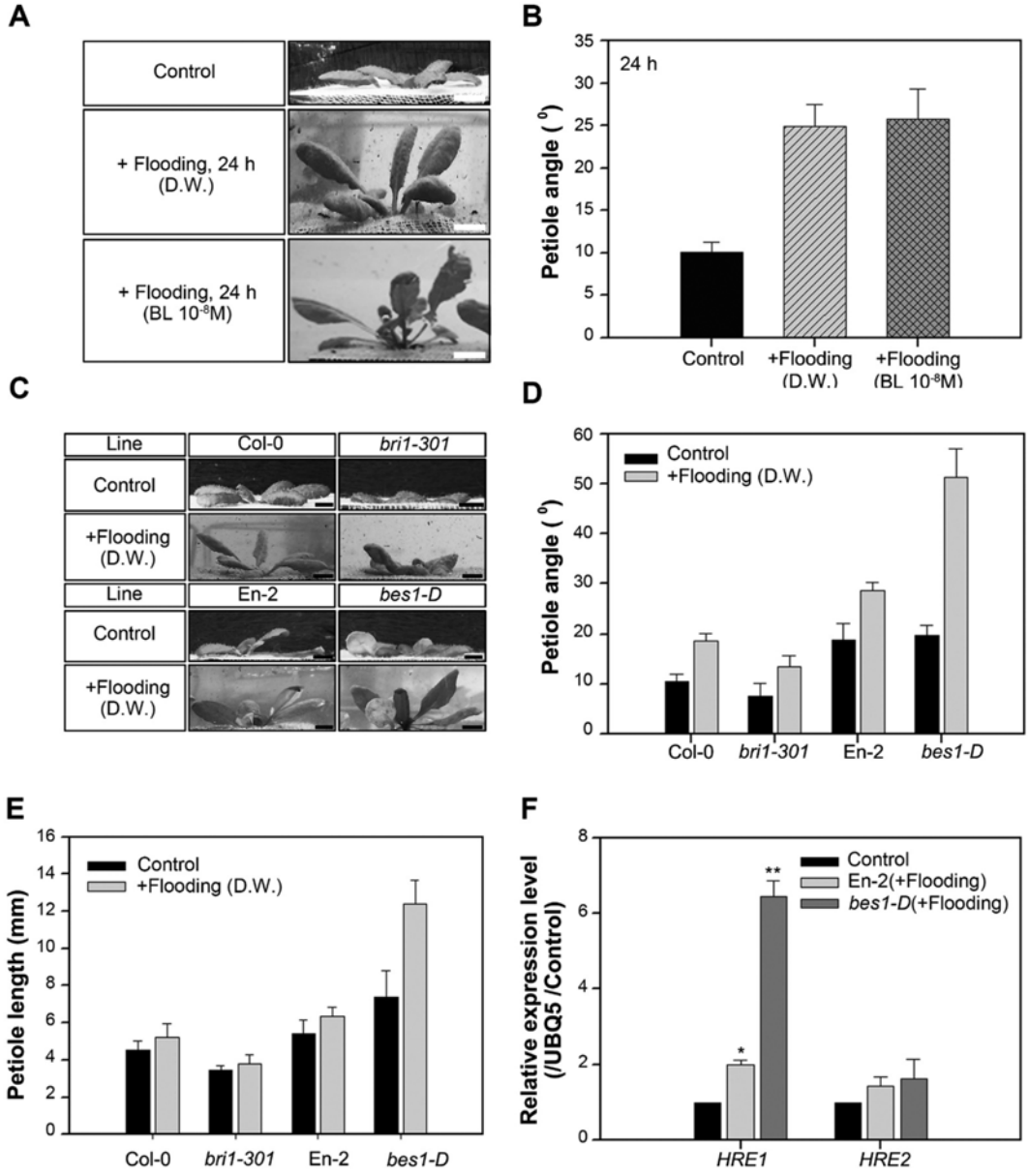

Fig. 1. Effects of BRs on the induction of hyponastic growth of Arabidopsis under flooding stress. (A, B) The hyponastic growth phenotype of 27 d-old Arabidopsis Col-0 plants exposed to control growth conditions, submerged in distilled water, or in 10^{-8} M BL-treated distilled water for 24 h. Scale bar is 10 mm. (C-E) Different hyponastic growth patterns under submergence conditions in a BR-related mutant (*bri1-301, bes1-D*) and in corresponding wild-type (Col-0, En-2) plants. Scale bar is 10 mm. After 24 h of submergence treatment, the petiole angles were measured as the angle between the ground and the third petiole. Data are means (n=5) ± S.E. More than five petiole lengths were measured per plant at the end of the experiment. Data are means ($n > 25$) ± S.E. In the data showing the petiole angle and length compared with control group, p-value is less than the value 0.1. (F) Relative expression levels of hypoxia marker genes (*HRE1, HRE2*) in 7 d-old seedlings of *bes1-D* and En-2 exposed to submergence for 24 h. Data are the means from three independent experiments ± S.E. Single asterisk (*), $P < 0.05$ compared to control. Double asterisk (**), $P < 0.01$ compared to control.

decreased expression of *CPD*, indicating that BRs were appropriately used in this study (Fig. 2E). Upon application of BL, expression of *ACS6* and *ACS8* was up-regulated compared to the levels seen in BL-untreated Arabidopsis (Fig. 2E). Expression of *ACO1* was greatly up-regulated, while expression of *ACO4* was not changed by BL treatment. These findings demonstrate that endogenous ethylene production in Arabidopsis can be increased by BRs via the enhanced expression of *ACS6* and *ACS8* followed by *ACO1*. The expression patterns of *ACS6, ACS8* and *ACO1* upon BR treatment match those induced by submergence (Fig. 2C, D), implying that BR-induced hyponastic growth is closely related to the expression of ethylene biosynthetic genes to produce ethylene under submergence in Arabidopsis.

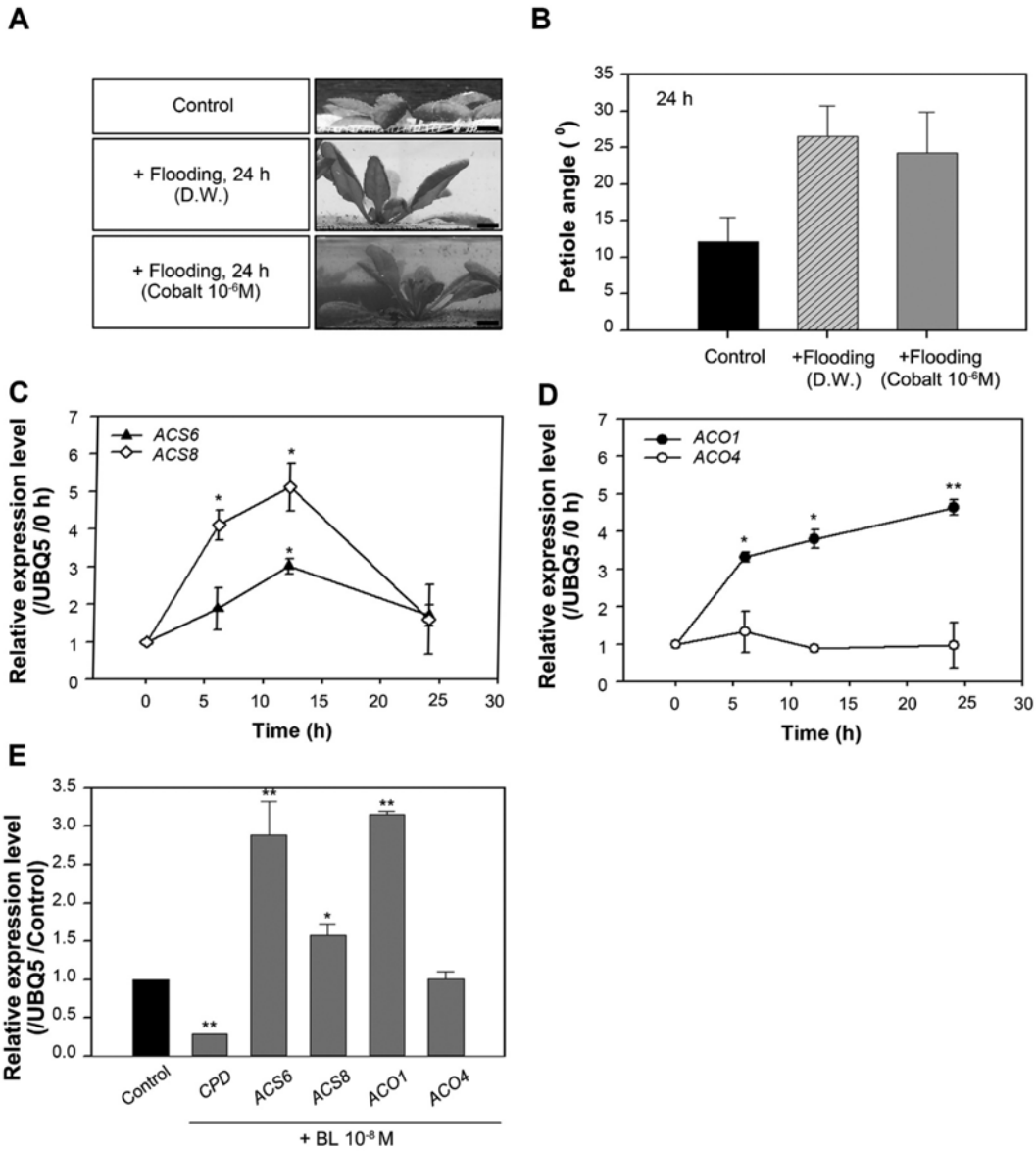

Fig. 2. BRs induced ethylene production under flooding stress in Arabidopsis. (A, B) 27 d-old Col-0 plants exposed to control growth conditions, submerged in distilled water, or in 10^{-6} M Cobalt-treated distilled water. Scale bar is 10 mm. After 24 h of submergence treatment, the petiole angles were measured as described above. Data are means (n=5) ± S.E. In the data showing the petiole angle compared with control group, p-value is less than 0.1. (C, D) Relative expression levels of ethylene biosynthetic genes (*ACS6, ACS8, ACO1* and *ACO4*) were measured according to the increase in submergence exposure time from 0 to 24 h. Single asterisk (*), $P < 0.05$ compared to 0 h. Double asterisk (**), $P < 0.01$ compared to 0 h. (E) Relative expression levels of ethylene biosynthetic genes (*ACS6, ACS8, ACO1* and *ACO4*) were measured in response to 10^{-8} M BL treatment for 2 h. RNA was isolated from 7 d-old seedlings exposed to flooding condition (±BL). *CPD* was used as a BR response marker gene. Data are the means from three independent experiments ± S.E. Single asterisk (*), $P < 0.05$ compared to control. Double asterisk (**), $P < 0.01$ compared to control.

As shown in Fig. 3A, in *bes1-D* which shows increased hyponastic growth, expression of *ACS6* and *ACS8* was slightly increased relative to wild type (En-2) plants after 24 h of submergence treatment. Expression of *ACO4* was not significantly altered. In contrast, the expression of *ACO1* was clearly increased. These results imply that BES1 positively regulates ethylene production mainly through *ACO1* when BR signaling is activated.

Upon application of cobalt ion under submergence, *bes1-D* showed an approximately 40% decrease in hyponastic growth, which is more severe than that observed in En-2, implying that hyponastic growth in *bes1-D* under flooding conditions is also mediated by the biosynthesis of ethylene (Fig. 3B). Therefore, we expected that an *ACO1* knockout mutant, *aco1-1*, would show reduced hyponastic growth relative to wild type Col-0. However, *aco1-1* exhibited

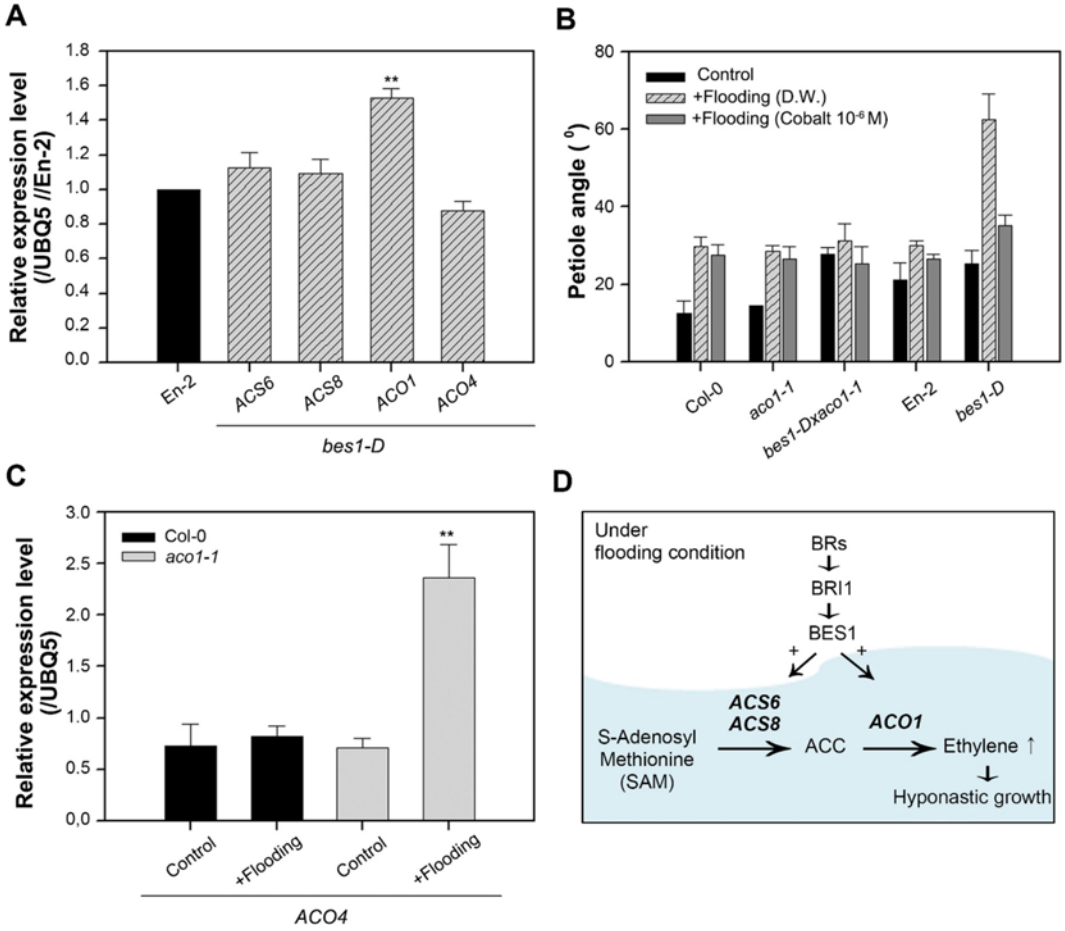

Fig. 3. BRs-induced hyponastic growth is mainly mediated by *ACO1* in Arabidopsis under flooding stress. (A) The relative expression levels of ethylene biosynthetic genes (*ACS6, ACS8, ACO1*, and *ACO4*) in 7 d-old seedlings of *bes1-D* and its corresponding wild-type En-2 were measured after 24 h of submergence treatment. Double asterisk (**), $P < 0.01$ compared to En-2. (B) 27 d-old mutants (*aco1-1, bes1-D* and *bes1-Dxaco1-1*) were exposed to submergence with 10^{-6} M cobalt-treated and untreated distilled water. After 24 h of submergence treatment, the petiole angles were measured as described above. Data are means (n=5) ± S.E. In the data showing the petiole angle compared with control group, *p*-value is greater than 0.05. (C) The relative expression level of *ACO4* was evaluated from 7 d-old seedlings of *aco1-1* under submergence conditions. Data are the means from three independent experiments ± S.E. (D) Schematic diagram showing that BRs function as a regulator of *ACO1* gene expression to induce hyponastic growth under the submergence condition used in this study. Double asterisk (**), $P < 0.01$ compared to control.

almost the same hyponastic response as Col-0, suggesting the presence of functional redundancy with other *ACOs* in *aco1-1* (Fig. 3B). Indeed, expression of *ACO4* was greatly increased in *aco1-1* under flooding conditions, suggesting that the endogenous level of ethylene seems to be maintained at the level of wild type in *aco1-1* by enhanced expression of *ACO4* (Fig. 3C). In a homozygous *bes1-Dxaco1-1* double mutant, the hypersensitive hyponastic growth observed in *bes1-D* was not observed under submergence (Fig. 3B). Additionally, the inhibitory effect of cobalt ion on the hyponastic growth of submerged *bes1-D* was significantly reduced to a level similar to that in *aco1-1* (Fig. 3B). These findings demonstrate that the hyponastic growth observed in submerged *bes1-D* is controlled via a downstream component, *ACO1,* in Arabidopsis.

As terrestrial plants, Arabidopsis is not the ideal model species to study submergence-related responses of plants. However, the hyponastic growth of leaf petioles, which is a typical response under submergence in wet and semi-wet plants, was observed in both Arabidopsis Col-0 and En-2 ecotypes (Fig. 1A, C). Given that Col-0 and En-2 are frequently used background Arabidopsis ecotypes for the production of mutants used to characterize the functions of genes, this suggests that the molecular mechanism of hyponastic growth of plants under submergence can be more precisely understood in Arabidopsis plants.

Although ethylene is known to be a key hormone for inducing hyponastic growth under submergence, how the ethylene level is maintained for hyponastic growth in a submerged leaf is poorly understood except in the case of the

up-regulation of *RpACS1* in *Rumex palustris* and *OsACS1* in *Oryza sativa* by submergence (Zarembinski and Theologis 1997; Rieu et al. 2005). In Arabidopsis, *ACS* is encoded by a multigene family. Among Arabidopsis *ACS* genes, *ACS6* and *ACS8* have the closest homologous relationship with *RpACS1* and *OsACS1*, suggesting that these *ACSs* may be involved in the production of ethylene in submerged Arabidopsis leaves. In fact, expression of both *ACSs* was clearly activated by submergence in the early stage of hyponastic growth of Arabidopsis, implying that S-adenosylmethionine can be converted into ACC by ACS6 and ACS8 in submerged Arabidopsis leaves (Fig. 2C). *ACO* is also encoded by a multigene family in Arabidopsis. Among Arabidopsis *ACO* genes, *ACO1, ACO2* and *ACO4* are known to be functional *ACOs* (Gomez-Lim et al, 1993; Raz and Ecker, 1999*)*. During submergence, expression of *ACO4* was not altered, but *ACO1* expression was greatly increased in a time-dependent manner in Arabidopsis (Fig. 2D). This indicates that ACC produced by ACS6 and ACS8 is likely to be converted mainly to ethylene by ACO1 in Arabidopsis under submergence (Fig. 3D).

The involvement of several plant hormones, such as ABA, GA and IAA, in hyponastic growth in submerged plants has been reported, suggesting that interactions between ethylene and other plant hormones are important for the hyponastic response in submerged plants (Bailey-Serres and Voesenek, 2008). In this study, we found that the hyponastic growth of submerged Arabidopsis leaves was significantly enhanced (Fig. 1), and that this enhanced hyponastic growth was dramatically reduced by application of an ethylene biosynthetic inhibitor in a BR dominant mutant, *bes1-D* (Fig. 2A, B). This indicates that BRs are involved in submergence-induced hyponastic growth as a positive regulator, and that the regulatory action of BRs is mediated by ethylene production in submerged Arabidopsis.

In the study, we found that *ACO1* was highly expressed in both submerged Arabidopsis leaves and in *bes1-D* (Fig. 2D, 3A). This implies that the enhanced hyponastic growth observed under flooding conditions occurs due to ethylene production via *ACO1* in *bes1-D*. In fact, the *bes1-Dxaco1-1* double mutant showed similar hyponastic responses as seen in *aco1-1*, demonstrating that BR-induced hyponastic growth is mediated by ACO1 in Arabidopsis (Fig. 3B). In Arabidopsis, the promoter region of *ACO1* (2 kb upstream sequences from the start codon) contains four *cis*-elements (E-boxes). EMSA and chromatin-IP assays revealed that BES1 binds directly to these E-boxes and alters *ACO1* expression in Arabidopsis (data will be published elsewhere). Therefore, in BR-induced hyponastic growth under submergence, BES1, functioning as a transcription factor in BR signaling, is likely to bind to E-boxes in the promoter region of *ACO1* to increase *ACO1* expression, resulting in the production of ethylene and the induction of the hyponastic growth of Arabidopsis leaves.

In conclusion, BRs function as activators of the hyponastic response under submergence in Arabidopsis. To increase hyponastic growth and thereby escape flooding stress, endogenous BRs are perceived by the BRI1 receptor on the plasma membrane, and BR signals are transduced to the transcription factor BES1. Then, BES1 up-regulates *ACS6* and *ACS8* followed by *ACO1*, resulting in the production of endogenous ethylene to induce hyponastic growth in submerged Arabidopsis leaves (Fig 3D). The hyponastic responses in *aco1-1, bes1-D* and *bes1-Dxaco1-1* demonstrate that BR signaling is likely to be an upstream response in ethylene-induced hyponastic growth under submergence in Arabidopsis.

Materials and Methods

Plant materials and Growth Conditions

Arabidopsis thaliana Columbia-0 (Col-0) and Enkheim-2 (En-2) were used as background wild-type plants in this study. The homozygous *ACO1* knockout mutant line, *aco1-1* (SALK_127963), was purchased from SALK. Seeds of *bes1-D* were obtained from Dr. Zhiyong Wang (Department of Plant Biology, Carnegie Institution for Science, Stanford University, CA, USA). Homozygous *bes1-Dxaco1-1* double mutant was generated through a cross of the two single mutant lines. Seeds were surface-sterilized with 70% ethanol (v/v) for 30 sec, washed with distilled water and stratified at 4°C for 3 days. Surface-sterilized seeds were planted on 0.8% agar (Phytagel; Sigma, St. Louis, MO, USA) containing 0.5X Murashige and Skoog salt medium (MS medium, Duchefa) and 1% (w/v) sucrose. The Arabidopsis seedlings were grown under a 16-h-light and 8-h-dark photoperiod at 22°C and 100 μE m^{-2} sec^{-1} in a plant growth chamber (Sanyo, Moriguchi City, Osaka, Japan) with 75% humidity. After a week of growth, seedlings were transferred to soil and grown for 3 weeks. Peat moss (Flora Gard, Berlin, Germany) and vermiculite (GFC, Seoul, Korea) mixed at a ratio of 2:1 were used in the potting soil.

Flooding Stress and Chemical Treatment

Seedlings were exposed to flooding stress in square dishes (SPL, Korea). After sowing the seeds, 7 d-old seedlings were completely submerged in distilled water or in chemical-treated distilled water for 24 h. In the case of adult plants, 27 d-old plants were imposed by placing the pot into a 7.8 L acryl box (26 × 15 × 20 cm). After fixing the pots on the bottom of acryl box using wires, the plants were completely submerged in distilled water up to a water depth of 15 cm from the soil surface. Chemicals (Brassinolide or $CoCl_2$) were added to distilled water at the concentrations described. Petiole lengths in 27 d-old Arabidopsis plants exposed to normal growth conditions or submerged for 24 h were measured. Petiole angles and lengths were measured at the end of the experiment.

Measurement of Petiole Angle and Length

Petiole angles and lengths were directly measured using a protractor

and ruler, respectively. After submergence exposure for 24 h, the angle between the soil surface and the third petiole was measured once in the water and once out of the water.

RNA Extraction and RT-qPCR

Leaf tissue and seedling samples of at least 100 mg were harvested in liquid nitrogen and homogenized using a hand grinder. RNA was extracted from the homogenized plant powder using the Qiazol Lysis Reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Total RNA from each sample was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on the iCycler iQTM detection system (Bio-Rad, USA). The PCR conditions were as follows: an initial incubation at 95ºC for 3 min (pre-denaturation), followed by 45 cycles of 95ºC for 10 sec (denaturation), 60ºC for 15 sec (annealing) and 72ºC for 15 sec (elongation). For normalization of gene expression, *UBQ5* (AT3G62250) was used as an internal control. The primer sequences used in this study are described in supplementary Table S1.

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Author's Contributions

SKK and JHY conceived and designed the experiments. JHY, SHK, JR, JEL and HSY performed the experiments. SKK and JHY analyzed the data. SKK and JHY wrote and revised the manuscript for publication.

Supporting Information

Table S1. Primer sequences used in this study.

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