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# Rapid stomatal response to fluctuating light: an under-explored mechanism to improve drought tolerance in rice

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**Abstract.** Light inside a canopy constantly fluctuates. Under fluctuating light (FL) conditions, stomatal conductance and photosynthetic rate constantly change. In this study, we explored whether this dynamics of stomata movements upon FL influenced the water use efficiency of rice in the field. We used a USDA-curated rice mini-core diversity panel consisting of 204 worldwide distributed accessions. *A priori* model on dynamic stomatal response to FL was utilised to identify kinetic parameters describing the stomatal delays during the closing ( $\tau_{cl}$ ) and the opening ( $\tau_{op}$ ) phase. Result showed that  $\tau_{cl}$  had a larger variation than  $\tau_{op}$  across the mini-core panel.  $\tau_{cl}$  was negatively correlated with water use efficiency (WUE) related traits, stem diameter, grain weight per tiller and heading time, but positively correlated with maximum annual temperature, carbon assimilation related traits and biomass (P < 0.05). We further showed a strong correlation of  $\tau_{cl}$  with the relative decrease of biomass under drought in 14 accessions with different  $\tau_{cl}$ . We discussed the adjustment of stomatal conductance under fluctuating light in light of the trade-off between optimising CO<sub>2</sub> uptake and optimising water saving. This study suggests that stomatal dynamics under fluctuating light is closely related to drought resistance and hence detailed study is needed to enable its application in breeding drought tolerance in rice.

Additional keywords: abiotic stress, acclimation, drought stress, photosynthetic rate, stomatal conductance, yield.

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# Introduction

Light levels in a canopy fluctuate frequently and rarely stay at constant levels. The passing cloud can create intermittent shade to leaves in a canopy (Knapp and Smith 1988). Leaves at the top of a canopy can shade leaves at lower layers of a canopy when the wind blows, leaves at lower layers of a canopy experience more fluctuating light (FL) levels or sunflecks (Amtmann 1985; Way and Pearcy 2012). Even if leaves in a canopy keep still, the gradual changes of the sun-earth geometry during a day can create FL on leaves at lower layers of a canopy (Myneni and Impens 1985). Although FL may only last from seconds to minutes, FL can contribute up to 20–80% of the total solar energy incident on understory leaves (Chazdon 1988; Pearcy 1990).

Possibly as a result of long-term plant evolution under dynamic light conditions, plants are equipped with a diverse set of mechanisms to gain increased fitness or to avoid damage under FL levels (Stitt and Zhu 2014). For example, under strong light, state transition can occur to transfer energy away from PSII to avoid photo-damage (Dietzel *et al.* 2011). Chloroplast movement is another commonly observed mechanism plants utilise to avoid photo-damage (Kasahara *et al.* 2002). In addition to these relatively slow structural or anatomical changes, plants also use an array of biochemical and biophysical mechanisms to cope with FL. Under strong light, the rapid decrease of lumen pH can lead to increased rate constant for heat dissipation, which can help quickly dissipate light energy that are in excess of the capacity of photochemistry (Ort 2001). When FL occur, the rate constant of heat dissipation decreases and helps gain increased canopy light use efficiency (Zhu *et al.* 2004). The understory plants are capable of accumulating large amount of 3-phosphate glycerate, which can be used to utilise FL (Pearcy 1990).

Recently, another mechanism that may be important for controlling plant light and WUE in the field has been recognised: the rapid rate of responses of stomata conductance to FL levels (Lawson and Blatt 2014). The theory is that when the light level decreases, there will be a potential decrease of WUE if stomatal conductance ( $g_s$ ) cannot decrease rapidly; however, when the light levels increase abruptly from low to high light levels, if  $g_s$  cannot increase rapidly, photosynthetic light use efficiency will be decreased. There are large variations in both the sensitivity and responsiveness of  $g_s$  to changing environments among different species (Lawson 2009; Lawson *et al.* 2003, 2012).

Applying a proper mathematical modelling to qualify dynamic response in  $g_s$  to FL is critical to identifying variations among or inter-species. There are several strategies to estimate dynamic changes in gs to FL treatments, such as piece-wise linear, logistic models, asymmetric sigmoid function and exponential models (Kirschbaum et al. 1988; Zipperlen and Press 1997; Naumburg et al. 2001; Vico et al. 2011; Vialet-Chabrand et al. 2013). Notably, Vico et al. (2011) first introduced two parameters –  $\tau_{cl}$  and  $\tau_{op}$  – to represent stomatal response delays during closing and opening phase respectively. Vico et al. (2011) obtained these parameters by exponential fitting on 40 dataset from the public resource. In most species, delays in stomatal opening appear to be shorter than delays in closing, that is  $\tau_{op} < \tau_{cl}$  (Ooba and Takahashi 2003; Vico *et al*. 2011). Time scales of stomatal delays in different species also exhibit a large degree of variation. This variation was reported to be commensurate with FL durations (Cardon et al. 1994: Naumburg et al. 2001) and to depend on the time of day and history of FL occurrence (Kaiser and Kappen 1997).

Laboratory studies have shown that leaves with more rapid responses of  $g_s$  exerted higher leaf-level water and light use efficiencies (Lawson and Blatt 2014). Considering the high level of temporal and spatial heterogeneities of light inside a canopy, it is predicted that plants with more rapid response to changes in light levels should have increased water and light use efficiencies in the field. In addition, plant functional types and

geographic origins are two important determinants of  $g_s$  delays in response to FL (Vico et al. 2011). Therefore, in this work, this hypothesis has been tested with a rice diversity panel that was developed by USDA to represent the genetic diversity of the 18000 accessions of the USDA-ARS rice (Oryza sativa L.) germplasm collection (Agrama et al. 2009). This collection contains 217 accessions which were originated from 76 countries covering 15 geographic regions. It consists of six groups, indica (35.4%), aus (18.7%), tropical japonica (18.2%), temperate japonica (15.2%), aromatic (3.0%) and their admixtures (9.6%) (Agrama et al. 2009; Li et al. 2010). This mini-core collection, with its large genetic diversity and large diversity of climates of their origins, represents an ideal choice of germplasm to study the natural variation of stomatal delays and its implication to both water and light use efficiencies in the field.

In this study, we aim to address three specific questions. (1) What are the levels of variability in stomatal responses to FL in the rice mini-core diversity panel? (2) Do plants with different stomatal responses to FL have different WUE? (3) Do plants with different stomatal responses to FL have different drought tolerance in the field?

# Materials and methods

#### Plant materials

The experiments were conducted in 2013 and 2014. In July 2013, a diversity panel composed of 204 accessions derived from 217 USDA mini-core rice accessions. The remaining 13 accessions in the mini-core accessions were not used due to long growth seasons.

# Geographic and climate information of the origins of different accessions

Data of geography such as latitude (LAT), longitude (LONG) and elevation (ELV) for the origin of each accession was

Table 1. Geographic and climate information in 12 accessions selected in 2014 Hainan experiments

Stomatal delays ( $\tau_{cl}$ ) were classified into two subgroups (fast and slow) based on previous observations in Beijing in 2013 experiments. Genetic stock number (GSOR) in Genetic Stock Oryza (http://www.ars.usda.gov/Main/docs.htm?docid\_=f8318, see text). Abbreviations: ACN, accession number; POP, population; LAT, latitude; LONG, longitude; PAR, total earth surface photosynthetically active radiation(PPFD); MDL, maximum daylength (h); MET, mean annual temperature (°F); MAT, maximum annual temperature (°F). Geographic and geoclimate information derived from the year that individual accession released (1914–97), and geoclimate information was collected from NOAA (https://gis.ncdc.noaa.gov/map/viewer/#apP=cdoandcfg= cdoandtheme=annualandlayers=1, see text)

GSOR	ACN	POP	LAT	LONG	PAR (PPFD)	MDL (h)	MET (°F)	MAT (°F)	$\tau_{cl}$ Group
311 258	N4129	TRJ	-0.41	26.62	54.56	12.02	83.65	95	Fast
310 337	C4030	TRJ	18.27	102.84	51.83	13.10	81.11	103.1	Fast
311 787	Y4211	TEJ	44.72	38.29	38.56	15.39	54.63	103.1	Fast
311 677	T4177	TEJ	42.16	24.17	40.19	15.08	31.17	72	Fast
311 745	X4203	IND	24.11	115.42	49.72	13.49	72.58	94.5	Fast
311 236	N4126	IND	-6.30	106.04	54.33	12.37	80.12	98.6	Fast
311 123	M4114	IND	41.71	12.31	40.47	15.03	59.29	103.3	Slow
311 141	M4116	AUS	12.85	-7.96	53.27	12.76	83.05	105.8	Slow
311 181	N4122	AUS	27.10	85.10	48.42	13.71	64.90	98.6	Slow
311 667	T4174	AUS	17.17	-2.38	52.16	13.03	80.92	114.8	Slow
311 153	M4118	IND	14.64	120.46	52.85	12.87	82.64	98.2	Slow
310 846	J4087	IND	22.51	121.95	50.35	13.38	78.52	104	Slow

collected from Genetic Stock Oryza (http://www.ars.usda.gov/ Main/docs.htm? docid\_=Í8318, accessed 3 May 2014), according to Genetic stock number (GSOR) of each accession. Annual climate information such as mean (MET) and maximum (MAT) annual temperature (°F), mean annual dew point (MEDP) and mean annual precipitation (PRCP) was downloaded from National Oceanic and Atmospheric Administration, NOAA (https://gis.ncdc.noaa.gov/, accessed 15 March 2015). All of these data were collected based on the year that each accession released (1914–97) to determine originated weather condition for each accession. Total photosynthetically active radiation (PAR), and maximum daylength (MDL) during growth season were determined by the solar elevation angle, which further depends on the originated information of LAT and LONG for each accession (Song *et al.* 2013).

## Growth conditions

The experiments using the diversity panel with 204 accessions were conducted in the Institute Genetics and Developmental Biology, Chinese Academy Sciences (CAS), Beijing (116.3943°E, 39.9820°N) in 2013. Rice seeds from the each accession were grown for ~30 days in a soil seed bed. Then, from early June, seedlings were transplanted into plastic pots (12 L volume) containing commercial peat soil (Pindstrup Substrate no. 4, Pindstrup Horticulture Ltd, Shanghai, China) and then grown under outdoor conditions. Three plants for each accession were seeded in one pot and each accession used two pots. Measurements were started from early July 2013. During this period, the average lower temperature range was 24~26°C, and the average higher temperature range was  $30 \sim 31^{\circ}$ C, whereas the humidity range was  $61 \sim 75\%$ . Before the measurements, plants were first moved into an environmentcontrolled growth room for 30-50 min to ensure full-adaptation of stomata. The growth room was maintained to have an air temperature of 27°C and a photon flux density of around  $600 \,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ . The experiments were conducted between 0830 to 1630 hours.

Based on the measured stomatal delays in response to fluctuating light (FL) levels (see gas exchange measurements for more details) from 2013, a subset of 204 rice accessions, i.e. 12 accessions with extremely contrasting  $\tau_{cl}$  was selected to determine whether different  $\tau_{cl}$  is able to affect a drought treatment study in 2014. This selection strategy is based on larger amplitude and longer duration in  $\tau_{cl}$  than that  $\tau_{op}$  (Ooba and Takahashi 200; Vico *et al.* 2011). Therefore, in 2014 experiments, our study focussed on  $\tau_{cl}$  instead of  $\tau_{op}$ . Among these 12 accessions, six were in fast  $\tau_{cl}$  group, six were in slow  $\tau_{cl}$  group (Table 1). Between two  $\tau_{cl}$  groups, the MAT were significantly different (P < 0.05). MAT in fast  $\tau_{cl}$  group exhibited a lower value compared with slow  $\tau_{cl}$  group.

In 2014, these 12 rice accessions with contrasting  $\tau_{cl}$  based on previous investigation in 2013 were grown under two conditions, i.e. an extreme drought stress condition (DS hereafter) and a normal irrigated condition (NORM hereafter) in Hainan (110.0375°E, 18.5060°N), China. For the drought treatment, rice was sheltered during rain. The mean daily air temperature between two locations during growth seasons (Beijing and Hainan) were 25.8 and 26.5°C. Mean daylengths

were 12.5 and 12 h respectively. These data was obtained from China Meteorological Administration (http://www.cma.gov.cn/, accessed 15 March 2015) recorded from 2013 to 2014. Two plots were used for the DS conditions and one plot was used for the NORM condition. To decrease the boundary effect, 36 plants were planted  $(6 \times 6)$  for each accession, with 15 cm between plants within each row and 20 cm between rows. The rice fields were managed according to standard local agronomic practice with the following fertiliser application guideline:  $48 \text{ kg N} \text{ ha}^{-1}$ ,  $120 \text{ kg } P_2O_5 \text{ ha}^{-1}$ , and  $100 \text{ kg } \text{ K}_2\text{O} \text{ ha}^{-1}$  as the basal fertiliser, and additional 86 kg N ha<sup>-1</sup> at the tillering stage and 28 kg N ha<sup>-1</sup> at the booting stage. Weeds in both DS and NORM fields were controlled by a combination of chemical and manual methods and insects were controlled chemically. For DS blocks, soil moisture was monitored with a time domain reflectometry method at a soil depth of 20-30 cm (Ledieu et al. 1986). For the DS blocks, the soil water content was  $8\sim 12\%$  (v/v) during photosynthetic measurements.

### Fluctuating light treatments

Experiments were conducted between 50 and 60 days after emergency (DAE) in both 2013 and 2014. The middle segment of the top fully expanded leaf on the main tiller was chosen during experiments. Four portable infrared gas exchange system



Fig. 1. Dynamics of photosynthetic CO<sub>2</sub> uptake rate (*A*), stomatal conductance ( $g_s$ ) and water use efficiency (WUE) to changing light levels. (*a*) Regions used to calculate WUE and also to model  $\tau_{c1}$  and  $\tau_{op}$ . (*b*) Measured dynamic changes of *A*,  $g_s$  and WUE under fluctuating light. The error bars of each trait indicate maximum s.e. throughout fluctuating light treatments. The light intensities used in the fluctuating light treatments were 1200 and 100 µmol m<sup>-2</sup>s<sup>-1</sup> for high light and low light conditions respectively. (*a*, *b*) *A*,  $g_s$  and WUE are depicted in green, red and white respectively.

(Li-6400XT, Li-Cor Inc., Lincoln, NE, USA) were pre-adjusted by manufacturer standard gas and used simultaneously during experiments. The chamber is  $2 \times 3$  cm in dimension and is equipped with a blue–red LED light source. The measurements were conducted on sunny days between 0900 and 1130 hours and between 1300 and 1500 hours. During the measurements, the leaf temperature was maintained at 25°C and the RH was maintained in range of 68 to 87%. The reference CO<sub>2</sub> concentration was set as 400 µmol mol<sup>-1</sup> during the experiment.

During the fluctuating light (FL) treatments, leaf was first exposed to a high light with a PPFD of  $1200 \,\mu\text{mol m}^{-2} \,\text{s}^{-1}$  for at least 5 min until it reaches a steady-state. Then the PPFD was switched to be  $100 \,\mu\text{mol m}^{-2} \,\text{s}^{-1}$ . In 2013, the plants were kept under this low light for 25 min while in 2014 plants were kept under low light for 20 min. The light level of  $100 \,\mu\text{mol m}^{-2} \,\text{s}^{-1}$  was chosen based on work by Lawson and Blatt (2014). Leaves were kept under low light until  $g_{\rm s}$  stabilised. After the  $g_{\rm s}$  stabilisation, the PPFD was recovered to  $1200 \,\mu\text{mol m}^{-2} \,\text{s}^{-1}$ . Leaves were kept under high light for 5 min. Four biological replicates from four different plants for each accession were determined. With the

measured A and  $g_s$ , we calculated the ratio of A (and  $g_s$ ) between DS and NORM conditions, i.e.  $A_{DS}/A_{NORM}$  and  $g_{sDS}/g_{sNORM}$ , where  $A_{DS}$  and  $A_{NORM}$  represents the A under DS and NORM conditions, and  $g_{sDS}$  and  $g_{sNORM}$  represents the  $g_s$  under DS and NORM conditions.

## Calculation of photosynthetic and physiological parameters

In this study, 13 photosynthetic traits during FL were determined including steady-states and instantaneous photosynthetic rates. The initial WUE,  $g_s$  and A under high light as well as these three photosynthetic parameters under low light were estimated as the average values of these parameter during the last five measurements before light switching. We estimated the ratio in A under high light over low light ( $A_{loss}$ ) as function of light use efficiency of A. Same equation was applied in WUE (WUE<sub>loss</sub>) as well. As  $g_s$  exhibits an asymmetric response to light changes, we used an exponential fitting via MATLAB R2010a (MathWorks Inc., Natick, MA, USA), to determine amplitude of stomatal delays when light switching according to Vico *et al.* (2011) using the following equation:

# Table 2. Phenotypic variations among 30 ecophysiological, geographical and biomass related traits for the 204 accessions used in the 2013 Beijing experiment

One-way ANOVA analyses were conducted using StatView, ver. 5.0.1 (see text). Abbreviations: CV, coefficient of variance; FD, fold differences. For further abbreviations see Table 1

EFD	Min	Max	FD	Mean	s.d.	CV	P-value
			Ecophysiolog	v			
$\tau_{cl}$ (min)	1.81	12.42	6.86	4.46	1.69	2.85	< 0.0001
$\tau_{op}$ (min)	1.21	3.93	3.24	2.06	0.44	0.19	< 0.0001
$\tau_{\rm cl}/g_{\rm s}~({\rm m}^2{\rm mmol}^{-1})$	0.28	2.36	8.42	0.70	0.29	0.09	< 0.0001
$A \ (\mu mol  m^{-2}  s^{-1})$	13.65	28.19	2.06	23.02	2.96	8.75	< 0.0001
$g_{\rm s} ({\rm mol}{\rm m}^{-2}{\rm s}^{-1})$	0.18	0.99	5.5	0.64	0.12	0.02	< 0.0001
WUE ( $\mu$ mol mol <sup>-1</sup> )	2.87	6.73	2.34	4.45	0.65	0.43	< 0.0001
$A_{\rm low} (\mu {\rm mol}{\rm m}^{-2}{\rm s}^{-1})$	2.56	6.62	2.58	4.87	0.64	0.41	< 0.0001
$g_{slow} \pmod{m^{-2} s^{-1}}$	0.05	0.26	5.2	0.12	0.04	0.001	< 0.0001
$WUE_{low}$ (µmol mol <sup>-1</sup> )	2.09	7.30	3.49	3.91	0.82	0.67	0.0002
A <sub>loss</sub>	0.15	0.33	2.2	0.23	0.03	0.001	< 0.0001
WUE <sub>loss</sub>	0.55	1.58	2.87	0.88	0.16	0.03	< 0.0001
$WUE_{total}$ (µmol mol <sup>-1</sup> )	2.20	5.76	2.61	3.73	0.63	0.40	< 0.0001
SPAD	27.13	65.2	2.40	37.42	4.87	23.75	< 0.0001
			Geography				
LAT (degrees)	-34.9	50.79	1.4	13.84	22.71	2161.6	< 0.0001
LONG (degrees)	-99.17	178.7	1.8	59.29	68.85	4710.8	< 0.0001
ELV (m)	-999	9140	9.1	415.28	827.06	684025.71	< 0.0001
PAR (PPFD)	34.5	54.56	1.58	48.61	5.16	26.62	< 0.0001
MDL (h)	12.02	16.28	1.35	13.59	0.97	0.95	0.6352
MET (°F)	31.17	91.8	2.94	70.01	12.44	154.81	< 0.0001
MAT (°F)	9.00	127.4	14.1	100.32	10.89	118.49	< 0.0001
MEDP	9.00	76.48	8.49	55.74	15.35	235.62	< 0.0001
PRCP (mm year <sup>-1</sup> )	0.003	2.03	676	0.21	0.21	0.05	< 0.0001
			Biomass				
FW (g)	48.6	350.29	7.20	99.36	32.76	1073.38	< 0.0001
DW (g)	9.75	80.08	8.21	25.7	9.29	86.23	< 0.0001
SBD (mm)	2.97	11.77	3.96	6.45	1.75	3.06	< 0.0001
TIR	4.67	25.67	5.49	12.1	4.14	17.13	< 0.0001
SPL (cm)	18.85	34.43	1.82	25.59	2.62	6.85	< 0.0001
YD (g)	13.88	120.55	8.68	51.3	16.9	285.55	< 0.0001
YD/TIR (g)	0.96	5.38	5.60	2.85	0.82	0.68	0.0003
HEAD (days)	64.00	150.0	2.34	112.95	23.41	548.06	< 0.0001

Stomatal dynamics and drought tolerance

$$g_{(t)} = g \times (\varphi) + [g_0 - g \times (\varphi)] \times \exp(-t/\tau_g), \qquad (1)$$

where  $\tau_{\alpha}$  takes different values depending on whether the leaf is responding to a sudden increase  $(\tau_{op})$  or decrease  $(\tau_{cl})$  in light. The regions used to calculate  $\tau_{cl}$  and  $\tau_{op}$  are shown in Fig. 1*a*. These two parameters represent stomatal delays in minute scale. Goodness of fit  $(R^2)$  after simulation in stomatal opening phase and stomatal closing phase were 0.88 and 0.95, respectively, which reveals a good fitting algorithm applied in this study. To decrease initial effects of  $g_s$  on  $\tau_{cl}$ ,  $\tau_{cl}$  over  $g_s$  was calculated. To compare the potential decrease of WUE due to difference in the  $g_s$  responses, we estimated WUE<sub>total</sub> by obtaining the averaged WUE throughout the light fluctuating experiment. We determined the SPAD value using the SPAD 502 Plus Chlorophyll meter (Spectrum Technologies Inc., Aurora, IL, USA). For each leaf, averaged SPAD values were recorded from five different positions in the middle section, and four replicates from four different plants were determined for each accession.

## Agronomic traits measurements

In 2013 large-scale measurements, four spike related traits, i.e. final tiller number (TIR), stem base diameter (SBD), spike length (SPL) and number per plant (SPN), and three grain weight related traits, i.e. yield per plant (YD), yield per tiller (YD/TIR) and heading time (HEAD) were determined. At least four replicates were conducted for each trait above.

The total aboveground biomass for each accession was measured with a sample size of four while the gas-exchange data were collected. After determining FW, samples were kept at 120°C for 1 h and then under 70°C for at least 24 h until constant weight in baking oven. In 2014 experiments, DW under NORM over drought conditions were calculated to determine biomass accumulation in response to DS at canopy levels.

# Isotope carbon ratio measurements

To evaluate the long-term WUE, we measured the carbon isotope ratio ( $\delta^{13}$ C) of the top fully-expanded leaf at the maturity stage (around 90 DAE). To do this, leaf materials were first dried at 70°C to constant weight, then grounded and analysed using Thermo Delta V advantage. The long-term standard deviation of the equipment is 0.1‰. The <sup>13</sup>C/<sup>12</sup>C ratio was expressed following the delta ( $\delta$ ) notation in parts per mill (‰) relative to the international V-PDB (Vienna PeeDee Belemnite) standard. The measurements were performed at the Mass Spectrometry Laboratory of Forestry Science Research Institute China. Four replicates were used for  $\delta^{13}$ C measurements.

#### Statistical analysis

To analyse the variance of 24 physiological traits and 9 originated climate information in 204 mini-core collection, one group variance analysis was conducted using StatView, ver. 5.0.1 (SAS Institute Inc., Cary, NC, USA). Coefficient of



**Fig. 2.** Natural variations of  $\tau_{cl}(a)$  and  $\tau_{op}(c)$ , and distribution of  $\tau_{cl}(b)$  and  $\tau_{op}(d)$ , in mini-core population. Abbreviations: Admix, admixture of subpopulation; ARO, *aromatic*; AUS, *aus*; IND, *indica*; TEJ, *temperate japonica*; TRJ, *tropical japonica*. Data from 204 accessions were used for the plot.

variance and standard deviation were calculated when comparing groups with or without same unit. The correlation between traits was calculated as Pearson's correlation coefficients among genotype means. Corrplot package in R software (http://www. r-project.org/, accessed 13 June 2015) was employed to calculate Pearson' coefficient index (r) or goodness of fit ( $R^2$ ).

### Results

# Natural variations of the rapidity of responses of g<sub>s</sub> to fluctuating light levels

During fluctuating light (FL), A,  $g_s$  and WUE showed dynamic changes (Fig. 1b). Steady-state A were  $23.02 \pm 2.96 \,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$  at 1200  $\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$  (Fig. 1b; Table 2). After decreasing PPFD from 1200 to 100  $\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ , A decreased immediately by 80% within 1 min and kept  $4.87 \pm 0.64 \,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ , whereas  $g_s$  showed an exponential reduction:  $g_{\text{slow}}$  across mini-core accession was  $0.12 \pm 0.04 \,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ , which accounts for 25% of the initial  $g_s$  (Fig. 1b). However, WUE increased in an opposite pattern as the change in  $g_s$ , and reached a maximum at around 13 min after switching to low light. When the light level was increased again, a rapid increase in A,  $g_s$  and WUE was observed.

In the 2013 experiments, we investigated nine ecophysiological traits and eight biomass related traits. To further analyse whether stomatal responses might be caused by different eco-niches in which plants were originally grown, we obtained the 13 geography and climate factors related to the growing regions of these rice accessions from NOAA database (https://gis.ncdc.noaa.gov, accessed 15 March 2015). The fold differences between the maximum and minimum for each geographical factor were more than those of the ecophysiological and biomass traits. Most of these traits showed a significant difference (P < 0.0001), except for maximum daylength (MDL) according to one-way ANOVA (Table 2). Across these geographical factors, annual precipitation (PRCP) and maximum air temperature (MAT) exhibited 676- and 14.1-fold differences between the maximum and minimum respectively. Among them, latitude (LAT) and longitude (LONG) were in the range from  $-34.9 \sim 50.8^{\circ}$ , and -99.2 ~178.7° respectively. Annual mean temperature (MET) ranged from 31.2 ~91.8°F. MAT ranged from 9 ~76.5°F.

There were large natural variations in  $\tau_{cl}$  and  $\tau_{op}$  (Fig. 2). The values in  $\tau_{cl}$  ranged from 1.81 to 12.42 with a normal distribution across these 204 rice accessions (Fig. 2; Table 2). Among subpopulation, AUS exhibited a relatively higher median and minimum values of  $\tau_{c1}$  and  $\tau_{op}$ , compared with two types of japonica (TEJ and TRJ) (Fig. 2). The median of  $\tau_{cl}$  and  $\tau_{op}$  across *indica* (IND) showed similar or higher rates than in *japonica*. Mean of  $\tau_{cl}$  was 4.46, which is two times higher than  $\tau_{\rm op}.$  Fold differences between minimum and maximum in  $\tau_{cl}$  and  $\tau_{op}$  were 6.86 and 3.24, respectively, the variance level is significant (P < 0.0001) (Table 2). To eliminate the effects of initial  $g_s$ ,  $\tau_{cl}$  was normalised ( $\tau_{cl}/g_s$ ) in this study. The  $\tau_{cl}/g_s$  ranged from 0.28 to 2.36 with an 8.42-fold difference (Table 2). With respect to fold difference in A,  $g_s$  and WUE under both high light and low light, the ranking from high to low is:  $g_s > WUE > A$ , i.e. a relatively higher fold difference was observed in  $g_s$  under high light and

low light, which were 5.5 and 5.2 respectively. Whereas *A* under high light and low light exhibited a relatively low differences, the fold difference is no more than 2.5. Among ecophysiological traits, SPAD values exhibited highest standard deviation and coefficient of variance.

Under different geography and climate conditions, biomass related traits exhibited a significant variation as well (P < 0.0001). FW and DW per plant ranged from 48.6 to 350.3 and 9.8 to 80.1 g respectively (Table 2). In terms of spike traits, i.e. SBD, TIR and SPL, the fold differences between the minimum and maximum were 3.96, 5.49 and 1.82 respectively. In contrast, YD and YD/TIR exhibited higher fold differences, i.e. 8.68 and 5.60 respectively. HEAD time ranged from 64 to 150 days. The mean of HEAD time was 113 days.



Fig. 3. Matrix of correlation coefficients between 24 ecophysiologcal and biomass-related traits and 9 geoclimate traits across 204 mini-core accessions. The colour gradients from red to blue represent the range of Pearson correlation coefficient (r) from -1.0 to 1.0. The black lines are used to separate variable categories. The P-values for the correlation between each pair of traits were shown in Table S1, available as Supplementary Material to this paper. Abbreviations:  $\tau_{cl}$ , stomatal delays during closing phase;  $\tau_{on}$ , stomatal delays during opening phase; A, photosynthesis rates;  $g_s$ , stomatal conductance; WUE, water use efficiency; Alow, photosynthesis rates under low light; gslow, stomatal conductance under low light; WUElow, water use efficiency under low light;  $A_{loss}$ , photosynthesis rates under low light over high light; WUE<sub>loss</sub>, water use efficiency under low light over high light; WUEtotal, totally mean WUE throughout FL treatments; SPAD, SPAD records; LAT, latitude; LONG, longitude; ELV, elevation; PAR, total earth surface photosynthetically active radiation; MDL, maximum daylength; MET, mean annual temperature; MAT, maximum annual temperature; MEDP, mean dew point; PRCP, precipitation; SBD, stem diameter; TIR, tiller number; SPL, spike length; YD, yield per plant; YD/TIR, yield per tiller; HEAD, heading time.

# Variation of $\tau_{cl}$ is linked to water and shadefleck use efficiency in the mini-core panel

Among the collected parameters, we calculated the Pearson correlation coefficient (*r*) between them (Fig. 3; see Table S1, available as Supplementary Material to this paper). Correlation *r* in photosynthetic traits during FL revealed a robust relationship between these traits.  $\tau_{cl}$  was positively correlated with  $\tau_{op}$ ,  $\tau_{cl}/g_s$ , *A*,  $g_s$ ,  $A_{low}$ ,  $g_{slow}$  and  $A_{loss}$  (*P*<0.05), but was negatively correlated with WUE and WUE<sub>total</sub> (*P*<0.05). Furthermore,  $\tau_{op}$  was positively correlated with WUE<sub>loss</sub>, WUE<sub>low</sub> and WUE<sub>total</sub> (*P*<0.05), but not significantly correlated with *A*,  $g_s$  and WUE. And  $\tau_{op}$  was negatively correlated with SPAD (*P*<0.05).

The diverse origin, from the perspective of geography and year of release, in the mini-core collection can affect stomatal evolution under FL. As shown in Fig. 3,  $\tau_{cl}$  and  $\tau_{op}$  displayed an intricate relationship in response to geography factors,  $\tau_{cl}$  and  $\tau_{cl}/g_s$  was positively correlated with MAT (P < 0.05), whereas  $\tau_{op}$  was positively correlated with LAT and MAL (P < 0.05) but negatively correlated with MEDP (P < 0.05) that is typically representing absolute air humidity.

For the biomass related traits, both  $\tau_{cl}$  and  $\tau_{op}$  were positively correlated with biomass accumulation (DW and FW) (P < 0.05), but no correlation was observed between  $\tau_{cl}/g_s$  and biomass (Fig. 3). In contrast,  $\tau_{cl}$ ,  $\tau_{op}$  and  $\tau_{cl}/g_s$  were all negatively correlated with SBD. In terms of spike traits,  $\tau_{op}$  was positively correlated with TIR and SPL, but negatively correlated with YD/TIR (P < 0.05). In contrast,  $\tau_{c1}$  was negatively correlated with HEAD time (P < 0.05).

# The relationship between $\tau_{cl}$ and drought resistance in the field

Because  $\tau_{cl}$  was negatively correlated with WUE related traits (Fig. 3) and showed a larger variation compared with  $\tau_{op}$  (Fig. 2), we selected 12 accessions for further experiment in 2014 to test whether difference in  $\tau_{cl}$  can lead to difference in WUE in the field (see material and methods for details). Among these 12 accessions, six accessions possessed extremely high six  $\tau_{cl}$  (Fast group) and six accessions with extremely low six  $\tau_{cl}$  (slow group).

In the 2013 experiments, A and  $g_s$  within the slow group were 30% and 80% higher than those in the fast group respectively before light was switched from high to low light (Fig. 4*a*, *b*). After light was switched to low light,  $g_s$  in the fast group declined and gained stable values within 8 min. In contrast, there was a greater lag in  $g_s$  in the slow group. *A* under low light didn't differ between two groups. When light was increased from low to high light levels,  $g_s$  and *A* in two groups increased rapidly. Before light switching, WUE in the fast group was ~75 µmol mmol<sup>-1</sup>, which is 50% higher than that in slow group. After light switching to low light levels, WUE dropped sharply then recovered back after a new balance between *A* and  $g_s$  was formed. WUE<sub>low</sub> in both groups were slightly lower compared with WUE under high light. However, WUE in fast group



**Fig. 4.** Dynamic changes of carbon assimilation (*a*), stomatal conductance (*b*), water use efficiency (*c*), and internal CO<sub>2</sub> over leaf surface CO<sub>2</sub> (*d*) during fluctuating light. Grey area represents low light treatments. Error bars indicate the maximum s.e. for each group. Closed circles in red and black show  $\tau_{cl}$  in the fast and slow groups respectively.

recovered to normal levels at a faster pace than that in the slow group. In terms of  $C_i/C_a$  (Fig. 4*d*), because of stomatal effects, a relatively higher  $C_i/C_a$  was induced in the slow group than the fast group after light switching. The dynamics in *A* under FL was similar between the fast and slow groups; however, the dynamics of  $g_s$  differ dramatically between the fast and slow group (Fig. 4, see Fig. S1, available as Supplementary Material to this paper).

The  $\tau_{cl}$  measured across 12 accessions under two growth locations (Beijing, BJ and Hainan, HN) were highly correlated (Fig. S3).  $R^2$  regarding to  $\tau_{cl}$  between BJ and HN was 0.65; similarly,  $R^2$  for  $\tau_{cl}/g_s$  between BJ and HN was 0.79 (P < 0.001) (Fig. S6). In addition,  $\tau_{cl}$  hold the higher values in slow group than that that of the fast group under two growth conditions (Fig. S3). A co-ordination in  $\tau_{cl}$  and a conserved ranking across accessions between NORM and DS condition were observed (Fig. 5, Fig. S3). In contrast, the  $\tau_{op}$  showed a slightly less conserved ranking between these two conditions (Fig. 5*b*). Furthermore, when the  $\tau_{cl}/\tau_{op}$  in the DS was plotted against the ratio in NORM (Fig. 5*c*), a slope <1 was obtained, i.e. we have a smaller  $\tau_{cl}/\tau_{op}$  under DS compared with the ratio under NORM.

Among accessions,  $\tau_{cl}$  appeared to be substantial differences. In particular, T4174 and C4030 showed an extremely contrasting  $\tau_{cl}$  with around 3-fold differences under both NORM and DS conditions (Fig. 6). Diverse response across accessions due to DS was observed. Drought treatments induced 2-fold differences in  $\tau_{cl}$  in T4174, but 50% fold differences in T4177 (Fig. 6). A positive correlation between  $\tau_{cl}$  and biomass (DW) under both NORM and DS conditions was observed ( $R^2 = 0.67$ and 0.37 respectively) (Fig. 7c and insert in Fig. 7c). Remarkably, both  $\tau_{cl}$  was negatively correlated with biomass drought ratio (Fig. 7d). The  $R^2$  is 0.69, while  $R^2$  of  $\tau_{cl}/g_s$  is 0.43 (Fig. S6). In contrast, A and  $g_s$  across 12 subset accessions were not significantly correlated with biomass drought ratio (Fig. S4).We further evaluated the correlation between biomass drought ratio with the changes of A and  $g_s$ under drought stress. Specifically, we calculated the ratio of A (and  $g_s$ ) between DS and NORM conditions, i.e.  $A_{DS}/A_{NORM}$ and  $g_{\rm sDS}/g_{\rm sNORM}$ , and the obtained the correlation between biomass drought ratio and  $A_{\rm DS}/A_{\rm NORM}$  and  $g_{\rm sDS}/g_{\rm sNORM}$ separately. The biomass drought ratio showed a higher  $R^2$ 



Fig. 5. Correlation of  $\tau_{cl}$ ,  $\tau_{op}$ , and  $\tau_{cl}/\tau_{op}$  between normal condition and drought stress condition. Closed circles in red and black show  $\tau_{cl}$  in the fast and slow groups respectively. Determination of coefficient is depicted as  $R^2$ .



Fig. 6. Comparison of  $\tau_{cl}$  among accessions under normal condition and drought stress condition (insert). Red and black bars represent  $\tau_{cl}$  of the fast and slow groups respectively. One-way ANOVA was used to compare the significant levels between accessions, and same letter represent no significant differences (P < 0.05).

with  $\tau_{cl}$  than either A, or  $g_s$  or  $A_{DS}/A_{NORM}$  or  $g_{sDS}/g_{sNORM}$  (Figs 7d, S5). These results suggested that  $\tau_{cl}$ , instead of either A or  $g_s$  or  $A_{DS}/A_{NORM}$  or  $g_{sDS}/g_{sNORM}$ , was closely related to drought tolerance. Biomass between 2013 and 2014 experiments showed a robust correlation ( $R^2$ =0.88) (Fig. S2).

Drought induced ~60% reduction in biomass on average across accessions (data not shown), but changes in biomass under DS differed among accessions obviously from each other (Fig. 7b). Among these accessions, biomass in T4174 showed a relatively higher values; in contrast, C4030 showed a lower biomass (Fig. 7a). In terms of biomass drought ratio, N4129 and Y4211 were around 0.75, which are 2-fold higher than T4174 and N4122 (Fig. 7b).

# Correlation between $\delta^{13}C$ and $\tau_{cl}$

To investigate whether  $\tau_{cl}$  can affect long-term leaf water use efficiency, carbon isotope ratio,  $\delta^{13}$ C in 12 accessions were determined. Result showed a negative correlation between  $\tau_{cl}$ and  $\delta^{13}$ C ( $R^2 = 0.5$ ) (Fig. 8 insert).  $\delta^{13}$ C across accessions were in ranges of -24.9 to -29.4%. Accessions in fast group exhibited a lower values in  $\delta^{13}$ C, compared with that in slow group (P = 0.05). In particular,  $\delta^{13}$ C of T4174 and J4087 in slow group were averaged around -29.0%, in contrast, the average  $\delta^{13}$ C of Y4211 and N4216 in fast group were -25.1% (Fig. 8). The  $\tau_{cl}$  showed a positive correlation with biomass and a negative correlation with the biomass drought ratio, i.e. the ratio of the biomass under drought to the biomass under control conditions (Figs 6, 7*c*, *d*).

### Discussion

Stomata always exhibited a lag before getting stabilised during fluctuating light (FL) regimes, whereas carbon assimilation (*A*)

responded to FL at a faster pace (Pfitsch and Pearcy 1989; Vico *et al.* 2011). This study used a rice mini-core panel consisting of 204 accessions collected by USDA to examine the significance of this phenomena to drought resistance in the field. The results suggest that there were large natural variations in the stomatal delays in response to FL and this variation was closely related to WUE and drought resistance in the field.

There were substantial natural variations in the stomatal delays to changes in light levels in the rice diversity panel (Fig. 2; Table 2), which is consistent with earlier reports (Chazdon 1988; Pearcy 1990; Ooba and Takahashi 2003). Simulation studies (Vico et al. 2011) using different species assembled from public database showed  $\tau_{op}$  is less than that  $\tau_{cl}$ during FL in graminoids, forbs and angiosperms, and there is a strong coordination between  $\tau_{cl}$  and  $\tau_{op}$ . Rice in our study showed the same trend as well (Fig. 3; Table 2). The value of  $\tau_{cl}$  across these 204 rice accessions varied between 1.81 and 12.42 (Table 2) whereas the values of  $\tau_{op}$  ranged between 1.21 and 3.93 (Table 2). This much higher value of  $\tau_{cl}$  compared with  $\tau_{op}$  (Table 2; Fig. 2) suggested that rice has the tendency of optimising carbon assimilation instead of conserving water under NORM conditions. This might be related to the fact that rice normally is grown in wet environments, therefore, the evolutionary pressure is on selection of faster stomatal opening to optimise the photosynthetic light use efficiency. Consistent with this, a survey of light saturated rate of photosynthesis in 24 accessions of rice  $(A_{\text{max}})$ , representing 17 species and 8 genomes, showed that  $A_{\text{max}}$  ranged from 25 to 45  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (Giuliani et al. 2013), which is relatively higher compared with Amax of other C<sub>3</sub> plants (Wright et al. 2004).

Compared with the NORM conditions, rice grown under DS conditions showed decreased  $\tau_{op}$  and  $\tau_{cl}$  (Fig. 5*a*, *b*). But the decrease in  $\tau_{cl}$  was higher than  $\tau_{op}$ , as shown by the decreased ratio of  $\tau_{cl}/\tau_{op}$  under DS conditions (Fig. 5*c*). This indicated that the internal regulatory mechanisms of the control of stomatal conductance conditions to optimise water conservation instead of carbon assimilation under DS. For rice grown in a paddy field, the relatively faster opening of stomata compared with the closing of stomata (i.e. high  $\tau_{cl}/\tau_{op}$ ) under FL can lead to a relatively less favourable WUE at the top of the canopy where the leaves experience relatively constant high light levels.

The observed variations of both parameters should be related to the long-term adaptation to the climatic conditions of the eco-niches, as demonstrated by the correlation between these parameters and the geographic and climate factors of the origin of rice accessions (Fig. 3). For example,  $\tau_{cl}$  was positively correlated with MAT (P < 0.05), but  $\tau_{op}$  was negatively correlated with MEDP (P < 0.05) and PRCP. The positive correlation between  $\tau_{c1}$  and MAT was in contrast to the expectation that under high temperature regions a lower  $\tau_{cl}$  would be desirable to ensure higher WUE. A higher dew point indicates higher humidity in the air. In our study, the negative correlation between  $\tau_{op}$  and MEDP suggested that areas with high humidity, i.e. high MEDP, will have a lower  $\tau_{op}$ , i.e. faster stomatal opening speed. In line with this, PRCP showed a negative correlation with  $\tau_{op}$ , i.e. under high precipitation, the  $\tau_{\rm op}$  would be lower. These are reasonable since under conditions without water stress, decrease  $\tau_{op}$ , i.e. increasing the stomatal



Fig. 7. Comparison on biomass (*a*) and biomass drought ratio (*b*) among accessions, and correlation of  $\tau_{c1}$  with biomass under normal (*c*) and drought (*c* insert) and biomass drought ratio (*d*). One-way ANOVA was used to compare the significant levels between accessions, and different letters represent significant differences (*P*<0.05). Determination of coefficient is depicted as  $R^2$ .

opening speed can help optimise photosynthetic light use efficiency, which is more important than decreasing the stomatal response time for water saving. Therefore, humidity and precipitation are dominant environmental parameters that influence the speed of stomatal dynamics under varying light conditions.

Large variations in  $\tau_{cl}$  and  $\tau_{op}$  between different species have been observed previously, this study showed that not only there is also a huge variation of  $\tau_{cl}$  and  $\tau_{op}$  within the same species, but also there are substantial variation between subgroups of rice. For example, the AUS showed relatively higher  $\tau_{cl}$  and  $\tau_{op}$  while TRJ showed low values of  $\tau_{cl}$  and  $\tau_{op}$  (Fig. 2). These rice accessions therefore provide a rich source of materials to study the potential driving force and molecular mechanisms controlling the evolution of these different features.

The large variation in  $\tau_{cl}$  is closely related to drought resistance in the field. The relationship between  $\tau_{cl}$  and WUE has been reported earlier at the leaf level (Lawson and Blatt 2014). However, the potential impacts of different stomatal dynamics on drought resistance in the field will depend the time scale of the dynamic changes of the light environments inside a canopy, which will be further determined by the canopy architecture, wind speed, cloud



**Fig. 8.** Comparison on  $\delta^{13}$ C and  $\tau_{cl}$  for each rice accession. Inserted panel showed correlation between  $\delta^{13}$ C and  $\tau_{cl}$ . One-way ANOVA was used to compare the significant levels between accessions, and different letters represent significant differences (*P*<0.05). Determination of coefficient is depicted as  $R^2$ .

movement, angle of incident radiation etc. (Chazdon 1988; Pearcy 1990). Data from this study demonstrate that accessions with higher  $\tau_{cl}$ , i.e. slower rate of stomatal closure, exerted a lower drought resistance, as demonstrated by the relatively higher biomass drought ratio (Fig. 7b, d). This is further confirmed by the changes of isotope carbon ratio,  $\delta^{13}C$ (Fig. 8), which reflects the long-term WUE of a plant (Farquhar and Richards 1984; Farquhar et al. 1989). We further tested whether the drought resistance might also be linked to the difference in A,  $g_s$ , or changes of A or  $g_s$  under drought conditions (i.e.  $A_{\rm DS}/A_{\rm NORM}$ , or  $g_{\rm sDS}/g_{\rm sNORM}$ ). Correlation analysis suggested that in the biomass drought ratio possessed much higher correlation with  $\tau_{cl}$  as compared with those with either A, or  $g_s$  or  $A_{DS}/A_{NORM}$ , or  $g_{sDS}/g_{sNORM}$  (Figs 7, S4, S5). The biomass ratio showed a  $R^2$  of 0.2 with  $g_{sDS}/g_{sNORM}$ . Therefore, though the data also indicated that  $g_s$  might also be related to the biomass drought ratio,  $\tau_{cl}$  is a parameter that are more closely related to drought resistance in the field.

Our findings suggest that  $\tau_{cl}$  exhibited a positive correlation with biomass accumulation, though decreased  $\tau_{cl}$  was linked to an increased drought resistance. Indeed, both  $\tau_{cl}$  and  $\tau_{op}$  were positively correlated with biomass accumulation (DW and FW) (Fig. 3). Concurrent with this positive correlation, we also found a positive correlation between  $\tau_{cl}$  and  $g_s$  and A (Fig. 3), i.e. those lines with a faster stomatal closing dynamics indeed performed on average lower  $g_s$  and A values. The observed correlation between  $\tau_{cl}$  and biomass therefore may be related to the overall higher A in those lines with higher  $\tau_{cl}$ . The molecular mechanism underlying the positive correlation between  $\tau_{cl}$  and A and  $g_s$  is unknown. However, these data suggest that for regions where drought is relatively a rare event, decreasing  $\tau_{cl}$  should not be used as a breeding target. In contrast, for regions with frequent severe drought, it would be more effective for rice productivity (Tuberosa and Salvi 2006). This is clearly demonstrated by the strong inverse relationship between  $\tau_{cl}$  and biomass drought ratio (Fig. 7*d*). For example, the line Y4211, which showed a  $\tau_{c1}$  of ~2 min, its decrease of biomass under DS was only ~20%; this is in great contrast with line T4174, which showed a  $\tau_{cl}$  of ~2 min of ~8.5 min and had a decrease of biomass by ~70% under DS (Figs 6, 7*d*). Though a positive correlation between  $\tau_{cl}$  and biomass is apparent in our used 12 accessions, we cannot eliminate the possibility that the observed positive correlation between  $\tau_{cl}$  and biomass is caused by other factors, such as changed root: shoot ratio or different construction cost for the biomass.

Torres *et al.* (2013) conducted a large scale drought tolerance using rice genebank germplasm for yield and found that many drought tolerant accessions belong to AUS or IND group. However, three accessions out of the 12 accessions used in 2014 belong to the AUS group, but in general they showed relatively low stomatal closing speed (Table 1). We emphasise here that although faster stomatal closing is shown here to be associated with drought tolerance, rapid stomatal closing is not the only mechanism that plant use to cope with drought. The AUS and IND groups used by Torres *et al.* (2013) may have other drought escape, recovery, and avoidance mechanisms to gain high drought tolerance. This points to a future direction of improving drought tolerance in AUS or IND cultivars, i.e. the stomatal response dynamics can be optimised in the AUS or IND rice cultivars to gain even higher drought tolerance.

In summary, this study demonstrated that there are large natural variations in  $\tau_{cl}$  and  $\tau_{op}$  in response to fluctuating light in one single species. We also noted the tendency of stomatal dynamics to optimise either carbon uptake or water conservation depending on water availability and temperature of origin of the rice accession. Our findings revealed that the biomass accumulation had a positive correlation with stomatal closing speed, even though this speed was positively correlated with drought resistance. All these findings suggest that stomatal movement has a complex influence on the fitness of plants in the field and its application in rice breeding needs to consider the severity of the water stress.

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