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REGULAR PAPER

Loss of heterophylly in aquatic plants: not ABA-mediated stress but exogenous ABA treatment induces stomatal leaves in *Potamogeton perfoliatus*

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Abstract Heterophyllous aquatic plants produce aerial (i.e., floating and terrestrial) and submerged leaves—the latter lack stomata—while homophyllous plants contain only submerged leaves, and cannot survive on land. To identify whether differences in morphogenetic potential and/or physiological stress responses are responsible for variation in phenotypic plasticity between two plants types, responses to abscisic acid (ABA) and salinity stress were compared between the closely related, but ecologically diverse pondweeds, *Potamogeton wrightii* (heterophyllous) and *P. perfoliatus* (homophyllous). The ABA-treated (1 or 10 μM) *P. wrightii* plants exhibited heterophylly and produced leaves with stomata. The obligate submerged *P. perfoliatus* plants were able to produce stomata on their leaves, but there were no changes to leaf shape, and stomatal production occurred only at a high ABA concentration (10 μM). Under salinity stress conditions, only *P. wrightii* leaves formed stomata. Additionally, the expression of stress-responsive *NCED* genes, which encode a key enzyme in ABA biosynthesis, was consistently up-regulated in *P. wrightii*, but only temporarily in *P. perfoliatus*. The observed species-specific gene expression patterns may be responsible for the induction or suppression of stomatal production during exposure to salinity stress. These results suggest that the two *Potamogeton* species have an innate morphogenetic ability to form stomata, but the actual production of stomata depends on ABA-mediated stress responses specific to each species and habitat.

Keywords Abscisic acid (ABA) · Adaptive phenotypic plasticity · Heterophylly · 9-cis-epoxycarotenoid dioxygenase · Pondweed · Salinity stress

Introduction

Many organisms have evolved sophisticated stress response mechanisms, and have acquired the ability to alter their phenotypes to adapt to environmental changes. Bradshaw (1965) suggested that plants should be more plastic, tolerate a broader range of environmental conditions, and experience more persistent natural selection than animals. To survive exposure to various stresses, some plants adopt physiological plasticity, such as acclimation, while others exhibit morphological plasticity. The responses of aquatic plants to changing environments are examples of phenotypic plasticity with adaptive value (Minorsky 2003; Sculthorpe 1967; Wanke 2011; Wells and Pigliucci 2000).

Aquatic plants are thought to have evolved from terrestrial ancestors, and to have adapted secondarily to aquatic habitats. They include both heterophyllous and obligate submerged homophyllous (non-heterophyllous) species (Cook 1990, 1999). Heterophyllous species produce submerged and aerial leaves (i.e., floating and/or terrestrial). The structure of the aerial leaves is similar to that of land plants, while the submerged leaves are thin, elongated and lack differentiation of the stomata, cuticle and mesophyll layers. Many environmental factors, such as water stress, photoperiod, and temperature, affect heterophyllous leaf formation (reviewed by Kuwabara and Nagata 2002; Wells and Pigliucci 2000).

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The plant hormone abscisic acid (ABA) plays an important role in mediating the adaptation to stress (i.e., drought, salinity and cold), including rapid regulation of stomatal behaviors that control water loss through transpiration. It has been suggested that ABA is a long-distance signaling molecule that is continuously transported from mature leaves to developing leaves to optimize performance under the prevailing environmental changes (reviewed by Chater et al. 2014). In the ABA biosynthesis pathway, a key regulated step is catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) (Zeevaart and Creelman 1988). The NCED is encoded by a multigene family, and stress-inducible members contribute a rate-limiting step of ABA biosynthesis (Finkelstein 2013). After exposure to water deficit, ABA concentration is increased 2- to 30-fold, which lead to stress responses and activation of ABA biosynthesis pathway, in particular, the expression of NCED genes is upregulated (Iuchi et al. 2001; Qin and Zeevaart 1999).

In aquatic plants, ABA content of submerged leaves is undetectable or very low (i.e., 0–6.5 ng g^{-1} fresh weight), as compared with that of terrestrial plant leaves (i.e., 20–400 ng g^{-1} fresh weight) (Milborrow and Robinson 1973; Qin and Zeevaart 1999; Wright 1977; Zeevaart 1980). When heterophyllous plants experience osmotic stress, ABA concentrations increase and aerial leaves are generated (Goliber and Feldman 1989; Milborrow and Robinson 1973). Additionally, the application of exogenous ABA induces new leaves to grow like aerial leaves, even under submerged conditions. The effects of ABA on leaf shape and structure have been described for phylogenetically diverse heterophyllous plants, including species from the genera *Potamogeton* L., *Callitriche* L., *Hippuris* L., *Ludwigia* L., *Ranunculus* L., and *Marsilea* L. (fern) (Anderson 1978; Kuwabara and Nagata 2002; Wells and Pigliucci 2000). However, the effects of exogenous ABA have not been examined in homophyllous aquatics, which produce only submerged leaves under natural conditions.

The genus *Potamogeton* L. (monocotyledonous pondweeds; family: Potamogetonaceae) is the largest exclusively aquatic genus that includes heterophyllous and homophyllous species (Bradshaw 1965; Iida et al. 2004, 2009; Les and Sheridan 1990). Our recent study investigated the differences in phenotypic plasticity and natural habitats among sister *Potamogeton* species; heterophyllous *P. wrightii* Morong (=*P. malaianus* Miq.) grows in shallow freshwater and sometimes on land, while homophyllous *P. perfoliatus* L. cannot survive on land and grows exclusively in deeper fresh or brackish water (Iida et al. 2007, 2013). Because they are closely related, homophyllous species may have the morphogenetic ability to be heterophyllic. However, recent genomic analyses showed that the submerged seagrass *Zostera* L., which belongs to a sister family of Potamogetonaceae, has lost genes for some regulators of stomatal development, i.e., SCRM2/ICE2, SPCH, MUTE, and FAMA (Golicz et al. 2015; Olsen et al. 2016).

The purpose of this study is to identify whether differences in morphogenetic potential and/or physiological stress responses are responsible for variation in phenotypic plasticity between heterophyllous and homophyllous aquatic plants. The responses under ABA and diluted seawater treatments were compared between *P. wrightii* (heterophyllous) and its allied *P. perfoliatus* (homophyllous). Firstly, we addressed how exogenous ABA affects morphological traits and expression of genes involved in stomatal development and ABA biosynthesis. Secondary, we analyzed response to salinity stress that may activate NCED gene expression.

Materials and methods

Plant materials and stress treatments

Potamogeton wrightii (*Potamogeton malaianus* Miq. [synonym]; Japanese name: Sasabamo) and *P. perfoliatus* (Hirohanoebimo) samples were collected from Lake Biwa in Shiga, Japan (35°7′N, 135°55′E). They were maintained and propagated vegetatively using rhizomes in experimental ponds at Kobe University. For stock plants, submerged and terrestrial shoots were cultivated following the procedures of Amano et al. (2012) and Iida et al. (2007).

Submerged shoots with several rhizome segments were treated with various stresses, including ABA (Sigma-Aldrich, Tokyo, Japan), salinity [artificial seawater (SW): SEALIFE; Marinetech, Tokyo, Japan], polyethylene glycol (PEG) 6000 (Nacalai, Kyoto, Japan), and cold (5 °C water temperature). Submerged shoots were precultivated in basal medium, which consisted of 0.1 % Hoagland's solution (pH 7.0) (Hoagland and Arnon 1950), at 25 °C under a 12-h photoperiod with a light intensity of 100 μmol $m^{-2}s^{-1}$. Treatments were completed in basal medium, except for salinity stress. The culture medium was changed every second day (for ABA treatments) or every week. For long-term cultivation, rhizome segments were planted in a container filled with sand or in a rock wool block (for ABA treatments). For short-term cultivation, submerged shoots were transferred to glass bottles and treated starting at 10:30 am. After treatments, several mature and young folded leaves (including apical meristems) were harvested, frozen in liquid nitrogen, and stored at −80 °C for gene expression analyses.

Gene expression analysis

The extraction of total RNA and synthesis of cDNA were completed as previously described (Amano et al. 2012).

Potamogeton homologs of genes involved in stomatal development; a positive regulatory peptide (STOMAGEN; Sugano et al. 2010) and basic helix-loop-helix transcription factors (i.e., ICE, SPCH, MUTE, and FAMA; Pillitteri and Torii 2012), and NCEDs were identified by *de novo* sequencing of RNA from developing leaves of terrestrial shoots (*P. wrightii*) and from mature leaves of submerged shoots treated with low temperature stress (15 °C) (*P. wrightii* and *P. perfoliatus*). The RNA sequencing was completed by the Takara *de novo* RNA-seq service (Takara Bio Inc., Kusatsu, Japan). Sequences obtained in this study were deposited in the DNA Data Bank of Japan, and the gene accession numbers are provided in Supplemental Table S1. Functional annotations were based on sequence similarity searches. Gene-specific primers for reverse transcription (RT)-PCR were designed according to the obtained sequences (Table S2).

The RT-PCR and quantitative real-time RT-PCR were conducted following standard protocols (Amano et al. 2012). The RT-PCR amplification was completed with 30 cycles (for *ACTIN* and *NCED1–4*) or 35 cycles (for *NCED5*, *NCED6*, *STOMAGEN*, *ICE*, *SPCH*, *MUTE*, and *FAMA*) of 95 °C for 30 s and 58–60 °C for 30 s using gene-specific primers (Table S2). *ACTIN* (Table S1) was used as a positive internal control. All experiments were repeated at least three times using independently prepared total RNA.

Data analysis

Mature expanded leaves that developed for 30 days after each treatment were used in morphological parameter measurements. Stomatal density on the leaf surfaces was calculated as described by Iida et al. (2007). Morphological differences among cultivation conditions and real-time RT-PCR analysis of *NCED* genes were assessed by ANOVA and Tukey's multiple comparison test.

Results

Effects of exogenous ABA on stomatal production

Plants were treated with different concentrations of ABA (Fig. 1; Supplemental Fig. S1, Table S3). As early as 4 days after 10 μM ABA treatment was initiated, the main shoots exhibited a species-specific response (Fig. S1). In *P. wrightii* plants, mature leaves were damaged, but new leaves with stomata continued to emerge from the main shoot. The majority of *P. perfoliatus* mature leaves remained green. Additionally, new leaves did not emerge from the main shoot, in which the stem near the top became damaged and broke off about 10 days after 10 μM ABA treatment. Thereafter, both species produced new rhizome buds with adventive roots, which sprouted and formed new shoots bearing leaves with stomata (Fig. 1d, e). Following treatment with 10 μM ABA, the culture medium was removed and plants containing leaves with stomata were exposed to the air and cultivated under moist conditions. *Potamogeton wrightii* plants continued to produce new leaves with stomata, but *P. perfoliatus* plants withered and died within 20–30 days. Damage to pre-existing leaves and shoots was not observed in either species following treatment with 1.0 μM ABA (Fig. S1). Additionally, new leaves with stomata were produced only in *P. wrightii* plants (Fig. 1g; Table S3).

In *P. wrightii* plants, ABA treatment (1.0 or 10 μM) resulted in the production of stomata-containing leaves that resembled terrestrial leaves, with a short petiole and oblong leaf-blade (Fig. 1b, d, f, g; Table S3). Furthermore, the effects of ABA on leaf shape and stomatal density were dose-dependent (Fig. 1f, g). Increasing ABA concentrations resulted in a decrease in the ratio of leaf blade length to width (L/W ratio) because the leaf shape changed from narrow to broad. Stomata were produced after 1.0 or 10 μM ABA treatments, and stomatal density increased from 0 ± 0 to 70 ± 13 to 96 ± 17 mm^{-2} with increasing ABA concentration (i.e., 0.1, 1.0, and 10 μM, respectively). The ABA dose response of *P. perfoliatus* plants was considerably different from that of *P. wrightii* plants (Fig. 1f, g; Table S3). Regardless of ABA concentration, leaf shape and the L/W ratio remained unchanged. Stomata were produced at a density of 17 ± 4 mm^{-2} only after treatment with 10 μM ABA (Fig. 1e, g).

Because continuous exposure to ABA is required for the stable production of underwater leaves with stomata (Goliber and Feldman 1989; Hsu et al. 2001), we examined the effects of temporary ABA treatment on stomatal formation. Plants were treated with 10 μM ABA for 4 days and transferred to ABA-free basal medium (Fig. S2). In both species, leaves with stomata were only produced when exogenous ABA was present. Additionally, the morphology of newly formed leaves changed from stomata-less and submerged to stomata-containing and terrestrial-like (following ABA treatment), and then back to stomata-less and submerged (after ABA was removed).

Response of regulators of stomatal development to ABA

To confirm stomatal production in *Potamogeton* species, gene expression analyses were completed using young folded leaves (apical meristem included). *Potamogeton* homologs of genes encoding regulators of stomatal development (i.e., *PotSTOMAGEN*, *PotICE1*, *PotICE2*, *PotSPCH*, *PotMUTE*, and *PotFAMA*) were identified (Table S1; Figs. 2, S3–S5). In plants grown in freshwater conditions (without ABA), *STOMAGEN* and *MUTE* were

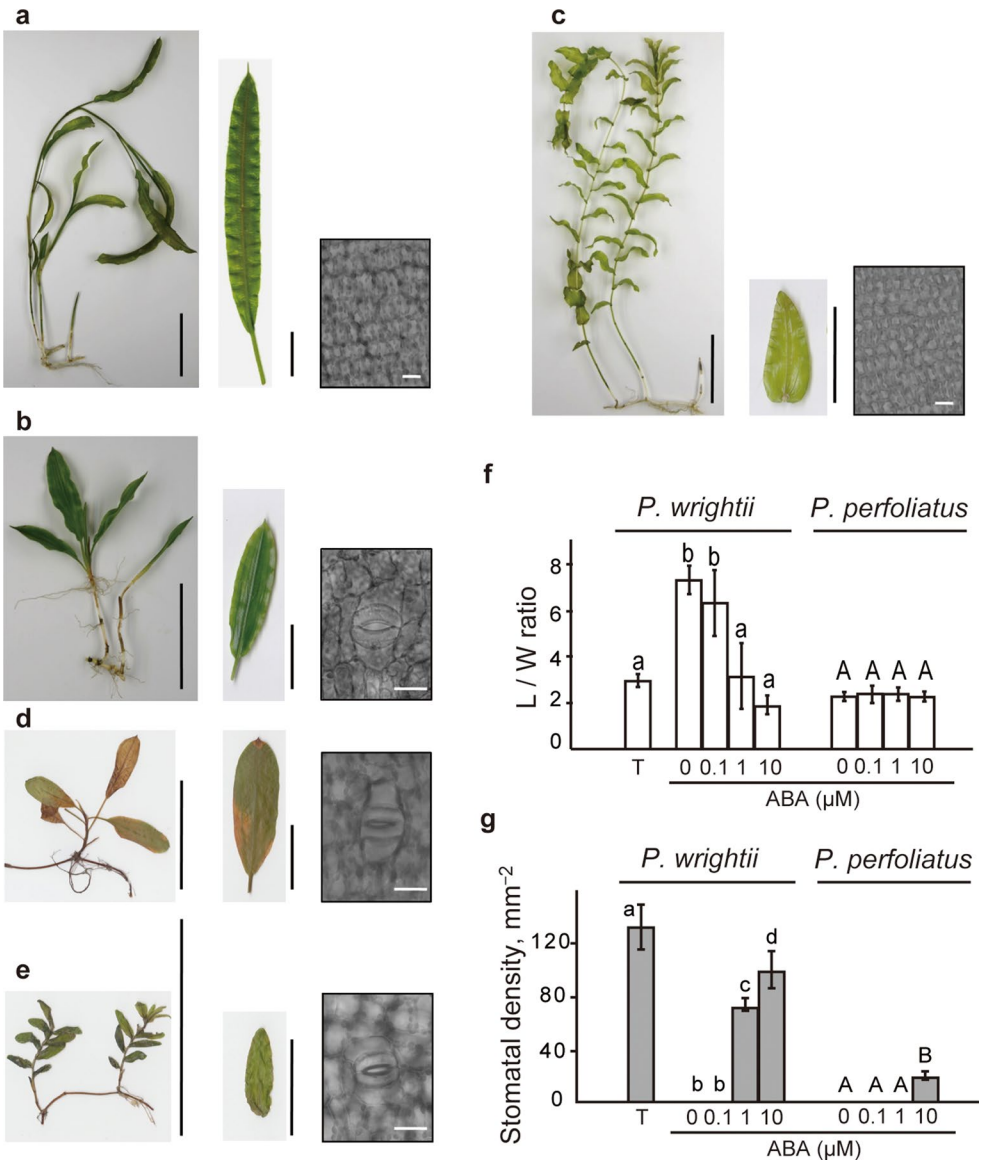

Fig. 1 Effect of exogenous abscisic acid (ABA) on morphological traits in *Potamogeton* species. Representative heterophyllous *P. wrightii* shoot, leaf, and adaxial leaf surface from underwater (control) (**a**) and terrestrial (**b**) environments. Homophyllous *P. perfoliatus* samples from underwater (control) conditions (**c**). *Potamogeton wrightii* (**d**) and *P. perfoliatus* (**e**) samples from plants cultivated in 10 μM ABA solution (for 30 days at 20 °C). Scale bars correspond to 5 cm (shoot), 2 cm (leaf), and 20 μm (leaf surface). Leaf shape determined by the leaf blade length to width ratio (L/W ratio) (**f**). Stomatal density on the adaxial leaf surface of plants cultivated for 30 days in ABA (0, 0.1, 1.0, and 10 μM) (**g**). T: data for terrestrial leaves adapted from Iida et al. (2007). Means followed by the same letter are not significantly different ($P < 0.05$; Tukey's multiple comparison test, n = 15). Error bars indicate the standard deviation

unexpressed (Fig. 2). In *P. wrightii*, *STOMAGEN* and all five transcription factor genes were expressed 2 days after ABA treatments, similar to the gene expression pattern in plants grown under terrestrial conditions (Fig. 2). The six genes were also expressed in *P. perfoliatus* treated with 10 μM ABA, and the gene expression patterns following exposure to 1 μM ABA were identical to those observed under freshwater conditions (Fig. 2).

Identification of *NCED* genes and analyses of expression

Six *NCED*-like genes were identified by *de novo* RNA sequencing, namely *PotNCED1–6* (Table S1; Figs. 3, S6–S8). Genomic PCR results revealed that the amplified fragments lacked intron sequences, which is a characteristic of *NCED* genes, for all genes except *NCED2* (Fig. S8; Tan et al. 2003). The sequences of *NCED1–3* were similar to that of *AtNCED4* (*CCD4*: carotenoid cleavage dioxygenase), which is not involved in ABA biosynthesis (Figs. S6, S7; Gonzalez-Jorge et al. 2013). The *NCED4–6* sequences were similar to those of other genes associated with ABA biosynthesis activity, including a sequence corresponding to a residue responsible for substrate specificity (Messing et al. 2010, Fig. S7).

Because ABA is synthesized and transported from mature to young leaves, the expression patterns of *NCED* genes were analyzed in mature leaves (Fig. 3). *NCED1*, *NCED2* (*P. perfoliatus*), and *NCED4* transcripts were barely detected (Fig. 3). Under osmotic stress conditions (i.e., PEG and SW treatments), *NCED2* (*P. wrightii*) and *NCED3* transcript levels decreased, whereas those of

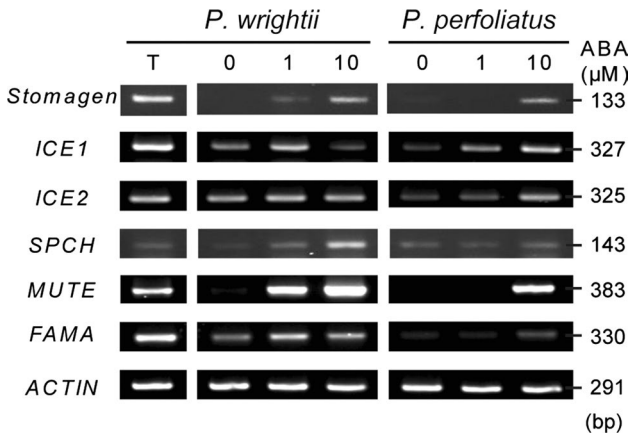

Fig. 2 Response of regulators of stomatal development to abscisic acid (ABA) in *Potamogeton* species. Total RNA was extracted from young folded leaves of *P. wrightii* terrestrial shoots (T) and submerged shoots of *P. wrightii* and *P. perfoliatus* 2 days after ABA treatment (0, 1, and 10 μM). Gene expression of regulators of stomatal development (i.e., *STOMAGEN*, *ICE1*, *ICE2*, *SPCH*, *MUTE*, and *FAMA*) were analyzed by reverse transcription PCR using gene-specific primers (Table S2). *ACTIN* served as a control. The numbers correspond to the length of specific regions. Results were consistent among three independent experiments

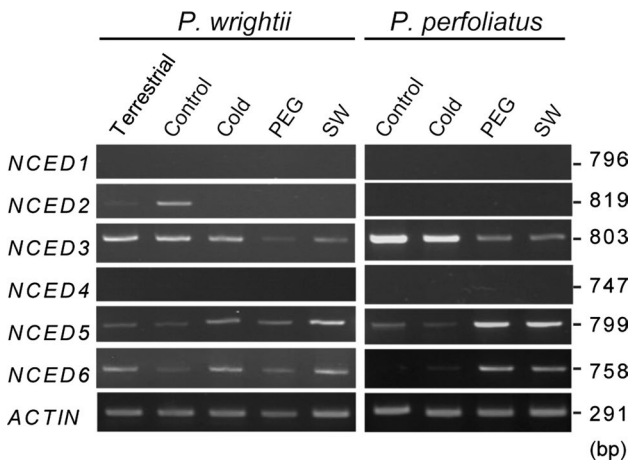

Fig. 3 *Potamogeton* species *NCED* gene expression levels in response to stress. Total RNA was extracted from mature leaves of submerged shoots 2 h after each treatment (Cold: 5 °C; PEG: 20 % polyethylene glycol 6000, solute potential: $\Psi\pi = -0.67$ MPa; SW: 1/3 strength seawater, solute potential: $\Psi\pi = -0.76$ MPa). Results for leaves from non-stressed terrestrial shoots (terrestrial) and submerged shoots (control) are also provided. The other details are the same as those of Fig. 2

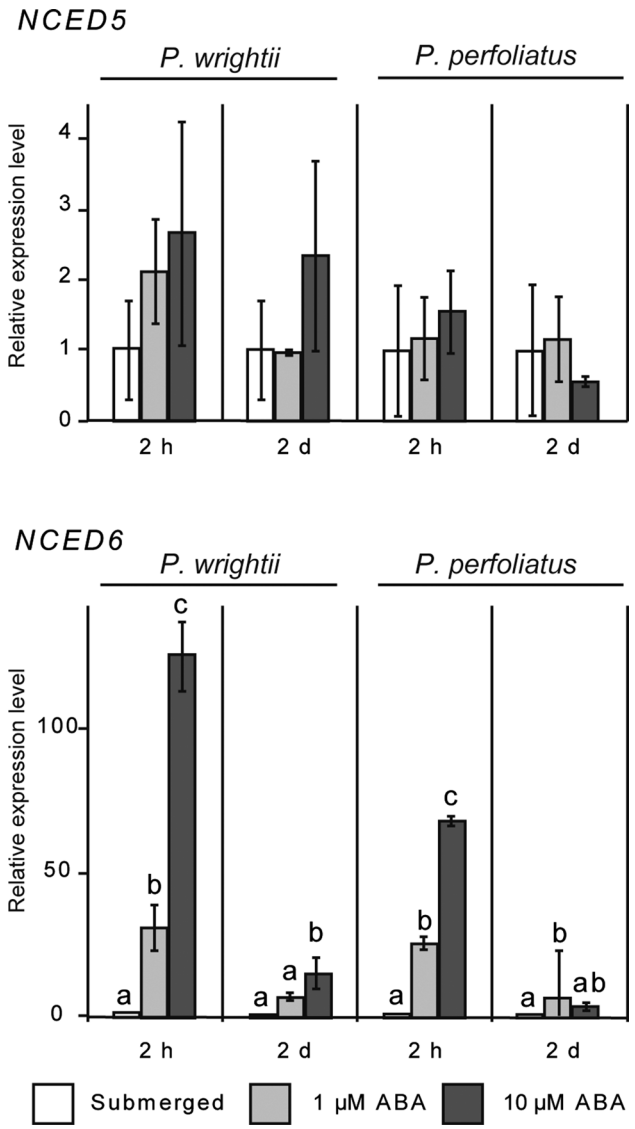

Fig. 4 Quantitative real-time PCR analyses of stress-responsive *NCED* genes in *Potamogeton* species following application of exogenous abscisic acid (ABA). Total RNA was extracted from mature expanded leaves 2 h or 2 days after ABA treatments (1 or 10 μM). Transcript expression levels were normalized to that of *ACTIN* and are provided as values relative to those of the submerged control. Data are expressed as the mean values of three individual experiments (n = 3). *Error bars* indicate the standard deviation. Significant differences among treatments were detected for *NCED6* in both species (ANOVA, $P < 0.001$). Means followed by the same letter are not significantly different (Tukey's multiple-comparison test, $P < 0.05$)

NCED5 and *NCED6* were up-regulated in both species (Fig. 3).

The expression of *NCED5* was relatively stable regardless of ABA concentration (1 or 10 μM) and treatment times (2 h or 2 days) in both species (Fig. 4). In contrast, *NCED6* expression in both species was highly induced 2 h after ABA treatment, but decreased to near control levels after 2 days (Fig. 4). Relative expression levels 2 h after treatments were positively correlated with exogenous ABA concentration (*P. wrightii*: 1 μM: 30.9 ± 7.8-fold and 10 μM: 125.5 ± 12.1-fold; *P. perfoliatus*: 1 μM: 25.4 ± 2.1-fold and 10 μM: 67.9 ± 16.5-fold) (Fig. 4).

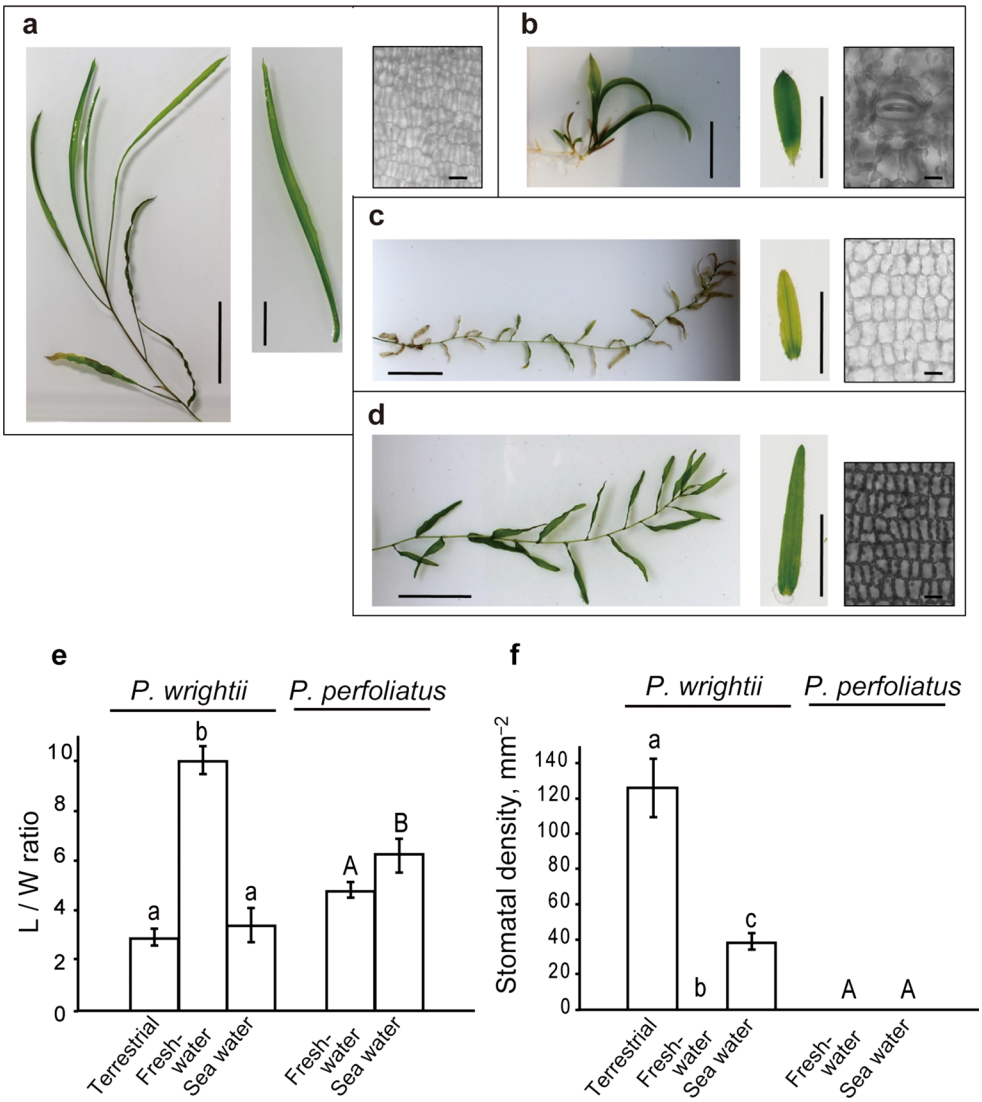

Fig. 5 Cultivation of *Potamogeton* species exposed to salinity stress. Shoot, leaf, and adaxial leaf surface of heterophyllous *P. wrightii* (**a**, **b**) and homophyllous *P. perfoliatus* (**c**, **d**) plants. Pre-existing (**a**, **c**) and newly formed shoots (**b**, **d**) were sampled following growth in artificial seawater (1/6 strength for 1 week followed by 1/3 strength for 30 days). Scale bars correspond to 5 cm (shoot), 2 cm (leaf), and 20 μm (leaf surface). Leaf shape (L/W ratio) (**e**) and stomatal density (**f**) on the adaxial surface of leaves on newly formed shoots. The other details are the same as those of Fig. 1

Comparison of the effects of salinity stress

Plants were initially cultivated in different concentrations of artificial SW (in 500 mL glass bottles). Under 1/6 strength SW (hereafter 1/6 SW), both species exhibited no obvious growth changes. This salinity level is much higher than that of the natural habitats of these species [i.e., maximum of 1/33 SW for *P. wrightii* and 1/16 SW for *P. perfoliatus*, based on Cl^- concentrations (Kadono 1982)]. The *P. wrightii* leaves were damaged, but not those of *P. perfoliatus*, following treatment with 1/3 SW for 1 week (with no pre-treatment).

Potamogeton wrightii plants pre-treated with 1/6 SW for 1–2 weeks exhibited no leaf damage following treatment with 1/3 SW (Fig. 5). Dwarf shoots with short internode stems emerged from the rhizome 2 weeks after 1/3 SW culture (Fig. 5b). The leaves on the dwarf shoots had short petioles, were smaller and rounder than normal submerged leaves, and bore stomata on the adaxial and abaxial sides. Additionally, stomatal density was lower than that of terrestrial leaves (Fig. 5b, e, f; Table S4). Salinity treatments caused pre-existing *P. wrightii* shoots to stop growing, and their leaf margins rolled inward (Fig. 5a). In contrast, salinity stress had no apparent effect on the growth of pre-existing or newly developed *P. perfoliatus* shoots (Fig. 5c, d). New leaves were narrow with a lack of stomata (Fig. 5c–f; Table S4). After exposure to salinity stress for 3 months, *P. perfoliatus* plants continued to produce submerged shoots, while *P. wrightii* shoots containing leaves with stomata exhibited delayed growth and eventually died. Because salinity stress involves ionic and osmotic components, plant responses to osmotic stress were examined using 20 % PEG 6000. The solute potential ($\Psi\pi = -0.67$ MPa) of this PEG concentration is similar to that of 1/3 SW treatment ($\Psi\pi = -0.76$ MPa). The mature leaves of *P. wrightii* were more severely damaged than those of *P. perfoliatus*

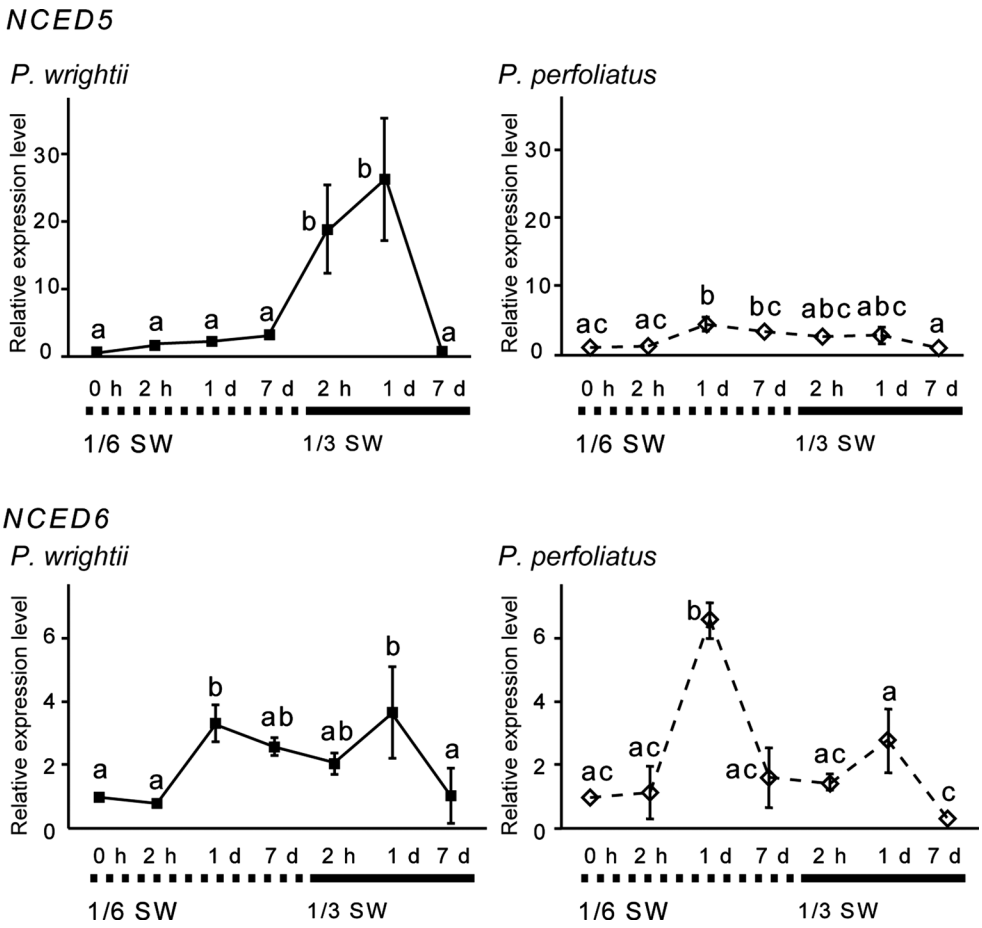

Fig. 6 Temporal stress-responsive *NCED* gene expression in *Potamogeton* species. Submerged shoots were pre-treated with 1/6 strength seawater (1/6 SW) for 1 week and transferred to 1/3 strength seawater (1/3 SW). Total RNA was extracted from mature expanded leaves at specific time points (1/6 SW: 0 h, 2 h, 1 day, and 7 days; 1/3 SW: 2 h, 1 day, and 7 days). Significant differences among treatments were detected for *NCED5* and *NCED6* in both species (ANOVA, $P < 0.001$). The other details are the same as those of Fig. 4

(Fig. S9). The PEG treatment induced the production of leaves with stomata at an average density of 47 ± 9 mm^{-2} (adaxial side, n = 5) in *P. wrightii* plants only (Fig. S9).

The expression of stress-responsive *NCED* genes was examined in mature leaves from the main shoot during the 1/6 SW pre-treatment period and from plants treated with 1/3 SW for 7 days (Fig. 6). In *P. wrightii*, *NCED5* expression increased considerably 1 day after treatment with 1/3 SW (26.2 ± 9.1-fold), whereas the *NCED 5* transcript level was consistently low (0.8–4.4-fold) in *P. perfoliatus* (Fig. 6). The expression of *NCED 6* was up-regulated in *P. wrightii* 1 day after 1/6 and 1/3 SW treatments (3.4 ± 0.6-fold and 3.7 ± 1.4-fold, respectively). In contrast, the up-regulation of *NCED6* in *P. perfoliatus* was not significant following treatment with 1/3 SW, but its expression increased 1 day after exposure to 1/6 SW (6.5 ± 0.9-fold) (Fig. 6).

Discussion

In this study, exogenous ABA application induced stomatal leaves in obligate submerged species *P. perfoliatus* as well as in heterophyllous *P. wrightii* (Fig. 1). However, under salinity stress, which often promotes ABA biosynthesis, stomata were induced in *P. wrightii*, but not in *P. perfoliatus* (Fig. 5). These results suggested that, between the two *Potamogeton* plants, differences in the ABA-mediated stress responses, but not in the morphogenetic potential, were responsible for variation in phenotypic plasticity under natural conditions.

ABA-mediated production of stomata in *Potamogeton* species

The induction of stomatal leaves by applying ABA was first reported in heterophyllous *Potamogeton nodosus* (Anderson 1978), and has since been demonstrated in a wide range of heterophyllous aquatics (Minorsky 2003). It has been shown that treatment with exogenous ABA (1.0–10 μM) effectively induces stomatal development, even in freshwater (Anderson 1978, 1982; Deschamp and Cooke 1983, 1984; Goliber and Feldman 1989; Kane and Albert 1987; Kuwabara et al. 2003; Liu 1984). To consistently produce leaves with stomata underwater, plants must be continuously exposed to a source of exogenous ABA (Goliber and Feldman 1989; Hsu et al. 2001). Even in homophyllous plants, the results of our exogenous ABA experiments are

consistent with those of previous studies (Figs. 1, S1, S2). In heterophyllous *P. wrightii* and its allied homophyllous *P. perfoliatus*, the continuous application of ABA led to the production of stomata on underwater leaves.

Although the expression patterns of regulatory genes for stomatal development were almost identical, the response to exogenous ABA differed between the two examined species (Figs. 1, 2; Table S3). In *P. wrightii*, stomata were produced by 1.0 μM of ABA treatment and the effect of exogenous ABA on leaf shape and stomatal density was dosage-dependent, but not in *P. perfoliatus* (Fig. 1; Table S3). These results indicate that *P. perfoliatus* was less sensitive to exogenous ABA. There are two possible explanations for the species-specific differences. The first is differences in positive feedback regulation of ABA. After exposure to osmotic stress, many ABA biosynthesis genes, including *NCEDs*, are up-regulated by a positive feedback mechanism, which results in the accumulation of ABA (Welsch et al. 2008; Xiong and Zhu 2003). It was found that exogenous ABA stimuli increases the expression of NCED genes in rice and certain ecotypes of *Arabidopsis*, but not in tomato and cowpea plants (Iuchi et al. 2000; Thompson et al. 2000; Welsch et al. 2008; Xiong et al. 2002). In *Potamogeton*, *NCED5* and *NCED6* were expected to be involved in ABA synthesis under abiotic stress conditions (Figs. 3, 4, 6, S6–S8). We suspect that positive feedback regulation through exogenous ABA operated insufficiently in *P. perfoliatus*. However, it was unclear whether there was a positive feedback effect on the transcription of *NCED5* in both examined species. Although the relative *NCED6* expression levels 2 h after treatments were positively correlated with exogenous ABA concentrations, the up-regulation of expression was transient (Fig. 4). The second explanation is that *P. perfoliatus* is more tolerant to ABA and thus less likely to respond to ABA. This is a more plausible explanation, because following treatment with 10 μM ABA, *P. wrightii* leaves were severely damaged, while *P. perfoliatus* leaves were relatively unaffected (Fig. S1). Additionally, the relative *NCED6* expression level was more increased in *P. wrightii* than that in *P. perfoliatus* (Fig. 4).

Previous studies of aquatic plants reported that ABA acts as an on/off switch for stomata (aerial leaf) formation depending on whether ABA is above the threshold for concentration and treatment time (Lin 2002; Wanke 2011). Although we did not measure ABA content, the consistent up-regulation of *NCED* expression in *P. wrightii* under salinity stress conditions may have resulted in the continuous accumulation of ABA, leading to the production of stomata on underwater leaves (Figs. 5, 6). In contrast, the induction of *P. perfoliatus NCED* expression was temporary, resulting in a lack of stomata (Figs. 5, 6). We conclude that heterophyllous *P. wrightii* and homophyllous *P. perfoliatus* have an innate morphogenetic ability to form stomata, but the actual production of stomata depends on ABA-mediated stress responses specific to each species.

Responses to environmental stresses and habitat specificity of *Potamogeton* species

Abscisic acid is synthesized *de novo* during exposure to osmotic stress conditions (i.e., drought, salinity, and cold), and plays important roles in stress tolerance. Submerged *P. wrightii* shoots were sensitive to salinity stress, and developed ABA-mediated morphological plasticity (Fig. 5; Table S4). Terrestrial shoots were produced in seawater, but the submerged leaves with stomata were unsuitable for photosynthesis. As revealed in other aquatic plants (Goliber and Feldman 1989), the plasticity was related to osmotic stress responses because the *P. wrightii* leaves with stomata were also formed following PEG treatment (Fig. S9). Previous studies have reported that *P. wrightii* is drought and heat tolerant and able to survive on land by producing terrestrial shoots (Amano et al. 2012; Iida et al. 2007). Similar to ABA and salinity stresses, osmotic stress during exposure to drought conditions induces phenotypic changes that enable *P. wrightii* to survive on land along the shores of freshwater bodies.

In contrast, *P. perfoliatus* is sensitive to drought and heat stress and unable to survive on land (Amano et al. 2012; Iida et al. 2007). However, it can spread in both fresh and brackish waters. Although the two *Potamogeton* species examined in this study inhabit the freshwater conditions of Lake Biwa, *P. perfoliatus* exhibited normal growth with undamaged leaves following 1/3 SW treatment. The submerged *P. perfoliatus* shoots may have an innate tolerance to salt and osmotic stress. Most plants respond to salinity-induced osmotic stress immediately after salt concentrations exceed a threshold value (approximately 40 mM NaCl; Munns and Tester 2008). Salt tolerant plants synthesize less ABA than sensitive plants, and can grow normally under medium salinity stress conditions (He and Cramer 1996; Kefu et al. 1991). The seedlings of the *AtNCED3*-deficient *A. thaliana* mutant, which is unable to accumulate ABA upon exposure to stress, grows more rapidly than wild-type seedlings following treatment with 160 mM NaCl (roughly corresponding to 1/3 SW) (Ruggiero et al. 2004). *NCED6* was up-regulated transiently (Fig. 6), suggesting that *P. perfoliatus* could rapidly acclimate to saline conditions, with no apparent growth effects or morphological plasticity. The suppression of stomatal development and preservation of submerged traits in salt tolerant *P. perfoliatus* plants is favorable for underwater life in fresh to brackish water, but not for life on land.

Terrestrial plants evolved from aquatic algae through the development of increasingly complex structures and

functions. Gaining the ability to produce stomata following exposure to abiotic stresses was a key evolutionary step that enabled plants to grow in terrestrial environments. Homophyllous aquatic plants, which permanently returned to underwater life, do not produce leaf stomata, and water and gas can pass directly across the leaf epidermis. Transitional states between exclusively aquatic or terrestrial lifestyles have been observed in heterophyllous aquatic plants that ordinarily grow submerged in water, but produce terrestrial shoots on land during seasonal drought conditions. Our findings suggest that by modifying responses to osmotic stress conditions, homophyllous plants can grow even in saline water. Such evolutionary changes may have generated ecological diversity that extended plant habitats from land to freshwater and even to marine environments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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