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Differences in the stimulation of cyclic electron flow in two tropical ferns under water stress are related to leaf anatomy

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Cyclic electron flow (CEF) plays an important role in photoprotection for angiosperms under environmental stresses. However, ferns are more sensitive to drought and their water transport systems are not as efficient as those of angiosperms, it is unclear whether CEF also contributes to photoprotection in these plants. Using *Microsorum punctatum* and *Paraleptochillus decurrens*, we studied the electron fluxes through both photosystem I (PSI) and photosystem II (PSII) under water stress and their leaf anatomies. Our goal was to determine if CEF functions in the photoprotection of these ferns and, if so, whether CEF stimulation is related to leaf anatomy. Compared with *P. decurrens*, *M. punctatum* had thicker leaves and cuticles and higher water storage capacity, but lower stomatal density and slower rate of water loss. During induced drought, the decrease in leaf water potential (Ψ_{leaf}) was more pronounced in *P. decurrens* than in *M. punctatum*. For both species, the decline in Ψ_{leaf} was associated with a lower effective PSII quantum yield, photochemical quantum yield of PSI and electron transport rate (ETR), whereas increases were found in the quantum yield of regulated energy dissipation, CEF and CEF/ETR(II) ratio. Values for CEF and the CEF/ETR(II) ratio peaked in *M. punctatum* at a light intensity of 500–600 μmol m^{-2} s^{-1} vs only 150–200 μmol m^{-2} s^{-1} in *P. decurrens*. Therefore, our results indicate that the stimulation of CEF in tropical ferns contributes to their photoprotection under water stress, and is related to their respective drought tolerance and leaf anatomy.

Introduction

Ferns (Pteridophytes) are important components of forest floras, having critical functions in ecosystem processes, especially in tropical rain forests (Watkins and Cardelús 2009). Compared with angiosperms, ferns have primitive vascular networks, smaller vessel and tracheid diameters, and lower stomatal densities (Carlquist and Schneider 2001). These traits are associated with lower water transport capacity and photosynthetic rates

Abbreviations – CEF, cyclic electron flow; DW, dry weight; ETR, electron transport rate; FAA, formaldehyde, acetic acid and alcohol; FW, fresh weight; LMA, leaf mass per unit area; LSD, least significant difference; NPQ, non-photochemical quenching; PPFD, photosynthetic photon flux density; PSI, photosystem I; PSII, photosystem II; RWC, relative water content; TW, turgid weight; XTBG, Xishuangbanna Tropical Botanical Garden.

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compared with angiosperm species (Brodribb et al. 2005, Watkins et al. 2010). Consequently, many ferns tend to occur in moist habitats under forest canopies. In tropical regions, epiphytic ferns are especially conspicuous component of wet forests (Li et al. 1996, Watkins and Cardelús 2009). Because epiphytic ferns grow on tree trunks, any periodic water deficits combined with high-light levels can affect their photosynthesis and growth (Hietz and Briones 1998). Li and Zhu (2005) have found that the size of a fern population in tropical monsoon forests is related to the relative air humidity and soil moisture. Ferns are very sensitive to disturbances in microclimate, especially changes in water availability (Hietz and Briones 1998). In fact, global climate change has increased the frequency and intensity of droughts in many areas of the world. Thus, the 385-million-year struggle by trees for light may now become a struggle for water (Hartmann 2011). Although investigating the photoprotection mechanisms of ferns under drought is essential for predicting ecosystem functioning, studies on the ecology of epiphytic ferns have been especially lacking (Watkins and Cardelús 2009, McElwain 2011).

Water stress is a major factor determining the productivity, growth and photosynthesis of terrestrial plants because a wide variety of growth-related processes is affected by water deficits (Flexas et al. 2004, Cai et al. 2010). Mild water stress inhibits photosynthetic carbon gain mainly through stomatal limitations, but non-stomatal factors may also hinder photosynthesis as drought worsens (Golding and Johnson 2003, Cai et al. 2010). By decreasing their rates of photosynthesis during droughty periods, plants may absorb more light energy than that can be consumed via photosynthetic carbon assimilation, leading to an over-reduction in the electron transport chain (Chow and Aro 2005, Sonoike 2006, Galmés et al. 2007). Both photosystem I (PSI) and photosystem II (PSII) can show photoinhibition under chilling and high-light stress (Barth et al. 2001, Zhang and Scheller 2004). Several mechanisms, for example, the anti-oxidative scavenging system, xanthophyll cycle and cyclic electron flow (CEF) around PSI, may be involved in protecting photosystems (Barth et al. 2001, Galmés et al. 2007, Huang et al. 2012). Lehtimäki et al. (2010) have suggested that the upregulation of components involved in ferredoxin-dependent CEF is an important mechanism for protecting PSI and PSII under drought stress.

Stimulation of CEF is an important protective mechanism against the photoinhibition of PSII under environmental stress (Golding and Johnson 2003, Johnson 2011, Huang et al. 2012). In doing so, CEF can generate a proton gradient across the thylakoid membrane to support non-photochemical quenching (NPQ) and protect the oxygen-evolving complex against photodamage (Munekage et al. 2002, Takahashi et al. 2009). In addition, CEF is a crucial means for protecting PSI from excess light stress by alleviating over-reduction on the PSI acceptor side and the over-accumulation of NADPH (Munekage et al. 2002, Huang et al. 2012). Takahashi et al. (2009) have suggested that the CEF-dependent generation of ΔpH across the thylakoid membrane helps to mitigate photoinhibition through at least two mechanisms: (1) thermal energy dissipation (qE), which blocks the inhibition of repairs to photodamaged PSII during protein synthesis or (2) suppression of photodamage to PSII through action that is independent of qE. Drought stress can accelerate the rate of $P700^+$ re-reduction, which indicates that cyclic electron transfer has occurred (Golding and Johnson 2003, Lehtimäki et al. 2010). However, no research has previously focused on the role of CEF in the photoprotection of ferns under various environmental stresses.

In addition to physiological adjustments, plants can alter their leaf structures when adapting to water deficit (Hietz and Briones 1998, Burghardt and Riederer 2003, Peña-Rojas et al. 2005). For example, ferns growing under more exposed conditions have thicker leaves, more but smaller stomata, stiffer cell walls and a higher turgor loss point (Hietz and Briones 1998). The potential transpirational demand by plants is primarily determined by both stomatal aperture and density. Water deficits can lead to an increase in stomatal density and a decrease in stomatal size (Xu and Zhou 2008). Plants with less densely distributed stomata usually can tolerate more arid environments than those with a higher density (Kebede et al. 1994). The cuticle is very hydrophobic or 'water-repelling', having a hydrophobic and flexible membrane composed of cutin and associated solvent-soluble lipids. One of its functions is to prevent water loss from the leaf interior (Helbsing et al. 2000). Specific cuticular properties and leaf structures are often correlated with transpirational demand in different habitats (Helbsing et al. 2000, Hao et al. 2010). For example, leaves tend to be thicker on plants growing on more arid sites (Cunningham et al. 1999). Because leaf structure plays a critical role in modulating the trade-off between the capacity for leaf water flux and tolerance to drought (Hao et al. 2010), it is reasonable to speculate that leaf anatomy affects photosynthetic processes and might be associated with CEF stimulation.

Here, we measured the chlorophyll *a* fluorescence of PSII and P700 of PSI in two tropical ferns at different intervals between the time that drought was introduced and re-watering began. *Microsorum punctatum* and *Paraleptochillus decurrens* grow in

limestone or montane forests (500–900 m elevation) in southwestern Yunnan Province, China. The former is a bark epiphyte or lithophyte while the latter grows mainly in soil and rarely on tree trunks (Li et al. 1996). These two ferns have significantly different leaf anatomies that can influence their physiological adaptation to water stress. We hypothesized that, although these species share similar habitats, their differences in leaf anatomy would affect their responses to drought treatment. Therefore, they were selected for an investigation of the role of CEF in photoprotection under water stress and to determine whether CEF stimulation is associated with leaf anatomy. We proposed that CEF would not be stimulated by such treatment because both species originated from tropical forests that are characterized by low levels of light.

Materials and methods

Plant materials

Plants of *M. punctatum* and *P. decurrens* (20 each) were collected from a montane forest at Xishuangbanna Tropical Botanical Garden (XTBG, 21°41′N, 101°25′E; altitude 570 m). They were grown at an XTBG greenhouse in plastic pots containing a bark mixture. The pots were shaded with nylon netting to allow about 20% full sunlight (300–400 μmol m^{-2} s^{-1}). The sampled region has a typical tropical monsoon climate, with a distinct dry season that occurs from November to April. Mean annual total precipitation is approximately 1560 mm, of which about 80% falls during the rainy season (May to October). The mean annual temperature is 21.7°C, ranging from 15.9 to 25.7°C during the year.

Plants were irrigated as needed to avoid water stress, and were fertilized once a month. After being cultivated for 6 months to recover their normal photosynthetic rate and water status, the experimental period began. First, leaf water potential, chlorophyll *a* fluorescence and P700 were recorded for these well-watered plants (D0). Afterward, irrigation was stopped for 8 days before re-commencing. The same measurements were made on days 2 (D2), 4 (D4), 6 (D6) and 8 (D8) after water stress was introduced, and on days 1 (R1) and 3 (R3) after re-watering began.

Physiological measurements

Pre-dawn water potential of the leaf (Ψ_{leaf}) was determined from five fronds per species per treatment, using a PMS 1000 pressure chamber (PMS Instruments Inc., Corvallis, OR).

Chlorophyll *a* fluorescence and P700 measurements were simultaneously recorded on each measuring date, using a dual channel fluorimeter (PAM-100; Heinz Walz, Effeltrich, Germany) that was connected to a computer with control software. Minimum fluorescence (F_o) and maximum fluorescence (F_m) were obtained from calculations of pre-dawn measurements from five dark-adapted leaves per species. After F_o was determined under weakly modulated light, a 0.8-s saturating light of 8000 μmol m^{-2} s^{-1} was shone on the dark-adapted leaves to determine F_m. Those leaves were then marked for gathering data for light-response curves after they were first light-adapted (300 μmol m^{-2} s^{-1}) for at least 20 min. Light-adapted fluorescence parameters were recorded from the same leaf after 2 min of exposure to increasingly greater photosynthetic photon flux density (PPFD; starting at 15 μmol m^{-2} s^{-1} and rising to 22, 31, 62, 104, 135, 225, 348, 540 and, finally, 834 μmol m^{-2} s^{-1}).

Fluorescence parameters for PSII were calculated as follows: maximum quantum yield of PSII $(F_v/F_m) = (F_m - F_o)/F_m$; coefficient of photochemical quenching $(qP) = (F_m' - F_s)/(F_m' - F_o')$; coefficient of NPQ $(qN) = (F_m - F_m')/(F_m - F_o')$; and effective PSII quantum yield $[Y(II)] = (F_m' - F_s)/F_m'$ (Genty et al. 1989). The terms F_o and F_o' represented the minimum fluorescence in the dark-adapted and light-adapted states, respectively, while F_m and F_m' indicated the dark-adapted and light-adapted maximum fluorescence, respectively. Term F_s was the light-adapted steady-state fluorescence and Y(NO) reflected the fraction of energy that was passively dissipated in the form of heat and fluorescence (calculated as F_s/F_m). A high Y(NO) value indicated the inability of a plant to protect itself against damage by excess light energy. Term Y(NPQ) corresponded to the fraction of energy dissipated in the form of heat via the regulated photoprotective NPQ mechanism, calculated as $F_s/F_m' - F_s/F_m$ (Kramer et al. 2004). The electron transfer rate of PSII was determined as $ETR(II) = L_{abs} \times PPFD \times 0.5 \times Y(II)$. Leaf absorbance ($L_{abs}$) was measured with a portable spectrometer attached to an integrated sphere (USB4000; Ocean Optics, Dunedin, FL). The values for L_{abs} from *M. punctatum* and *P. decurrens* were 0.911 and 0.902, respectively. Maximum electron transport rate [ETR(II)] of each leaf was estimated by a non-rectangular hyperbola (Prioul and Chartier 1977).

The P700 redox state was measured by a Dual PAM-100 with a dual wavelength unit (830/875 nm), per the method of Klughammer and Schreiber (2008). Saturation pulses (8000 μmol m^{-2} s^{-1}) were applied for assessing P700 parameters. The maximum change in P700 at the fully oxidized state (P_m) was determined by applying a saturation pulse after pre-illumination with far-red light. As a defined optical property, the amplitude of P_m depended upon the maximum amount of photo-oxidizable P700, which reflected the quantity of an

efficient PSI complex. The maximum change in P700 in a given light state (P_m') was determined similarly to that for P_m, except that background actinic light was used instead of far-red illumination. The photochemical quantum yield of PSI, Y(I), was defined as the fraction of overall P700 that was reduced and not limited by the acceptor side in a given state, calculated as $(P_m' - P)/P_m$. The non-photochemical quantum yield of PSI, [Y(ND)], was calculated as $1 - P700_{red}$, indicating the fraction of overall P700 that was oxidized in a given state. That yield can be enhanced by a trans-thylakoid proton gradient and photodamage to PSII. Term Y(NA), representing the fraction of overall P700 that could not be oxidized by a saturation pulse in a given state due to a lack of acceptors, was calculated as $(P_m - P_m')/P_m$. The electron transfer rate of PSI [ETR(I)] was calculated in the same manner as for ETR(II). Finally, CEF around PSI was estimated from the difference in electron flow between PSI and PSII (Miyake et al. 2005).

The leaf transpiration rate and stomatal conductance under normal irrigation were measured from fully developed leaves after 15 min of light induction (PPFD: 300 μmol m^{-2} s^{-1}) between 10:00 and 11:00 h on a clear day using a Li-Cor 6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE).

Leaf relative water content (RWC) is commonly used to assess plant water status. Here, six mature leaves per species were collected from different plants in the evening, and were immediately weighed [fresh weight (FW)]. After they were floated in distilled water inside a closed petri dish for 12 h, residual water was wiped from their leaf surfaces with tissue paper and they were re-weighed to determine turgid weight (TW). Their leaf areas were then measured with a Li-Cor 3000A area meter. Finally, the samples were oven-dried at 70°C for 48 h to obtain dry weight (DW). RWC (%) was calculated as $[(FW - DW)/(TW - DM)] \times 100$. The degree of sclerophylly was calculated as DW/area, and the degree of leaf succulence was expressed as the water content per unit leaf area [(FW − DW)/area] (Burghardt and Riederer 2003).

Water loss curves were created by progressively drying the leaves and measuring their masses at intervals (Burghardt and Riederer 2003). As described above, six mature leaves per species were collected from different plants in the evening. Values for FW were immediately recorded and TW was then obtained after the samples were floated in distilled water for 12 h. Afterwards, their petioles were sealed with paraffin wax, and the leaf samples were maintained under dim light (around 20 μmol m^{-2} s^{-1}) at room temperature. During this dehydrating process, weights were recorded every 30 min. At the end of this experiment, leaf areas were determined before the leaf samples were oven-dried at 70°C for 48 h to obtain their DW values. Linear regression equations were fitted to plots showing the amount of water lost per unit leaf area vs time. Rates of water loss (g min^{-1} m^{-2}) were obtained from the slopes of the graph (Helbsing et al. 2000).

Observations of leaf anatomy

The adaxial and abaxial epidermises from the middle portion of mature fronds were peeled from fresh leaves, and images were taken with a digital camera (DFC 295; Leica, Solms, Germany) mounted on a microscope (DM 2500; Leica). We counted the stomata from 60 randomly selected fields per species to determine their densities. The lengths and widths of their guard cells were recorded as stomatal lengths and widths.

Tissues from mature leaves were fixed in a solution of FAA (formaldehyde, acetic acid and 70% alcohol; 5:5:90, v:v:v) for 1 day. They were then dehydrated in an alcohol series and embedded in paraffin wax for sectioning. Transverse sections were imaged with the DFC 295 digital camera mounted on our DM 2500 microscope. Five slides per species were imaged at 10× or 20× magnification, and 10 images were taken per slide. Thicknesses of the leaf, mesophyll and cuticle were measured via the ImageJ program (http://rsb.info.nih.gov/ij/).

Statistical analysis

Statistical analysis was performed with SPSS 13.0 (SPSS Inc., Chicago, IL). Differences in physiological parameters among treatments were tested by one-way ANOVA and LSD (least significant difference) multiple-comparison tests, while differences in physiological parameters and leaf anatomy between species were assessed by independent *t*-tests.

Results

Leaf structure and stomatal traits differed significantly between species (Fig. 1, Table 1). *Microsorum punctatum* had significantly fewer stomata per mm^2 on the leaf epidermis (46.3 ± 0.6) compared with *P. decurrens* (56.0 ± 2.4) ($P < 0.01$), but stomatal area from the former were significantly larger (1539.0 ± 16.7) than from the latter (1330.5 ± 16.0) ($P < 0.001$). However, stomatal conductance and transpiration rate did not differ significantly between species. Leaves of *M. punctatum* had a comparatively thicker mesophyll, cell walls, cuticle and LMA. Although RWC values were similar between species under normal irrigation, *M. punctatum*

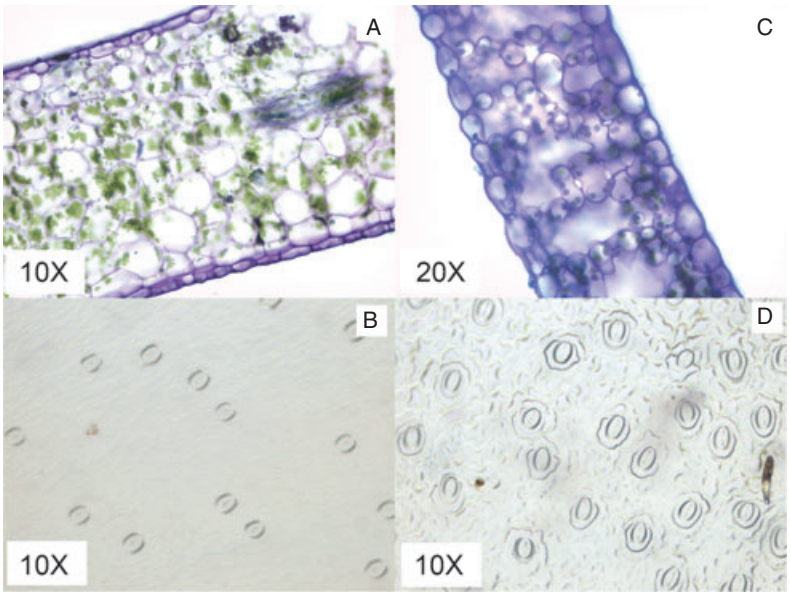

Fig. 1. Stomatal anatomy. (A) Leaf transverse section of *Microsorum punctatum*; (B) Stomata on lower leaf surface of *M. punctatum*; (C) Leaf transverse section of *Paraleptochillus decurrens*; (D) Stomata on lower leaf surface of *P. decurrens*.

Table 1. Comparisons of stomatal and leaf traits for *Microsorum punctatum* and *Paraleptochillus decurrens*. Values are means ± 1 SE.

	Microsorum punctatum	*Paraleptochillus decurrens*	*t*	*P*
Stomatal length (μm)	51.77 ± 0.43	54.22 ± 0.47	3.845	<0.001
Stomatal width (μm)	37.86 ± 0.24	31.25 ± 0.24	19.897	<0.001
Stomatal area (μm^2)	1539.0 ± 16.7	1330.5 ± 16.0	9.023	<0.001
Stomatal density (number, mm^{-2})	46.33 ± 0.58	56.00 ± 2.40	3.920	<0.01
Leaf thickness (μm)	570.5 ± 8.2	234.9 ± 2.2	39.494	<0.001
Mesophyll thickness (μm)	507.2 ± 7.5	186.5 ± 2.0	41.209	<0.001
Cell wall thickness (μm)	3.763 ± 0.070	2.116 ± 0.037	20.820	<0.001
Cuticle thickness (μm)	1.74 ± 0.03	1.05 ± 0.03	15.830	<0.001
Leaf mass per unit area ($g\ m^{-2}$)	86.99 ± 4.61	55.47 ± 2.12	6.217	<0.001
Degree of succulence ($g\ m^{-2}$)	487.7 ± 26.3	153.9 ± 1.5	11.656	<0.001
Degree of sclerophylly ($g\ m^{-2}$)	88.81 ± 4.30	55.47 ± 2.12	6.585	<0.001
Relative water content (%)	91.25 ± 1.85	93.04 ± 0.99	0.858	>0.05
Water loss rate ($g\ min^{-1}\ m^{-2}$)	0.050 ± 0.004	0.069 ± 0.002	4.210	<0.01
Stomatal conductance ($mol\ m^{-2}\ s^{-1}$)	0.064 ± 0.007	0.056 ± 0.003	0.971	>0.05
Transpiration rate ($mmol\ m^{-2}\ s^{-1}$)	0.593 ± 0.054	0.721 ± 0.038	1.931	>0.05

had a higher degree of succulence and sclerophylly than *P. decurrens*. Its rate of water loss was also slower (Fig. 2, Table 1). After 32 h of natural desiccation, the leaf RWC decreased from 100 to 77.1% for *M. punctatum* vs 100 to 15.4% for *P. decurrens*.

Pre-dawn leaf water potential (Ψ_{leaf}) decreased over the stress period for both species, but the magnitude of change was more pronounced for *P. decurrens* (Fig. 3). For example, values for Ψ_{leaf} from *M. punctatum* and *P. decurrens* decreased from −0.57 to −0.65 MPa, respectively, before drought was induced to −1.63 and −3.61 MPa, respectively, on day 8 of treatment. The maximum quantum yield of PSII (F_v/F_m) varied with Ψ_{leaf}. Compared with the pre-treatment status, values for F_v/F_m in the two ferns declined significantly on days 6 and 8, respectively, of the drought period. These results indicated that severe water stress resulted in photoinhibition in both species.

Light-response curves showed that qP, Y(NO) and Y(II) in the PSII of *M. punctatum* and *P. decurrens* decreased with increasing PPFD for all treatments, while qN and Y(NPQ) increased with PPFD (Fig. 4). However, the maximum qN peaked at 150 μmol $m^{-2}\ s^{-1}$ PPFD for *P. decurrens*, but at 600 μmol $m^{-2}\ s^{-1}$ for *M. punctatum*. As PPFD increased, the reduction in Y(II) was more rapid in *P. decurrens* than in *M. punctatum*. Furthermore, qP

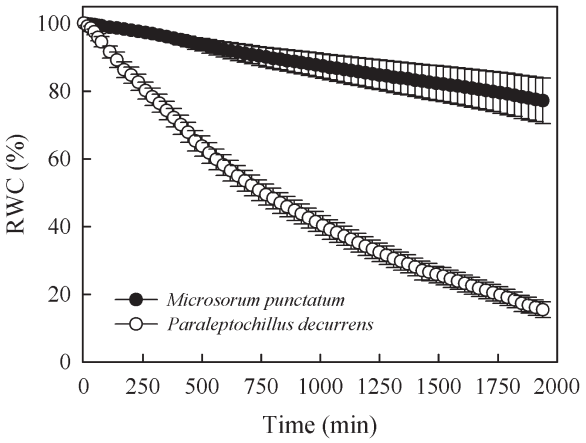

Fig. 2. Water loss curves for leaves of *Microsorum punctatum* and *Paraleptochillus decurrens*. Each point represents mean ± 1 SE for five measurements from different leaves.

and Y(II) for both species declined as stress became prolonged but then recovered considerably after re-watering began. In contrast, qN and Y(NPQ) showed the reverse trend. In PSI, Y(NA) and Y(I) in *M. punctatum* and *P. decurrens* decreased with higher PPFD while Y(ND) increased (Fig. 5). Compared with pre-stress readings, both Y(NA) and Y(I) for these two ferns declined markedly by day 8 after treatment began.

ETR(I), ETR(II), CEF and the CEF/ETR(II) ratio increased with PPFD for both species (Fig. 6). Whereas ETR(I) and ETR(II) were downregulated during water stress (Fig. 7), values for those parameters were higher in *M. punctatum* regardless of treatment. Although there were some differences in light intensities at which the maximum ratio of CEF to ETR(II) occurred among treatments, the highest ratio was observed at 500–600 μmol m^{-2} s^{-1} PPFD for *M. punctatum* and at 150–200 μmol m^{-2} s^{-1} PPFD for *P. decurrens* (Fig. 6). In addition, the induction times required to achieve that maximum ratio were shorter in *P. decurrens* (Figs 6 and 7).

Discussion

Our results revealed that CEF is important for enabling the photoprotection of two tropical fern species under water stress. The faster stimulation of CEF in stressed *P. decurrens* than in *M. punctatum* was linked to their differences in leaf structures and drought tolerance.

Correlation of leaf structure with drought tolerance

Plants must minimize their rate of water loss in order to be drought tolerant (Burghardt and Riederer 2003). This loss can occur through either the stomata or cuticle. Although stomatal size and density differed significantly between *M. punctatum* and *P. decurrens* (Table 1), stomatal conductance and transpiration rate did not when plants received normal irrigation. Saadu et al. (2009) also have reported no positive correlation between stomatal size and transpiration rate in tropical tuber species. Consequently, we cannot conclude that stomatal traits can be used as a factor in predicting drought tolerance by our two fern species.

Not only did the leaves of *M. punctatum* have thicker mesophyll, cell walls and cuticle, but they also showed a greater degree of succulence and sclerophylly (Table 1). This indicated that *M. punctatum* had a higher capacity to store water than did *P. decurrens*. The rate of water loss was also lower from the leaves of *M. punctatum* (Fig. 2). Watkins et al. (2007) have suggested that gametophyte morphology influences water-holding capacity in ferns. A leaf with a high LMA has a greater ability to store water and maintain more stable leaf hydraulic functioning during droughty periods (Bucci et al. 2004). The cuticle can efficiently inhibit water loss from the leaf interior because its properties are generally correlated with the transpirational demand associated with different habitats (Helbsing et al. 2000, Hao et al. 2010). Both leaves and epidermises tend to become thicker as local aridity increases (Cunningham et al. 1999). Because the stomata generally close within the first 30 min after a leaf is detached, water loss from a detached leaf is mainly regulated by leaf epidermal conductance (Muchow and Sinclair 1989). Here, the thicker cuticle from *M. punctatum* resulted in a slower loss of water. Along with that, its greater capacity for water storage contributed to more drought tolerance than was displayed by *P. decurrens*.

The role of CEF in protecting photosystems under water stress

Values for ETR(I) and ETR(II) in *P. decurrens* and *M. punctatum* decreased during water stress (Figs 6 and 7), indicating that drought conditions caused electron transport to be downregulated. These findings are consistent with those previously reported that linear electron flow around PSII is diminished under such stress (Cornic 1994, Golding and Johnson 2003, Galmés et al. 2007). We suggest that this response was due to a decrease in qP and the downregulation of effective quantum yields for both PSI and PSII. The large decline in qP under drought showed that more electrons returned immediately to PSI to reduce $P700^+$ and other components of the inter-system while the greater abundance of electrons in the intermediates

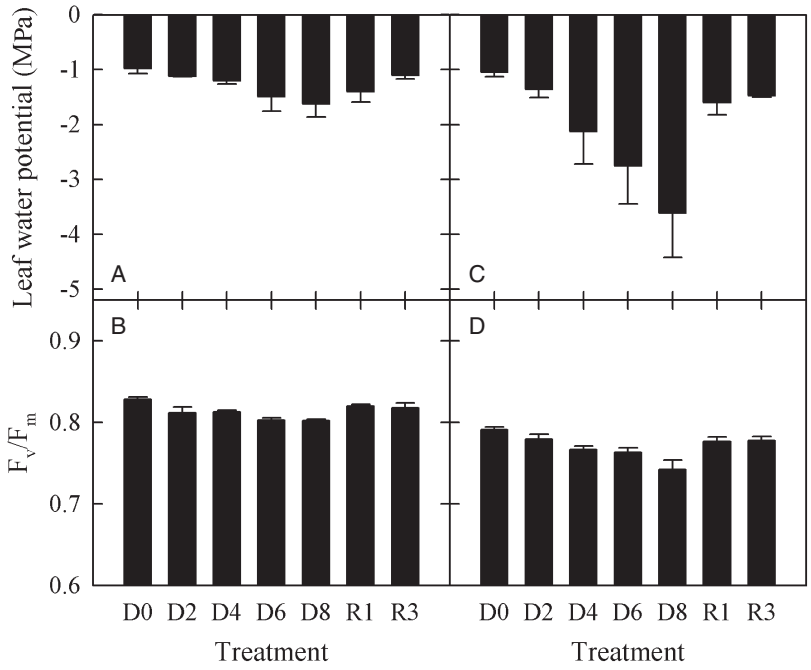

Fig. 3. Changes in leaf water potential and maximum quantum efficiency of PSII (F_v/F_m) in *Microsorum punctatum* (A, B) and *Paraleptochillus decurrens* (C, D) during drought stress and re-watering. Vertical bar indicates error of mean for five measurements from different plants.

between those photosystems minimized the functioning of the primary quinone electron acceptor of PSII (QA). Drought stress inhibits photosynthetic carbon fixation through either stomatal or non-stomatal limitation (Cornic 1994, Cai et al. 2010). In our study, this downregulation of electron flow in the two ferns was due to stomatal limitations, caused by an increase in leaf water potential during the early stage of water stress. This was evidenced by the steady values calculated for F_v/F_m (Fig. 2). However, that maximum quantum yield declined significantly during the late stage of stress, suggesting that photoinhibition could be an important factor limiting electron transport.

CEF in the two ferns was stimulated by either high or low light levels associated with drought. Lehtimäki et al. (2010) have also suggested that drought stress can accelerate the rate of $P700^+$ re-reduction, which may indicate induction of CEF. Over-reduction of the electron transport chain and photoinhibition often occurs under environmental stress when the light energy absorbed by a plant exceeds consumption via photosynthetic carbon assimilation (Chow and Aro 2005, Sonoike 2006, Galmés et al. 2007). Although the inhibition of linear electron flow can increase the risk of PSII photoinhibition, previous studies have shown that CEF around PSI is an alternative mechanism protecting PSII from photoinhibition (Golding and Johnson 2003, Sonoike 2006, Huang et al. 2012). For example, Golding and Johnson (2003) have proposed that CEF stimulates the formation of ΔpH and activates NPQ. This is based on their findings that linear electron flow, dependent on the generation of a proton gradient across the thylakoid membrane (i.e. ΔpH), is restricted under severe drought when associated with high light. NPQ can dissipate excess light energy in PSII under water stress (Galmés et al. 2007). However, NPQ is dependent on the formation of a proton gradient across that thylakoid membrane. Stimulation of CEF increases that ΔpH gradient, thereby activating NPQ and protecting PSII against excess excitation pressure (Munekage et al. 2002). The CEF-dependent building up of ΔpH not only serves for photoprotection but also assists in ATP synthesis (Munekage et al. 2004). Drought stress induces a greater demand for ATP. This possibly results in a higher NADPH/ATP ratio that can activate the NDH-mediated cyclic electron pathway, thus helping to dissipate excess energy and provide additional ATP to maintain active CO_2 fixation (Rumeau et al. 2007). In our experiments, CEF stimulation was accompanied by the significant stimulation of NPQ, indicating that CEF in our two ferns contributed to the formation of a high ΔpH gradient across the thylakoid membrane as well as enhanced photoprotection.

CEF around PSI is also thought to protect it by preventing over-reduction of its acceptor side and by maintaining a pH gradient across the thylakoid

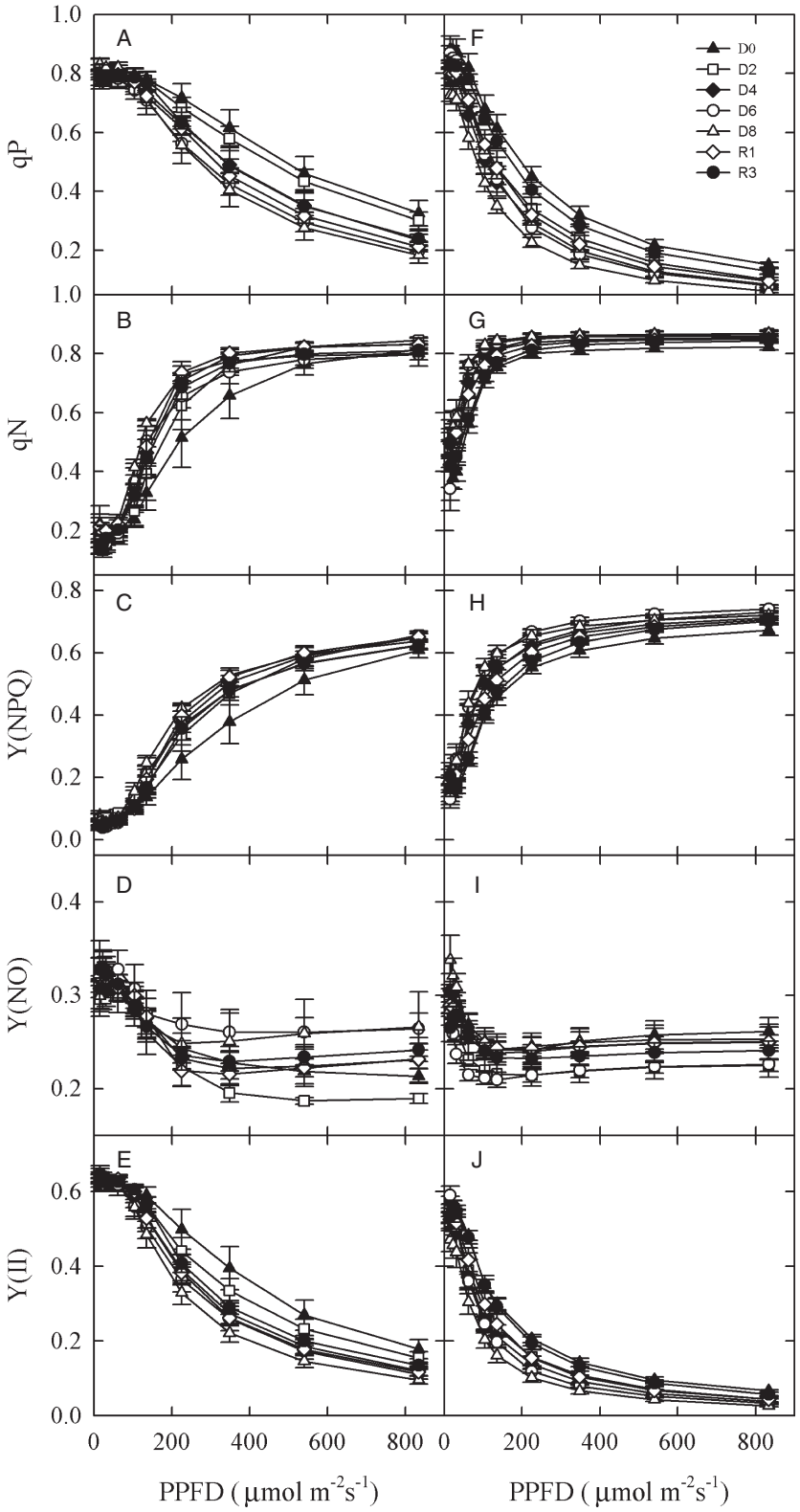

Fig. 4. Light-response changes in coefficient of photochemical quenching (qP), coefficient of NPQ (qN), quantum yield of regulated energy dissipation [Y(NPQ)], quantum yield of non-regulated energy dissipation [Y(NO)] and effective PSII quantum yield [Y(II)] in *Microsorum punctatum* (A–E) and *Paraleptochillus decurrens* (F–J) during drought stress and re-watering. Each point represents mean ± 1 SE for five measurements from different plants.

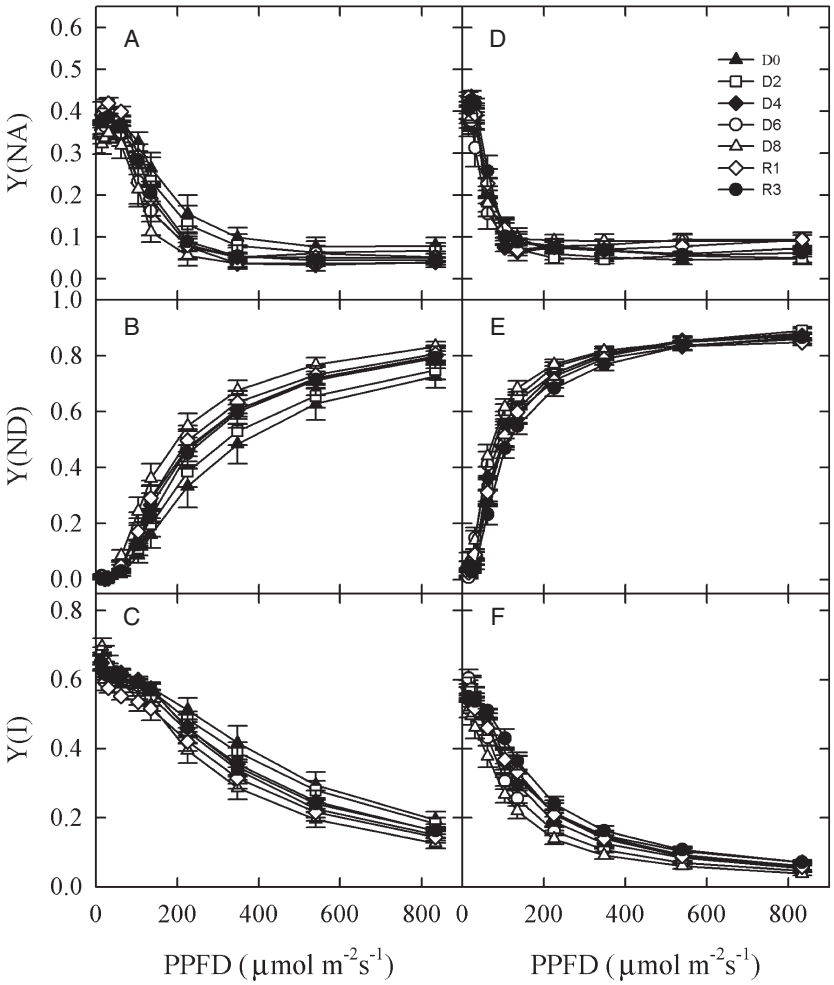

Fig. 5. Light-response changes in fraction of overall P700 that cannot be oxidized in a given state [Y(NA)], fraction of overall P700 oxidized in a given state [Y(ND)] and photochemical quantum yield [Y(I)] of PSI in *Microsorum punctatum* (A–C) and *Paraleptochillus decurrens* (D–F) during drought stress and re-watering. Each point represents mean ± 1 SE for five measurements from different plants.

membrane that downregulates PSII (Cornic et al. 2000, Huang et al. 2012). Photoinhibition of PSI is mainly promoted by this over-reduction and the over-accumulation of reducing power NADPH on that acceptor side of PSI, which leads to the generation of hydroxyl radicals that damage PSI complexes (Sonoike 2006). As drought stress induces a decrease in stomatal conductance, the inhibition of CO_2 assimilation can lead to the over-accumulation of NADPH and over-reduction of the PSI acceptor side. Values calculated for Y(NA) in our *P. decurrens* and *M. punctatum* leaves were maintained at a low level (0.1) under drought-associated high light, indicating that such over-reduction was blocked by strong CEF stimulation. Furthermore, oxidized P700 ($P700^+$) can harmlessly dissipate excess light energy as heat and thereby alleviate the photoinhibition of PSI (Nuijs et al. 1986). The value found here for Y(ND) was >0.7 under high light in our stressed ferns, showing that CEF has a role in protecting PSI against drought when associated with moderate or high-light intensities.

Stimulation of CEF in relation to leaf anatomy

Compared with *M. punctatum*, CEF of *P. decurrens* was stimulated by lower light, with the maximum CEF/ETR(II) ratios being 500–600 μmol m^{-2} s^{-1} PPFD for the former and 150–200 μmol m^{-2} s^{-1} PPFD for the latter. Concurrently, the induction times required to achieve those respective maximums were 4 and 6 days after the onset of water stress. These data indicated that CEF stimulation was more sensitive to high light and drought in *P. decurrens*. However, tissues for both two species were collected from the same

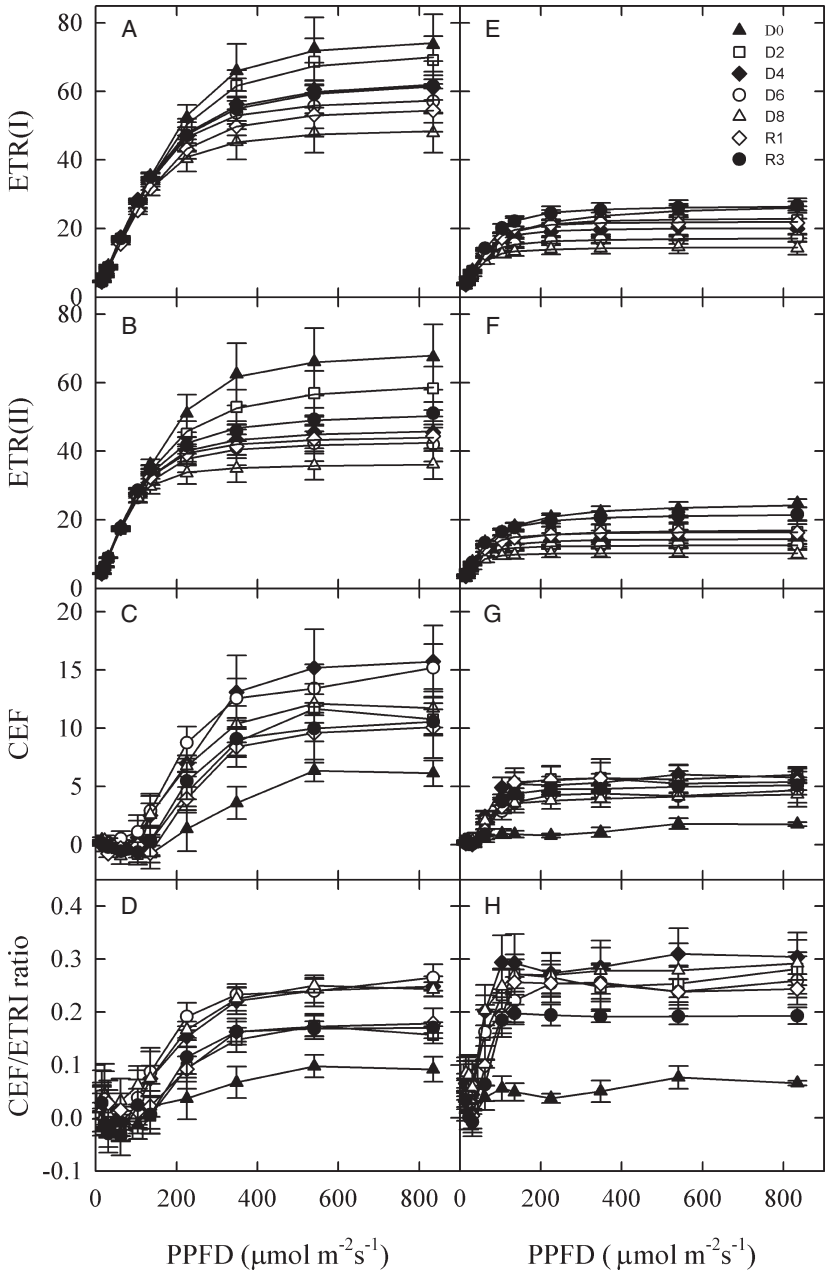

Fig. 6. Light-response changes in electron flow via PSI [ETR(I)], electron flow via PSII [ETR(I)], CEF and ratio of CEF to ETR(II) in *Microsorum punctatum* (A–D) and *Paraleptochillus decurrens* (E–H) during drought stress and re-watering. Each point represents mean ± 1 SE for five measurements from different plants.

microhabitat and cultivated within the same controlled environments. Therefore, this difference in their CEF stimulation reflected their genetic attributes.

The physiological response of a plant to its environment is often linked to leaf anatomy (Peňa-Rojas et al. 2005, Zhang et al. 2011). Hao et al. (2010) have highlighted the correlations of leaf structure with water flux capacity and drought tolerance. Photosynthetic induction is a function of leaf structure and stomatal density – the slow photosynthetic response of *Paphiopedilum armeniacum* to light is related to its lack of guard cell chloroplasts and peculiar stomatal anatomy (Zhang et al. 2011). Photosynthetic induction is slower in epiphytic ferns than in terrestrial ferns (Zhang et al. 2008). That is, the stomata primarily regulate water loss and CO_2 uptake, and conserve water during

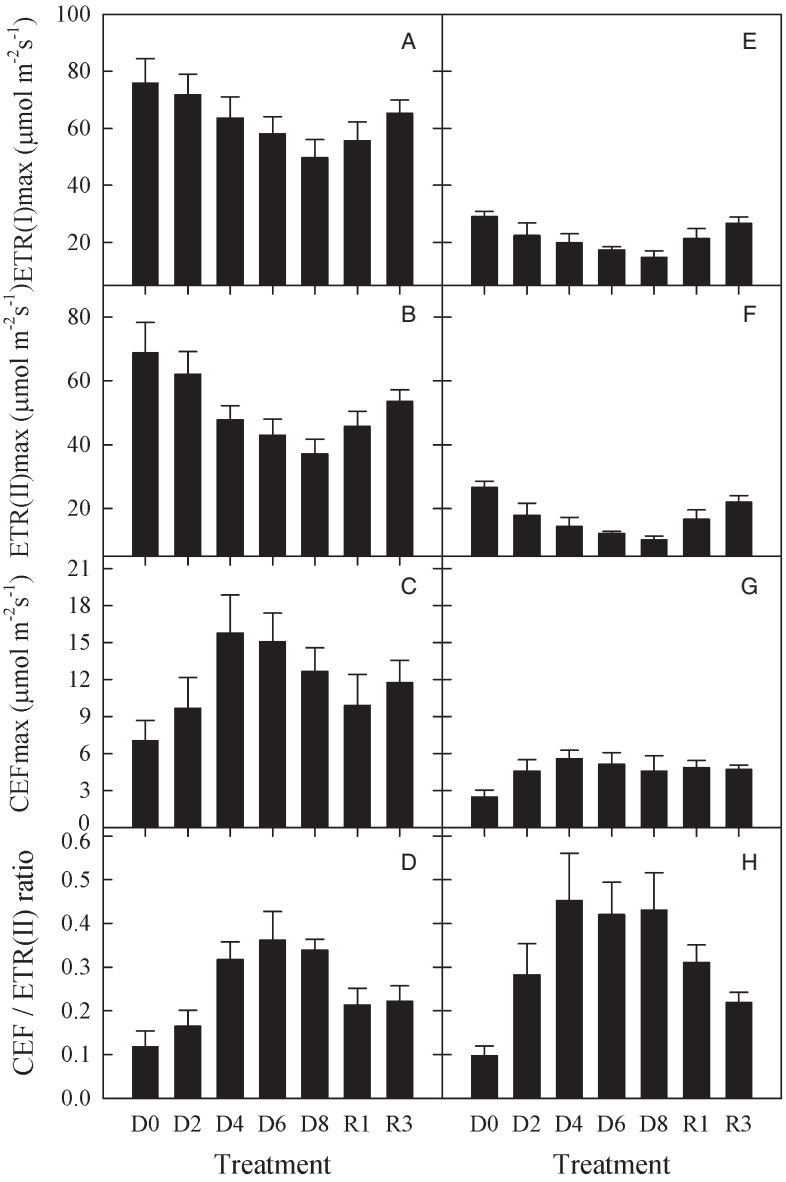

Fig. 7. Changes in maximum electron flow via PSI [$ETR(I)_{max}$], maximum electron flow via PSII [$ETR(I)_{max}$], CEF_{max} and ratio of CEF to ETR(II) in *Microsorum punctatum* (A–D) and *Paraleptochillus decurrens* (E–H) during drought stress and re-watering. Vertical bar indicates error of mean for five measurements from different plants.

drought. Stomatal conductance plays a dominant role in photosynthetic downregulation in drought-stressed species (Cornic 1994). Although stomatal size and density were not determining factors for the difference in drought tolerance by our two ferns, its greater water storage capacity and slower rate of water loss improved the water balance in *M. punctatum* under drought conditions, thereby delaying stomatal closure and photosynthetic downregulation. Conversely, the lower storage capacity and faster rate of water loss increased the sensitivity of *P. decurrens* to water stress. This greater sensitivity meant that those plants utilized less light for photosynthesis during the drought period. Consequently, that species required a more effective photoprotection mechanism to dissipate excess light energy. Therefore, these results show that leaf structure is linked to drought tolerance and photoprotection.

In conclusion, CEF has an important role in conferring photoprotection for both *M. punctatum* and *P. decurrens* under drought conditions. However, their differences in CEF stimulation are related to variations in their leaf structures, which directly influence their adaptability

to water stress. Our results will contribute to further understanding of the evolution and global distribution of fern species.

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