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Chlorophyll fluorescence, leaf gas exchange, and genomic analysis of chromosome segment substitution rice lines exposed to drought stress

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Abstract

This research aims to evaluate the photosynthesis-related parameters in rice chromosome segment substitution lines (CSSL), containing drought-tolerant region from DH212 in a Khao Dawk Mali105 genetic background. Screening at seedling stage indicated that CSSL4 was more tolerant to drought stress than KDML105 with the higher maximal quantum yield of PSII photochemistry. After withholding water, the decline in light-saturated net photosynthetic rate due to drought stress occurred simultaneously with the decrease in electron transport rate and effective quantum yield of PSII photochemistry values, suggesting that stomatal changes affect light-saturated net photosynthetic rate (P_{Nmax}) during the initial drought response. KDML105 rice showed the highest level of electron transport rate/P_{Nmax} ratio. This suggested that KDML105 has the lowest ability to use reducing power in photosynthesis process under drought stress conditions. Loci containing single nucleotide polymorphisms between CSSL4 and KDML105 were subjected for co-expression network analysis with 0.99 correlation. The co-expression between calmodulin-stimulated calcium-ATPase and C2H2 zinc finger protein was detected. This locus may contribute to the maintenance ability of photosynthesis process under drought stress conditions.

Additional key words: field capacity; maximum efficiency of PSII; *Oryza*; photosynthesis performance index; water stress.

Introduction

Drought is a complex environmental factor that limits plant yield. Rice production in many parts of Asia is adversely affected by drought stress (Polthanee *et al.* 2014). Farmers attempt to mitigate the effects of low water availability using several strategies, including irrigation, water management, crop diversification, and the use of drought-tolerant rice cultivars to prevent drought-induced yield losses (Bouman and Tuong 2001, Cattivelli *et al.* 2008, Lin 2011).

During the past decade, marker-assisted experimental approaches have resulted in the identification of various quantitative trait loci in rice exposed to drought conditions (Hemamalini *et al.* 2000, Jena and Mackill 2008). Khao Dawk Mali105 (KDML105) rice is one

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Abbreviations: C_{i} – intercellular CO_2 concentration; CMS – cell membrane stability; CSSL – chromosome segment substitution line; E – transpiration rate; ETR – electron transport rate; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v/F_m – maximal quantum yield of PSII photochemistry; FC – field capacity; g_s – stomatal conductance; LDS – leaf drying score; LRS – leaf rolling score; PI – photosynthesis performance index; P_{Nmax} – light-saturated net photosynthetic rate; RWC – relative water content; Φ_{PSII} – effective quantum yield of PSII photochemistry; Ψ – leaf water potential; Ψ_{p} – leaf turgor potential; Ψ_{tsoil} – soil matric potential; Ψ_{π} – leaf osmotic potential.

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of the popular rice cultivars known as jasmine rice. To obtain high quality rice, this cultivar is grown in the northeastern part of Thailand, where the irrigation is limited. Therefore, the drought-tolerant cultivar that can produce high quality rice as KDML105 is needed. Major drought tolerance-related quantitative trait loci in rice have been detected on chromosomes 1, 3, 4, 8, and 9 (Lanceras *et al.* 2004). CSSLs containing putative drought-tolerance gene regions in a KDML105 rice genetic background have also been generated. These regions were obtained from doubled haploid donors and transferred to the KDML105 genome (Kanjoo *et al.* 2011). Some CSSLs with substitutions on chromosomes 1, 4, and 8 produce higher grain yields under drought conditions compared with those of KDML105 plants (Kanjoo *et al.* 2012). Based on gene co-expression network analyses, CSSLs with varying sizes of chromosome 1-derived segments carry two node genes that are co-expressed with several genes associated with photosynthetic activities under salt stress conditions (Khrueasan *et al.* 2013).

Drought stress negatively influences photosynthetic processes. The severity of the detrimental effects depends on the intensity, duration, and rate of progression of the drought stress (Chaves *et al.* 2009). Decreased plant water status often causes the stomata closure. Investigations of stomatal conductance and leaf water potential have revealed that plant water status is correlated with stomatal conductance and transpiration under drought stress conditions (Tardieu and Davies 1993, Medrano *et al.* 2002). Moreover, stomata close in response to drought-induced chemical signals from roots exposed to dry soil (Davies and Zhang 1991, Comstock 2002). These phenomena restrict the diffusion of CO_2 to the chloroplasts, and subsequently increase the difference between intercellular CO_2 concentration (C_i) and chloroplast CO_2 concentration (C_c). This ultimately decreases the net photosynthetic rate (P_N) (Cornic *et al.* 1989, Renou *et al.* 1990, Flexas *et al.* 2007). Furthermore, drought stress downregulates photosystem activities, alters quantum yield (Méthy *et al.* 1996, Peltzer *et al.* 2002, Yin *et al.* 2006), and decreases the ETR, resulting in inhibited photosynthesis (Peeva and Cornic 2009, Li *et al.* 2012, Caulet *et al.* 2014).

We selected four CSSLs containing a substitution on chromosome 1 in a KDML105 rice genetic background (Kanjoo *et al.* 2011). The objective of this study was to analyze drought-tolerant rice CSSLs to identify genetic resources potentially useful for rice breeding programs.

Materials and methods

Plant materials and growth conditions: Four CSSLs (CSSL1–4) with a KDML105 rice (*Oryza sativa* L.) genetic background and their parents (KDML105 and DH212) (Kanjoo *et al.* 2011) were obtained from the Rice Gene Discovery Unit, National Center for Genetic Engineering and Biotechnology, Thailand. These four lines contain putative drought-tolerant region between RM212 to RM5310 markers on chromosome 1 of DH212 rice, which is the double haploid line generated from the cross between CT9993 and IR62266 rice. To generate chromosome substitution lines, the marker-selected lines were backcrossed to KDML105 rice for five generations.

In this research, to screen CSSLs at the seedling stage (November–December 2013), rice seeds were germinated for 5 d in plastic cups filled with distilled water. Seedlings were then transferred to 12.5-cm (in diameter) plastic pots, filled with 0.9 kg of clay soil. Seedlings were grown for 25 d inside a netted greenhouse at the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. They were exposed to 100, 75, or 50% soil field capacities (FCs). FC of 100% was determined according to the method of Veihmeyer and Hendrickson (1931) by determining the amount of water held in the soil after the excess gravitational water was drained away. The various FCs were maintained by the addition of the required amount of water to attain specific masses. The mean daytime and nighttime temperatures (12-h period) were 32.8 and 27°C, respectively. The mean photosynthetic photon flux density was 900–1,000 μmol m^{-2} s^{-1}, while the average daytime and nighttime relative humidity levels were 60 and 71%, respectively. Weeds were manually removed from pots every week. The plants were treated with drought stress for 12 d.

For leaf gas-exchange evaluations at the vegetative stage (August–October 2014), CSSLs seedlings and parental lines were transferred to 30-cm plastic pots filled with 5 kg of clay soil. After germination period of 25 d, each pot received 156.25 kg h^{-1} compound fertilizer (NPK 15-15-15). Rice plants were grown in a greenhouse under natural conditions at the Tropical Vegetable Research Development Center at Kasetsart University, Kamphangsaen Campus, Nakhon Pathom, Thailand. Plants were regularly watered to simulate normal conditions, while drought stress conditions were induced by withholding water. Plants were exposed to drought stress at 40 d after germination. The drought experiment was performed for 15 d. The average daytime and nighttime temperatures were 30.7 and 25.1°C, respectively. The average photosynthetic photon flux density was 1,100 μmol m^{-2} s^{-1}. The average daytime and nighttime relative humidity levels were 56.8 and 82.1%, respectively. Additionally, the air vapor pressure deficit was maintained under 2.5 kPa to prevent stomatal closures on rice leaves. The soil water potential was also determined using the *Watermark Soil Moisture Sensor* with data logger (*6450WD*, *Spectrum Technologies*, Aurora, USA). The soil moisture sensor probe diameter was 2.1 cm and it was placed in 10 cm depth from the top of the soil. The pot was 22 cm high.

Evaluation of seedling drought stress responses: The responses of CSSL seedlings to drought stress were investigated after water was withheld from 25-d-old seedlings until the required FC was maintained (*i.e.*, 75 or 50%) for 0, 6, and 12 d. The analyzed traits were leaf drying score (LDS), leaf rolling score (LRS), chlorophyll (Chl) content, cell membrane stability (CMS), F_v/F_m, and PI.

LDS and LRS determination: The LDS was determined according to the standard evaluation system for rice (IRRI 1996, Table 1S, *supplement*), while Chl content was measured with the *SPAD-502 Plus* chlorophyll meter (*Konica Minolta*, UK). The LRS was based on a scale of one to five, which was adopted from O'Toole and Moya (1978), with one indicating a lack of rolling symptoms and five corresponding to a completely rolled leaf. The Chl content was measured at three positions on the youngest fully expanded leaves, and the average values were calculated.

CMS measurement: The CMS was determined by cutting the youngest fully expanded leaf (0.05 g) into smaller pieces and soaking in distilled water. After 2 h, electrical conductivity was measured with a digital conductivity meter *S230 SevenCompact*™ (*Mettler Toledo*, USA) (*i.e.*, EC_0 value). The leaf samples were subsequently autoclaved for 15 min, and electrical conductivity was measured again (*i.e.*, EC_1 value). The CMS was calculated using the following equation (Lal *et al.* 2008): CMS [%] = $[1 - (EC_0/EC_1)] \times 100$.

Relative water content (RWC): RWC was collected from the first fully expanded leaves. The cut leaves were immediately weighed to get fresh mass. Then, it was placed in 1.5-ml microcentrifuge tube filled with sterilized water for 24 h. Then, the leaves were weighed again for turgid mass. Finally, the leaf tissues were dried at 60ºC for three days to get dry mass. RWC was calculated as [(fresh mass − dry mass)/(turgid mass − dry mass)] × 100.

Predawn water potential: Water potentials were measured in first fully expanded leaves. Leaf total potential (Ψ) was determined at predawn (4:00–6:00 h) with a pressure chamber (*SoilMoisture Equipment*, USA). Afterwards, the leaf blade was placed in a plastic tube, immediately frozen in liquid nitrogen, and stored until the leaf osmotic potential (Ψ_π) was measured. To obtain leaf sap, the samples were thawed and the contents of the tube were pressed to squeeze out the sap for measurement of its osmolality using a *Vapro5520* osmometer (*Wescor*, USA). Ψ_π was calculated following the van't Hoff equation: $\Psi_\pi = -RTC_s$, where R is the gas constant ($R = 8.314\ 10^{-3}$ cm^3 MPa mol^{-1} K^{-1}), T [K] is the ambient temperature, and C_s is the total solute concentration [mmol kg^{-1}]. Leaf turgor potential (Ψ_p) was calculated as the difference between the total and osmotic potentials ($\Psi_p = \Psi_t - \Psi_\pi$).

Chl *a* fluorescence parameters: The ground fluorescence in the dark-adapted state (F_0), maximum Chl fluorescence at a saturating radiation pulse in the dark-adapted state (F_m), variable component relating to the maximum capacity for photochemical quenching (F_v), nonphotochemical maximum yield (F_0/F_m), maximum quantum efficiency of PSII (F_v/F_m), energy conversion efficiency absorbed (F_v/F_0), and photosynthesis performance index (PI) of the first fully expanded leaves were measured using the *Pocket PEA* chlorophyll fluorimeter (*Hansatech Instrument*, King's Lynn, UK) after plants were incubated in darkness for 30 min. PI was derived according to the Nernst equation. Three components, the force due to the concentration of active reaction centers, the force of the light reactions, which is related to the quantum yield of primary photochemistry and the force related to the dark reactions were used for the calculation (Strasser *et al.* 2000).

Leaf gas-exchange parameters and Chl *a* fluorescence: Leaf gas-exchange measurements for the first fully expanded leaves were recorded from 8:00–12:00 h using the *LI-6400* portable photosynthesis system (*LI-COR*, Lincoln, NE, USA) with the *LI-6400-40* leaf chamber fluorometer (*LI-COR*). The light-saturated net photosynthetic rate (P_{Nmax}) was analyzed under specific conditions as described by Utkhao and Yingjajaval (2015). Stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were also determined based on the P_{Nmax} data.

The F_v/F_m measurements were recorded for the first fully expanded leaves using a pulse amplitude-modulated fluorometer (*PAM2100*, *Heinz Walz*, Effeltrich, Germany) at predawn (*i.e.*, 4:30–5:30 h). Chl fluorescence was calculated as described by Cregg *et al.* (2004), with F_0 and F_m corresponding to the minimal and maximal fluorescence yields of the dark-adapted state, respectively, and F_v referring to variable fluorescence (*i.e.*, $F_v = F_m - F_0$).

The following Chl *a* fluorescence parameters were determined and calculated: effective quantum yield of PSII photochemistry [$\Phi_{PSII} = (F_m' - F_s)/F_m'$] as described by Genty *et al.* (1989), and electron transport rate (ETR = $\Phi_{PSII} \times$ PPFD $\times 0.5 \times 0.84$) according to Björkman and Demmig (1987), where F_m' corresponds to the maximal fluorescence yield of the light-adapted state, F_s refers to the steady-state fluorescence yield under light conditions, PPFD corresponds to the photosynthetic photon flux density (*i.e.*, 1,500 μmol m^{-2} s^{-1}), 0.5 is the proportion of the excitation energy distributed to PSII, and 0.84 is the fraction of the incident light absorbed by the leaf.

Genomic sequencing and bio-informatic analysis: Whole genome sequencing of CSSL and KDML105 rice was performed according to Chutimanukul *et al.* (2018). Briefly, genomic DNA was extracted and the genomic libraries were prepared for sequencing according to the *Illumina HiSeq200* protocol. The reference rice genome provided in the MSU database (Kawahara *et al.* 2013) was indexed using *SAMtools* (Li *et al.* 2009) and *BWA* (Li and Durbin 2009) was used to map the sequence reads to the reference genome. The single nucleotide polymorphisms (SNPs) between CSSL4 and KDML105 genome were identified according to Chutimanukul *et al.* (2018). The loci containing at least ten SNPs per locus were retrieved for gene enrichment analysis using

ClueGO (Bindea *et al.* 2009) obtained from *Cytoscape App Store* (Lotia *et al.* 2013). The loci containing SNPs within the drought-tolerant QTL on chromosome 1 was subjected to co-expression network analysis tool in *Rice Expression Database* (Xia *et al.* 2017) to find the connection between the genes in QTL region and other genes in the genome.

Statistical analysis: The seedling evaluations involved a randomized complete block design with four replicates. A completely randomized design was used for vegetative stage samples. Data were analyzed using *SPSS Statistics 20* software (*IBM Corp.*, Armonk, NY, USA). Analysis of variance and *Duncan*'s multiple range test were used to compare the means of parameters at $p=0.05$.

Results

Response to drought: To evaluate drought responses of CSSLs containing the putative drought tolerance genetic region between RM212 and RM5310 on chromosome 1, four CSSLs with a KDML105 genetic background were evaluated for LDS, LRS, Chl content, CMS, F_v/F_m, and PI at seedling stage (21-d-old plants). Values were compared with those for the KDML105 and DH212 donor rice parental lines. In the 100% FC, relative water content (RWC) of all lines was similar throughout the experimental period (Fig. 1*A*). The condition of 75% FC did not cause the difference in RWC between the lines as much as 50% FC did (Fig. 1*B*,*C*). The drought conditions at 75% and 50% FC resulted in significant increases in LDS and LRS in all tested rice lines, relative to the corresponding results for the 100% FC controls. At 75% FC, there were no significant differences in LDS between the tested lines during the 12-d drought treatment. However, at 50% FC, the lowest and highest LDS values after 6 d of drought treatment were observed for lines DH212 and CSSL1, respectively. After 12 d, CSSL4 and CSSL2 had the lowest and highest LDS values, respectively (Table 1). These results suggest the CSSLs differ in their responses to drought.

A significant leaf-rolling phenotype was observed in all lines exposed to drought conditions (50% FC) for 6 d. The KDML105 rice plants had the highest LRS values, which were significantly higher than the corresponding values for CSSL3 plants. However, after 12 d of treatment, the highest LRS values were observed for CSSL2, with scores that were significantly higher than those for CSSL3 and CSSL4. The LRS values of all lines exposed to 75% FC were similar. In contrast, drought stress at 75% FC resulted in Chl content differences, as determined by SPAD values. After a 6-d exposure to 75% FC, CSSL4 plants had the highest SPAD values, which were significantly higher than those of KDML105 plants. This finding implies that CSSL4 plants exhibit a greater ability to maintain Chl contents. A similar trend was observed in plants grown at 50% FC (Table 1). However, CSSL1 and CSSL2 plants were the least able to maintain stable Chl contents under drought-stress conditions.

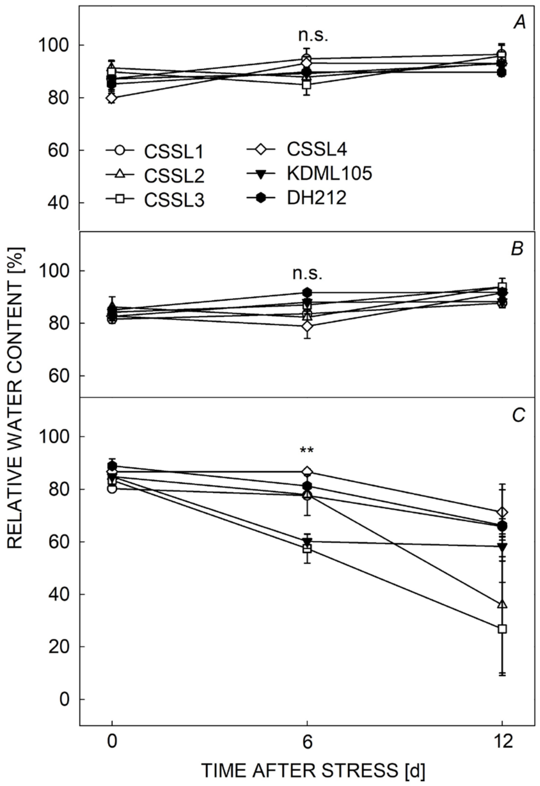

Fig. 1. Relative water content of leaves of chromosome segment substitution line rice plants (CSSL1–4) and their parental lines, KDML105 and DH212, at seedling stage under 100% field capacity (normal) (*A*), 75% field capacity (*B*), and 50% field capacity (*C*). Field capacity control was started when seedlings were 25-d old. Values are provided as the mean ± SE ($n = 4$). ** – significant difference between lines at $p<0.01$, ns – no significant difference was found between lines.

Cell membrane stability can be used to assess drought tolerance in many species, including rice (Beena *et al.* 2012, Iseki *et al.* 2014, Lima *et al.* 2015). Exposure to drought stress conditions led to decreased CMS in all lines. After 6 d of 50% FC stress, DH212 had the highest CMS value, which was significantly higher than the CMS value for KDML105. Additionally, CSSL2 and CSSL4 plants were able to maintain CMS levels better than the other lines. However, there were no significant differences in CMS at all FC levels between all lines after 12 d of drought treatment (Table 1).

Chl fluorescence and photosynthesis performance index: The F_v/F_m value for the first fully expanded leaf of all rice lines was approximately 0.8 throughout the experimental period under normal conditions (Fig. 2*A*). A slight decrease in the F_v/F_m value was detected under mild drought stress conditions (75% FC), while it decreased considerably at 50% FC (Fig. 2*B*,*C*). The F_v/F_m value of stressed CSSL4 leaves was higher than the corresponding values for the other lines after 6 d of mild drought conditions (Fig. 2*B*). Moreover, the F_v/F_m

Table 1. Responses of chromosome segment substitution line rice plants (CSSL1–4) and their parental lines, KDML105 and DH212, to drought stress. The leaf drying score (LDS) and leaf rolling score (LRS) are provided as the mean ± SE ($n = 4$). The SPAD and cell membrane stability (CMS) values are provided as the mean ± standard error ($n = 3$). FC – field capacity. 0, 6, 12 – day after treatment. *Different lowercase letters* indicate the significant difference between lines at $p<0.05$. The ns means no significant difference between lines at that time point.

FC	Line	Leaf drying score			Leaf rolling score			SPAD			Cell membrane stability [%]		
[%]		0^{ns}	6	12	0^{ns}	6	12	0	6	12	0	6	12^{ns}
100	CSSL1	0.50 ± 0.29	2.75 ± 0.63^{abc}	1.25 ± 0.63^{a}	1	1.00 ± 0.00^{a}	1.50 ± 0.29^{ab}	35.1 ± 0.54^{d}	31.5 ± 0.23^{abc}	32.5 ± 1.01^{abcde}	93.72 ± 0.19^{cde}	90.96 ± 1.01^{bc}	90.61 ± 0.69
	CSSL2	0.25 ± 0.25	2.00 ± 0.58^{ab}	2.00 ± 0.58^{abc}	1	1.00 ± 0.00^{a}	1.75 ± 0.25^{abc}	34.0 ± 0.43^{bcd}	33.3 ± 0.41^{abcd}	32.4 ± 0.62^{abcde}	92.40 ± 0.08^{ab}	91.60 ± 0.48^{bc}	92.27 ± 0.12
	CSSL3	0.50 ± 0.29	2.00 ± 0.58^{ab}	0.75 ± 0.25^{a}	1	1.00 ± 0.00^{a}	1.50 ± 0.29^{ab}	34.8 ± 0.29^{d}	32.3 ± 2.29^{abcd}	37.6 ± 3.33^{e}	91.53 ± 0.68^{a}	91.16 ± 0.26^{bc}	93.42 ± 0.13
	CSSL4	0	1.25 ± 0.63^{a}	0.75 ± 0.25^{a}	1	1.00 ± 0.00^{a}	1.25 ± 0.25^{a}	35.1 ± 0.54^{d}	35.4 ± 0.74^{bcdef}	33.9 ± 0.84^{cde}	93.54 ± 0.14^{cde}	92.10 ± 0.97^{bc}	93.44 ± 0.87
	KDML105	0.75 ± 0.48	1.75 ± 0.75^{ab}	1.50 ± 0.87^{ab}	1	1.00 ± 0.00^{a}	1.25 ± 0.25^{a}	33.9 ± 0.60^{bcd}	33.7 ± 0.86^{abcde}	33.8 ± 1.76^{cde}	93.79 ± 0.39^{cdef}	92.55 ± 0.47^{c}	91.47 ± 0.30
	DH212	0.75 ± 0.25	1.25 ± 0.63^{a}	1.25 ± 0.63^{a}	1	1.00 ± 0.00^{a}	1.25 ± 0.25^{a}	35.6 ± 1.13^{d}	37.2 ± 0.30^{def}	35.5 ± 2.32^{de}	94.31 ± 0.04^{ef}	93.08 ± 0.50^{c}	91.69± 0.63
75	CSSL1	0.50 ± 0.29	3.00 ± 0.82^{abcd}	2.00 ± 0.58^{abc}	1	1.50 ± 0.29^{ab}	2.25 ± 0.48^{abc}	31.9 ± 1.24^{ab}	35.0 ± 1.83^{bcdef}	27.5 ± 2.17^{ab}	92.99 ± 0.48^{bcd}	92.79 ± 0.58^{c}	90.17 ± 0.69
	CSSL2	0.75 ± 0.25	2.00 ± 0.58^{ab}	2.00 ± 0.58^{abc}	1	1.25 ± 0.25^{ab}	1.75 ± 0.25^{abc}	35.3 ± 0.94^{d}	32.6 ± 1.37^{abcd}	29.1 ± 1.64^{abc}	93.57 ± 0.35^{cde}	90.54 ± 0.76^{bc}	92.17 ± 0.39
	CSSL3	0.50 ± 0.29	2.00 ± 0.58^{ab}	1.25 ± 0.25^{a}	1	1.75 ± 0.25^{abc}	1.50 ± 0.29^{ab}	34.4 ± 0.32^{cd}	36.5 ± 0.78^{cdef}	32.7 ± 1.05^{abcde}	92.39 ± 0.24^{ab}	92.30 ± 0.09^{bc}	92.76 ± 0.38
	CSSL4	0.50 ± 0.29	2.25 ± 1.11^{ab}	2.75 ± 0.63^{abc}	1	1.50 ± 0.29^{ab}	2.25 ± 0.63^{abc}	36.0 ± 0.57^{d}	39.0 ± 0.65^{ef}	34.4 ± 1.54^{cde}	94.76 ± 0.49^{f}	92.61 ± 1.06^{c}	91.11 ± 0.33
	KDML105	0.75 ± 0.25	2.50 ± 0.50^{abc}	2.50 ± 1.50^{abc}	1	2.00 ± 0.41^{abc}	2.00 ± 0.41^{abc}	34.0 ± 0.54^{bcd}	32.3 ± 0.07^{abcd}	32.0 ± 1.79^{abcde}	93.79 ± 0.29^{cdef}	92.68 ± 0.74^{c}	92.30 ± 0.53
	DH212	0.75 ± 0.47	2.00 ± 0.41^{ab}	2.25 ± 0.75^{abc}	1	1.25 ± 0.25^{ab}	1.75 ± 0.25^{abc}	35.8 ± 0.26^{d}	33.8 ± 1.54^{abcde}	35.9 ± 1.80^{de}	92.81 ± 0.12^{bc}	92.13 ± 1.11^{bc}	90.35 ± 0.51
50	CSSL1	0.25 ± 0.25	5.50 ± 0.65^{e}	6.50 ± 0.96^{de}	1	3.75 ± 0.25^{e}	3.75 ± 0.48^{de}	31.5 ± 0.54^{a}	31.6 ± 2.49^{abc}	26.8 ± 1.59^{a}	94.08 ± 0.43^{ef}	83.79± 8.05^{bc}	90.27 ± 0.83
	CSSL2	0.50 ± 0.29	5.00 ± 1.08^{de}	7.00 ± 1.22^{e}	1	3.75 ± 0.25^{e}	4.50 ± 0.29^{e}	32.5 ± 0.31^{abc}	39.9 ± 3.40^{f}	26.6 ± 3.84^{a}	93.94 ± 0.08^{def}	91.98 ± 0.16^{bc}	74.18 ± 17.95
	CSSL3	0.50 ± 0.29	5.00 ± 0.82^{de}	5.00 ± 1.41^{cde}	1	2.50 ± 0.65^{cd}	3.00 ± 0.91^{bcd}	31.0 ± 0.72^{a}	30.5 ± 3.53^{ab}	33.1 ± 2.90^{bcde}	92.88 ± 0.51^{bc}	45.94 ± 4.03^{a}	68.15 ± 22.20
	CSSL4	0.50 ± 0.29	4.50 ± 0.50^{cde}	3.75 ± 1.11^{abcd}	1	3.25 ± 0.48^{de}	2.25 ± 0.25^{abc}	35.3 ± 0.62^{d}	35.4 ± 0.41^{bcdef}	35.9 ± 1.59^{de}	93.97 ± 0.11^{def}	90.79 ± 0.33^{bc}	92.19 ± 0.69
	KDML105	0.75 ± 0.25	4.00 ± 0.71^{bcde}	5.00 ± 1.41^{cde}	1	4.00 ± 0.29^{e}	3.00 ± 0.85^{cde}	31.6 ± 0.70^{a}	28.8 ± 1.80^{a}	35.5 ± 0.90^{de}	92.89 ± 0.32^{bc}	82.01 ± 8.75^{b}	89.59 ± 2.11
	DH212	0.75 ± 0.48	3.25 ± 0.85^{abcd}	4.50 ± 0.96^{bcde}	1	2.25 ± 0.63^{bc}	3.25 ± 0.48^{cde}	35.6 ± 0.92^{d}	31.2 ± 1.17^{abc}	31.1 ± 1.27^{abcd}	93.57 ± 0.26^{cde}	92.64 ± 0.76^{c}	72.22 ± 16.43

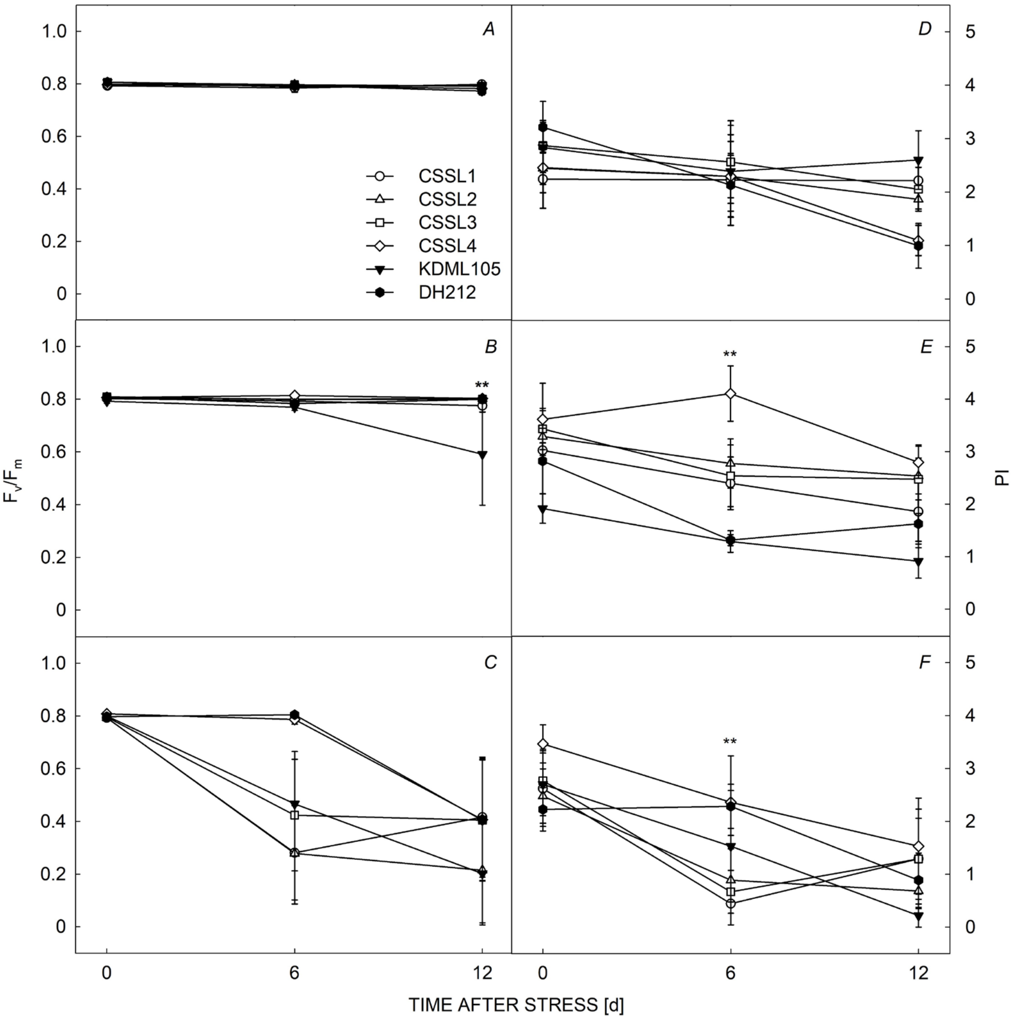

Fig. 2. Maximal quantum yield of PSII photochemistry (F_v/F_m) (*A–C*) and photosynthesis performance index (PI) (*D–F*) for chromosome segment substitution lines (CSSLs) 1–4 and their parental lines, KDML105 and DH212, under normal (100% field capacity) (*A*,*D*), mild drought stress (75% field capacity) (*B*,*E*), and drought stress (50% field capacity) (*C*,*F*) conditions. Values are provided as the mean ± SE ($n = 4$). ** – significant difference between lines at $p<0.01$.

values for CSSL4 and DH212 were relatively stable after 6 d at 50% FC, whereas the values for KDML105 and CSSL1–3 rapidly decreased (Fig. 2*C*).

Drought stress caused the reduction in the minimal fluorescence yield of the dark-adapted state (F_0), the maximal fluorescence yield of the dark-adapted state (F_m) and variable fluorescence (F_v), including the area above the fluorescence curve in all plant lines tested (Table 2). Although these lines had the significantly different F_0, F_m, and F_v at the beginning of the experiment (day 0), the maximal quantum yield of PSII photochemistry (F_v/F_m), energy conversion efficiency absorbed (F_v/F_0), and nonphotochemical maximum yield (F_0/F_m) of all lines were similar, suggesting a similar PSII efficiency among these lines in normal conditions. After 6 d of drought stress, F_m of CSSL4 and DH212 was higher than those of other lines, while F_0 of all lines was similar, leading to the significantly higher maximal quantum yield of PSII photochemistry of CSSL4 and DH212 (Table 2; Fig. 2*B*,*C*). Moreover, energy conversion efficiency absorbed (F_v/F_0) of these both lines was higher, while the nonphotochemical maximum yield of these two lines was lower than that of others.

The changes in PI were similar to those in F_v/F_m. No significant difference in PI was observed for seedlings grown at 100% FC (Fig. 2*D*). In contrast, after exposure to 75% FC for 6 d, CSSL4 plants had significantly higher PI values than KDML105 plants (Fig. 2*E*). At 50% FC, the differences in PI values between all lines were less pronounced, although the CSSL4 plants had higher values than the other lines (Fig. 2*F*).

Chl *a* fluorescence: In order to evaluate the response in more details, the vegetative stages of all lines were investigated. Drought stress response of rice plants at vegetative stage were evaluated by stopping watering when the plants were 40 d old. Withholding of water caused the decrease of soil matric potential (Ψ_{tsoil}) as shown in Fig. 3. Soil matric potential started to decrease after 3 d of water withholding. It reached

Table 2. Photochemical indices of chromosome segment substitution line rice plants (CSSL1–4) and their parental lines, KDML105 and DH212, after drought stress at 50% FC for 0, 6, and 12 d. *Different lowercase letters* indicate the significant difference between lines at $p<0.05$. The ns means no significant difference between lines at that time point.

Photochemical indices	Drought stress [d]	CSSL1	CSSL2	CSSL3	CSSL4	KDML105	DH212
Area (× 10^3)	0	451.9^{ab}	454.0^{ab}	479.6^{ab}	551.2^{bc}	459.4^{ab}	469.7^{ab}
	6	94.2^{a}	110.3^{ab}	156.4^{abc}	317.7^{cde}	209.2^{abcd}	378.2^{de}
	12	163.3^{abcd}	78.4^{ab}	156.2^{abcd}	167.8^{abcd}	44.3^{a}	86.1^{abc}
F_0 (× 10^3)	0	5.42^{abcd}	5.55^{abcde}	5.46^{abcd}	5.57^{abcde}	5.66^{abc}	6.05^{e}
	6^{ns}	5.90	5.78	5.52	5.54	6.17	5.76
	12	4.96^{bc}	2.60^{a}	3.62^{abc}	4.18^{abc}	3.24^{ab}	5.20^{c}
F_m (× 10^3)	0	26.28^{a}	26.95^{ab}	26.77^{a}	29.01^{bcde}	28.39^{abcd}	29.87^{cde}
	6	11.67^{a}	12.40^{a}	16.13^{a}	$26.72b^{c}$	18.06^{ab}	29.45^{c}
	12	15.81^{b}	7.87^{a}	13.71^{b}	15.51^{b}	7.42^{a}	15.85^{b}
F_v (× 10^3)	0	20.86^{a}	21.39^{ab}	21.31^{ab}	23.44^{bcde}	22.72^{abcde}	23.82^{cde}
	6	5.77^{a}	6.62^{a}	10.62^{a}	21.18^{c}	11.89^{ab}	23.68^{c}
	12	10.85^{abc}	5.27^{ab}	10.09^{abc}	11.33^{abc}	4.19^{a}	10.65^{abc}
F_0/F_m	0^{ns}	0.207	0.207	0.204	0.192	0.200	0.203
	6	0.720^{b}	0.722^{b}	0.576^{b}	0.214^{a}	0.534^{b}	0.196^{a}
	12	0.584^{ab}	0.785^{b}	0.596^{ab}	0.593^{ab}	0.798^{b}	0.596^{ab}
F_v/F_m	0^{ns}	0.793	0.793	0.796	0.808	0.800	0.797
	6	0.281^{a}	0.278^{a}	0.424^{a}	0.787^{b}	0.466^{a}	0.804^{b}
	12	0.416^{ab}	0.215^{a}	0.404^{ab}	0.407^{ab}	0.202^{a}	0.404^{ab}
F_v/F_0	0^{ns}	3.866	3.857	3.909	4.209	4.018	3.949
	6	1.043^{a}	1.240^{a}	1.893^{ab}	3.774^{cd}	2.168^{abc}	4.121^{d}
	12	2.146^{ab}	1.101^{a}	2.011^{ab}	2.101^{ab}	0.926^{a}	2.045^{ab}

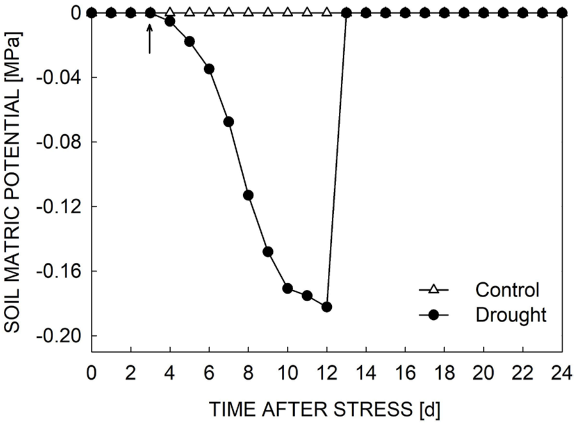

Fig. 3. Soil matric potential in normal and drought treated pots by water withholding. *An arrow* indicates the last point before the decrease of soil matric potential.

–0.2 MPa after 9 d. After 15 d, plants were rewatered and soil matric potential increased.

Drought increased F_0 of all rice lines, but only F_0 of CSSL4 could be adjusted to the original level after 15 d of water withholding (Fig. 4*A*). Both CSSL4 and DH212 could maintain F_m during 15 d of drought, while the other two lines, CSSL1 and KDML105, could not. Significantly lower F_m of CSSL1 and KDML105 was detected after 15 d of drought stress (Fig. 4*B*). The F_v/F_m value remained at 0.82–0.83 in all plants during the vegetative stage under normal conditions (*i.e.*, watered regularly). Withholding water for 12 d led to a minor decrease in F_v/F_m values, while a more obvious decrease was observed after 15 d of drought treatment. The PSII efficiency of CSSL4 and DH212 plants was significantly different from that of KDML105 and CSSL1 plants after 15 d of drought stress (Fig. 4*C*). CSSL4 and DH212 showed the ability to maintain F_v/F_0 (Fig. 4*D*) and F_0/F_m (Fig.4*E*), while CSSL1 and KDML105 did not have this ability.

Leaf gas exchange: The P_{Nmax} of all rice lines started to decrease 3 d after watering was stopped and decreased further after 6 and 9 d. The P_{Nmax} values for CSSL1 and CSSL4 were higher than the corresponding values for their parents after 9 d of drought stress. Additionally, there were no significant differences in P_{Nmax} between lines at all time points. However, the P_{Nmax} for all lines subjected to drought stress for 12 d decreased and eventually reached 0 (Fig. 5*A*).

The g_s and E values exhibited similar patterns throughout the study period. Although DH212 and the CSSLs had higher g_s and E values than that of

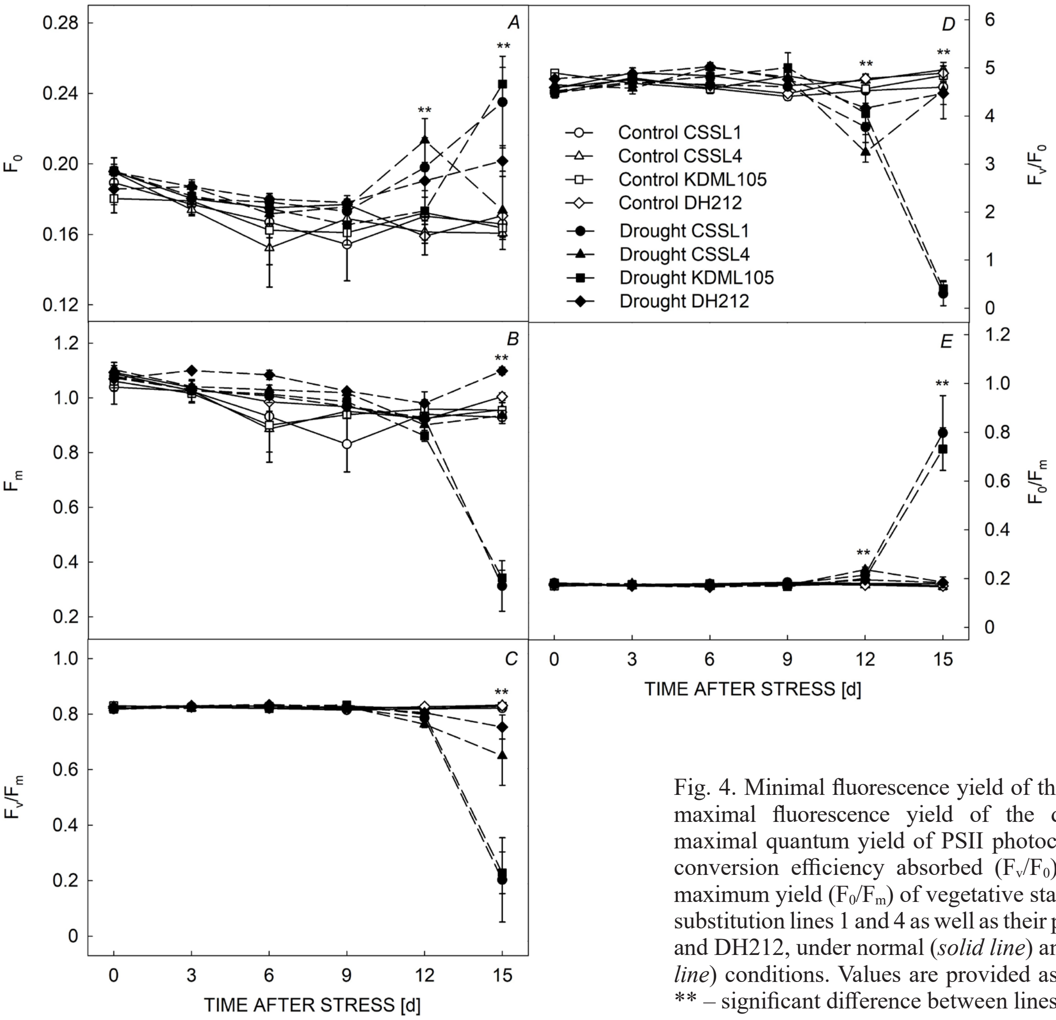

Fig. 4. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), maximal quantum yield of PSII photochemistry (F_v/F_m), energy conversion efficiency absorbed (F_v/F_0), and nonphotochemical maximum yield (F_0/F_m) of vegetative stage chromosome segment substitution lines 1 and 4 as well as their parental lines, KDML105 and DH212, under normal (*solid line*) and drought stress (*dashed line*) conditions. Values are provided as the mean ± SE (*n* = 4). ** – significant difference between lines at $p<0.01$.

KDML105 throughout the 12 d of drought stress, the differences were not significant (Fig. 5*B*,*D*). The g_s values of all lines increased by 28–34% 3 d after watering was stopped, but subsequently decreased. The smallest and largest increases in g_s were observed in CSSL4 and KDML105, respectively (Fig. 5*B*). Additionally, a considerable decrease in g_s values occurred in rice plants exposed to drought conditions for 9 d, resulting in decreases in *E* and P_{Nmax} values (Fig. 5*A*,*B*,*D*). Interestingly, the g_s values of all rice lines after 12 d of drought stress were unchanged from the values at 9 d after treatment, while the P_{Nmax} values decreased.

The most stable parameter was C_i. Under drought conditions, C_i was maintained at 300–350 µmol(CO_2) mol^{-1}. It decreased 3 d after watering was stopped, but rebounded to 370–390 µmol(CO_2) mol^{-1} after 12 d of treatment. No significant differences were observed between lines under normal and drought conditions (Fig. 5*C*). The ETR and Φ_{PSII} values represent the electron transport activity during photosynthesis, and were relatively stable during the early drought treatment period (*i.e.*, 0–6 d). However, after a prolonged period of drought stress, both ETR and Φ_{PSII} decreased considerably, although the CSSL4 plants maintained ETR and Φ_{PSII} values better than the other lines (Fig. 5*E*,*F*).

ETR/P_{Nmax} of rice plants under drought stress was investigated. After 6 d of water withholding, no significant difference in ETR/P_{Nmax} between tested lines was found, but after that ETR/P_{Nmax} started to increase and it was significantly higher than ETR/P_{Nmax} of the plants grown in normal condition after 9 d of water withholding. After 12 d of drought stress, ETR/P_{Nmax} of KDML105 rice was the highest and that of CSSL1 was the lowest (Fig. 5*G*). P_{Nmax}/C_i ratio responded in the opposite direction to ETR/P_{Nmax} ratio under drought stress. Drought caused the decrease in P_{Nmax}/C_i ratio and became the lowest after 12 d of water withholding. The significantly lower P_{Nmax}/C_i ratio due to drought stress was found after 9 d of water withholding (Fig. 5*H*).

As CSSL4 showed the higher drought ability than KDML105 rice, the investigation of water potential of this line in comparison with parental lines was done. Predawn leaf water potential (Ψ) of CSSL4 was similar to parental lines (Fig. 6*A*). Solute accumulation was increased by drought stress in all lines and DH212 had the highest level of solute accumulation (Fig. 6*B*), which led to the lowest osmotic potential (Ψ_π) of DH212 (Fig. 6*C*). After 14 d of drought stress, DH212 was the

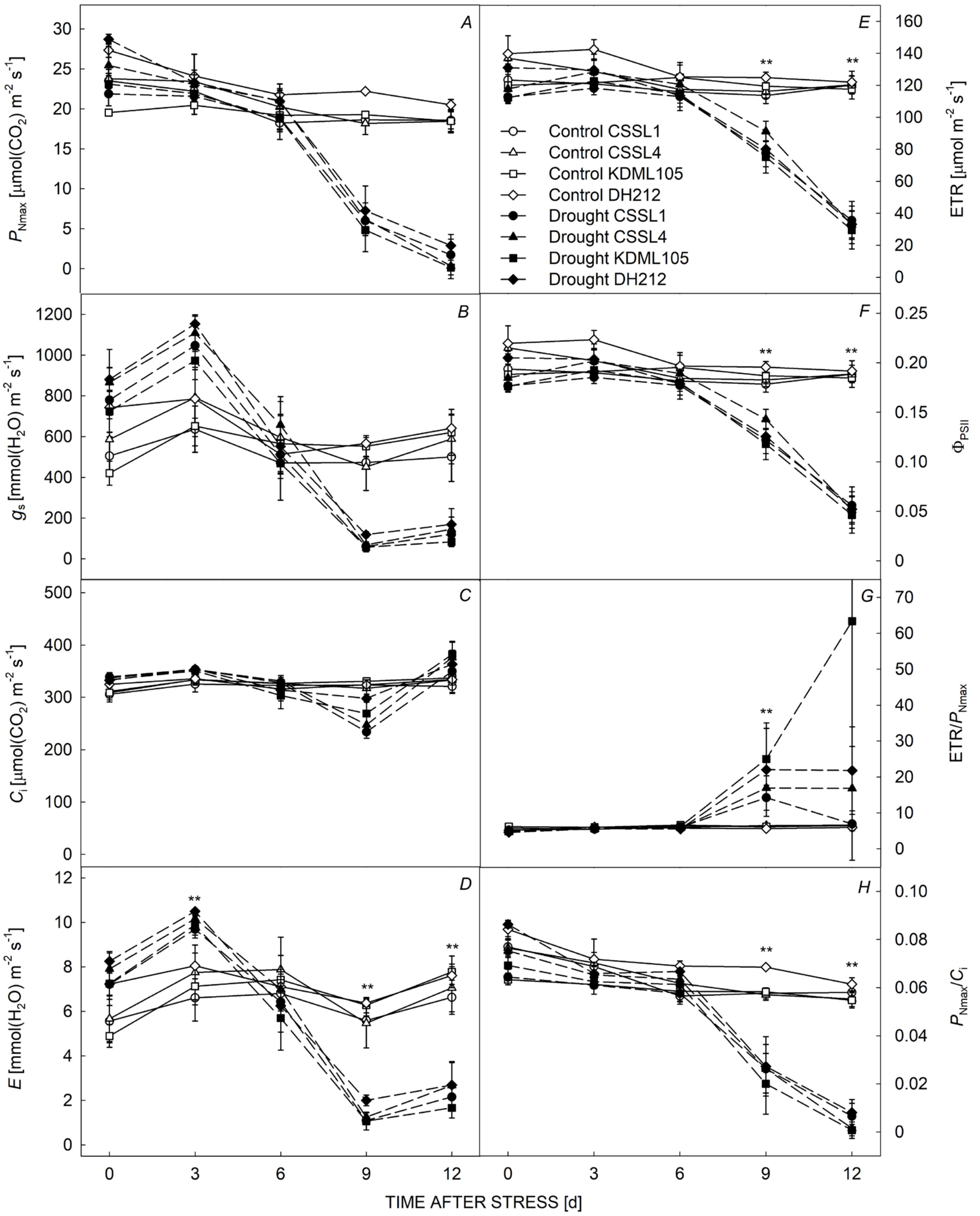

Fig. 5. Light-saturated net photosynthetic rate (P_{Nmax}) (*A*), stomatal conductance (g_s) (*B*), intercellular CO$_2$ concentration (C_i) (*C*), transpiration rate (*E*) (*D*), electron transport rate (ETR) (*E*), effective quantum yield of photosystem II photochemistry (Φ_{PSII}) (*F*), ETR/P_{Nmax} ratio (*G*), and P_{Nmax}/C_i ratio (*H*) of 40-d-old vegetative stage chromosome segment substitution line plants (1 and 4) as well as KDML105 and DH212 rice plants under normal (*solid line*) and drought stress (*dashed line*) conditions. Day 0 was the first day water was withheld from plants. Values are provided as the mean ± SE (*n* = 3). ** – significant difference between lines at $p<0.01$.

only line that could maintain turgor pressure, indicating by the positive pressure potential (Ψ_p) (Fig. 6*D*).

Genome comparison and the drought-tolerant gene prediction: The genomic sequences of CSSL4 and KDML105 rice were compared to identify SNP difference between two genomes. The dense clusters of SNPs were on chromosome 1, especially with the drought-tolerant QTL from DH212. However, SNPs were also distributed on other chromosomes. A total of 3485 loci containing more than 10 SNPs per locus were listed in Table 2S, *supplement*.

All loci containing more than 10 SNPs per locus were subjected for gene enrichment analysis to identify the biological process involved in these loci using *ClueGO*. These loci are enriched in six biological processes;

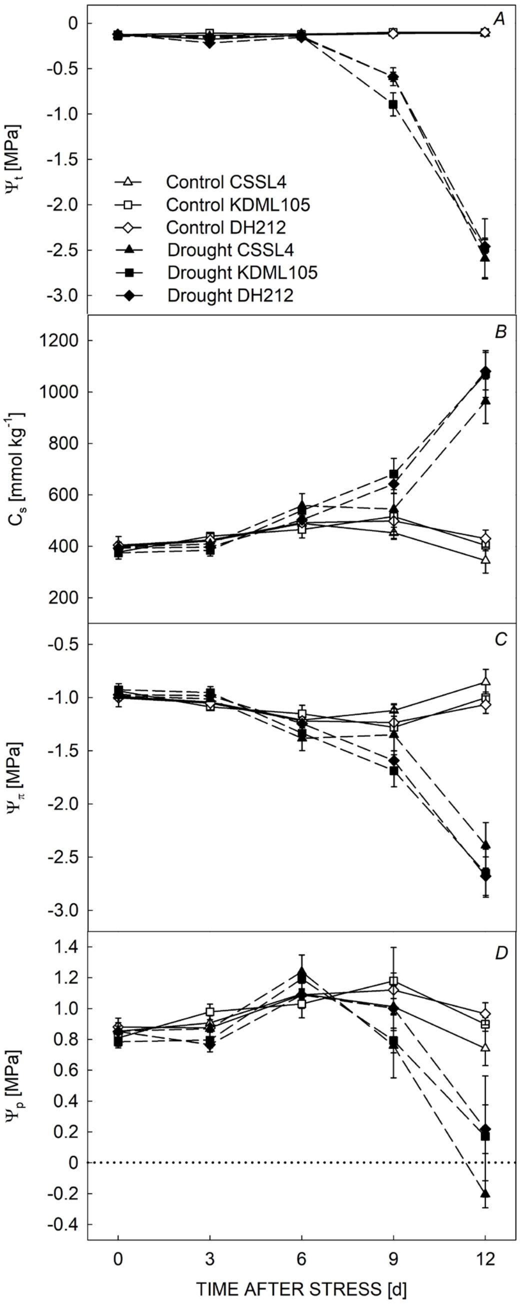

Fig. 6. Leaf water potential (Ψ_t) (*A*), solute accumulation (C_s) (*B*), osmotic potential (Ψ_π) (*C*), and pressure potential (Ψ_p) (*D*) of CSSL4, KDML105 and DH212 rice lines, measured at predawn in normal (*solid line*) and drought stress (*dashed line*) conditions. Values are provided as the mean ± SE ($n = 4$).

nucleobase-containing compound metabolic process, cellular protein modification process, transport, carbohydrate metabolic process, proteolysis, and generation of precursor metabolites and energy (Fig. 8).

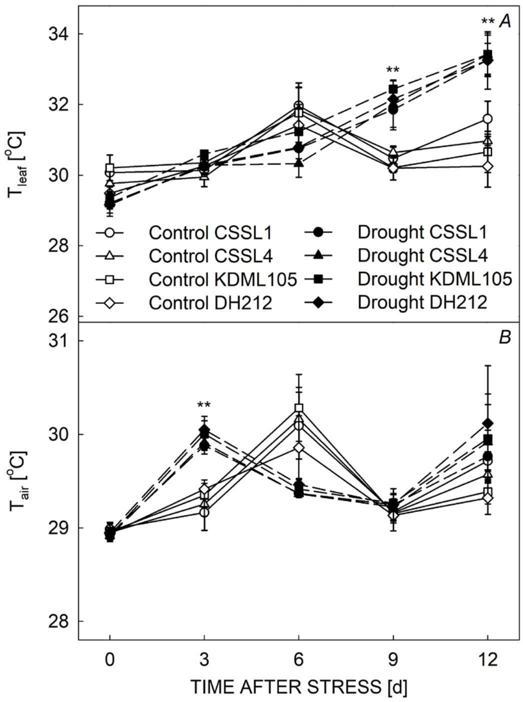

Fig. 7. Leaf temperature (T_{leaf}) (*A*) and air temperature (T_{air}) (*B*) of CSSL1, CSSL4, KDML105, and DH212 rice lines during the experimental period of plants at vegetative stage in normal (*solid line*) and drought stress (*dashed line*) conditions. Values are provided as the mean ± standard error ($n = 3$). ** – significant difference between lines at $p<0.01$.

In order to predict the crucial gene responsible for the drought-tolerant phenotype in CSSL4, loci with at least ten SNPs on chromosome 1 were subjected to co-expression analysis with *Pearson*'s *r* value of 0.99 to identify the potential genes involved in drought tolerance. The co-expression network was shown in Fig. 9. Interestingly, the highly correlated expression was found between *LOC_Os01g71240*, calmodulin-stimulated calcium-ATPase, and *LOC_Os02g44120*, which is zinc finger transcription factor with C2H2-type domain.

Discussion

Based on seedling morphological and physiological responses, CSSL4 may be considered a drought-tolerant line. It had higher PI values (Fig. 2*E*) and maintained F_v/F_m values (Fig. 2*B*) better than KDML105 rice seedlings under drought stress conditions. Moreover, F_m of CSSL4 was higher than KDML105 and CSSL1 (Table 2). This phenomenon was similar to that found in rice under high light stress, thus, Chl *a* fluorescence parameters can be used to distinguish between the stress-tolerant and stress-susceptible lines (Mirshad and Puthur 2016, Faseela and Puthur 2017). The photosynthetic characteristics of CSSL1 and CSSL4 plants were compared to those of their parents, KDML105 and DH212, to investigate whether the maintenance of PSII efficiency under drought stress conditions at the seedling stage continued in the vegetative stage (*i.e.*, tillering). Analysis of Chl fluorescence under drought stress conditions during the seedling and vegetative stages revealed that

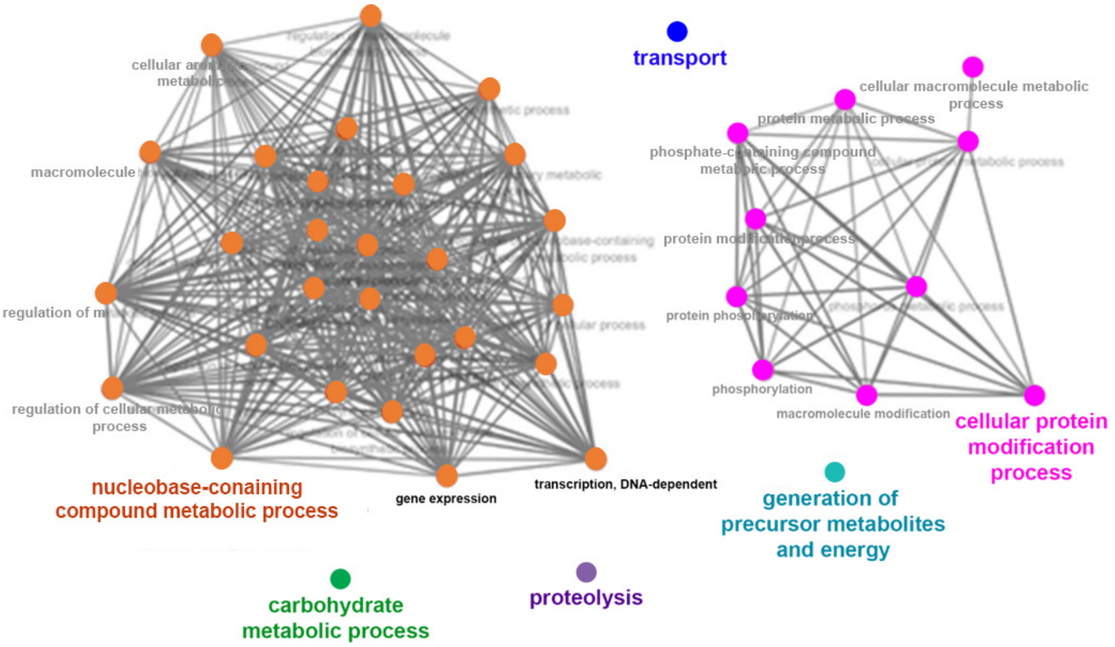

Fig. 8. Gene enrichment analysis of the loci containing more than ten single nucleotide polymorphisms (SNPs) per locus using *ClueGO* (Bindea *et al*. 2009).

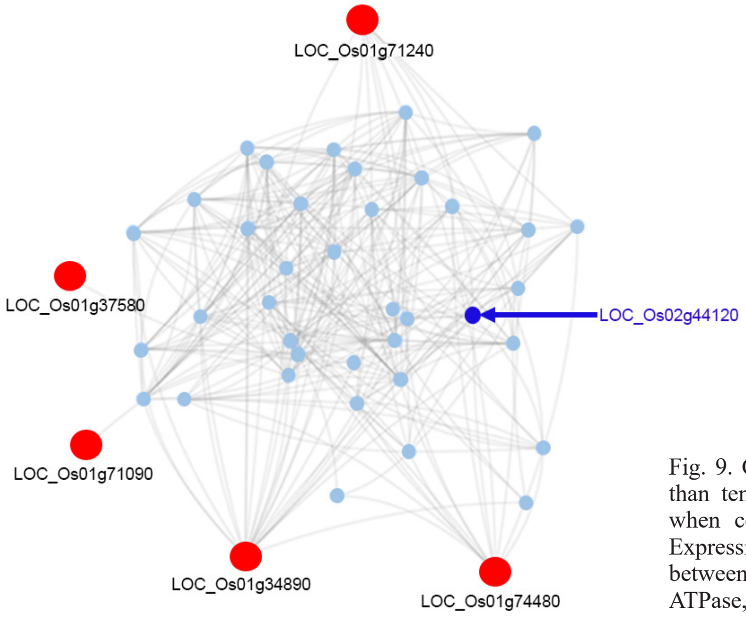

Fig. 9. Co-expression network analysis of the genes with more than ten single nucleotide polymorphisms (SNPs) in CSSL4, when compared to KDML105 rice, using the data in Rice Expression Database (Xia *et al*. 2017) showing the connection between LOC_Os01g71240, calmodulin-stimulated calcium ATPase, and LOC_Os02g44120, C2H2 zinc finger protein.

CSSL4 plants maintain F_v/F_m values much better than KDML105 or CSSL1 plants. The apparent decrease in F_v/F_m values observed in rice plants subjected to severe drought stress is consistent with the results of a previous study (Souza *et al*. 2004). Moreover, the reduction of area above the fluorescence curve and F_m was similar to the response of *Bruguiera cylindrica* (L.) Blume under salt stress (Palliyath and Puthur 2018). In wheat, the high-yielding varieties under drought stress were shown to have higher F_m, F_v/F_m, and F_v (Paknejad *et al* 2007) and Chl fluorescence can be used to determine drought tolerance in barley (Li *et al* 2006).

With increasing soil water stress, the decrease in F_v/F_m values was much smaller than that of P_{Nmax} values, suggesting CSSLs respond to drought stress by stabilizing PSII efficiency. The results of an investigation of turf grass [*Stenotaphrum secundatum* (Walt.) Kuntze] indicate that drought stress affects photosynthesis and plant growth more than photosystem activities (Lu *et al*. 2013). A previous study of *Coffea robusta* confirmed that PSII efficiency is more stable than P_N under drought-stress conditions (Da Matta *et al*. 1997). Although several reports have implied that Chl fluorescence is a reliable indicator of plant status following exposure to stress (Schreiber *et al.* 1994, Baker and Rosenqvist 2004, Li *et al.* 2006), it is not representative of the whole photosynthetic process (Kositsup *et al.* 2013).

The P_{Nmax} values progressively declined during the 12 d of drought treatment, while the lowest g_s value occurred after 9 d. These results imply that stomatal behavior is not the only factor limiting P_{Nmax}. The ETR and Φ_{PSII} values also continuously decreased under drought conditions (Fig. 3*E*,*F*), suggesting that ETR and Φ_{PSII} are major factors affecting the decrease in P_{Nmax} during prolonged periods of drought stress.

The increase in g_s and E after 3 d of water withholding was unexpected. Therefore, we checked leaf temperature and air temperature during our experimental period and found that leaf temperature of all lines was similar (Fig. 7*A*), but air temperature was higher in the drought-treated ones (Fig. 7*B*). After water withholding for 3 d, soil matric potential remained at the same level as normal condition and declined afterward. Therefore, it was possible that with the higher air temperature and availability of water in soil, plants adapted by widen the stomatal opening and increasing of transpiration. Drought stress responses in rice occur in a specific order, with stomatal closure being the first step (Chaves 1991, Subrahmanyam *et al.* 2006). This results in limited photosynthetic activities during exposure to mild or moderate drought stress (Flexas and Medrano 2002, Medrano *et al.* 2002), although the light reaction and Chl fluorescence parameters (*i.e.*, F_v/F_m, ETR, and Φ_{PSII}) are unaffected. These observations confirm that ETR and Φ_{PSII} are resistant to mild drought stress (Kaiser *et al.* 1981, Sharkey and Badger 1982, Dias and Brüggemann 2010). Under more severe drought conditions, the light reaction of photosynthesis is clearly affected, as indicated by decreased ETR and Φ_{PSII} values, leading to decreased photosynthetic activities. Prolonged exposure to drought stress downregulates PSII functions and damages the associated apparatus, resulting in near fatal consequences for rice plants. However, CSSL4 plants have slightly higher ETR and Φ_{PSII} values than other rice lines, suggesting they are better able to maintain a functional photosystem apparatus. Moreover, when we calculated for ETR/P_{Nmax} ratio, the value of KDML105 rice was higher than that of CSSLs and DH212, indicating the difference in the reducing power usage from electron transport. These data suggested that KDML105 rice had the higher photorespiration rate than other lines. The alternate electron sink could help plants to cope with the excess of electron flow due to stomatal closure in drought conditions (Peguero-Pina *et al.* 2009). The lower ETR/P_{Nmax} ratio in CSSLs suggested the lower oxidative stress. Drought stress can cause oxidative stress. The lower of electron transport can avoid reactive oxygen species (ROS) generation (Geissler *et al.* 2015). The reduction of ETR/P_{Nmax} ratio in CSSLs implies that CSSLs have the mechanism of oxidative stress avoidance under drought conditions.

The consistent phenotypic responses to maintain PSII efficiency at seedling and vegetative stages under drought stress implies that the adaptive mechanisms in photosynthesis process in both stages are similar. The evaluation of photosynthesis parameters could be done more efficiently at vegetative stage due to the larger leaf size, and the response in details, such as P_{Nmax}, g_s, E, C_i, ETR, and Φ_{PSII} can be done. The difference response could be detected after 9 d of water withholding. However, no significant differences between lines could be detected. In contrast, the evaluation with F_v/F_m in plants at seedling or vegetative stages revealed the significant differences of PSII efficiency. However, the difference was detected after 15 d of drought treatment, which was later after photosynthesis decline. These suggested that it was convenient to use F_v/F_m as the parameter for drought tolerance screening with the precaution for the appropriate timing as it is not the fast response, compared to other parameters involved in photosynthesis.

The gene enrichment of the loci containing SNPs different between CSSL4 and KDML105 revealed that the regulation of gene expression at transcriptional level and protein modification should be the important processes responsible for drought-tolerant phenotype of CSSL4. This was also supported by the co-expression network analysis, showing the co-expression of *LOC_Os01g71240*, calmodulin-stimulated calcium-ATPase, and *LOC_Os02g44120*, which is ZOS2-13 – C2H2 zinc finger transcription factor. C2H2 zinc finger proteins have been reported to be involved in abiotic stress responses including drought (for review *see* Wang *et al.* 2019). This type of transcription factor was the target of miRNA to regulate drought tolerance in upland rice cultivar, KMJ 1-12-3 (Awasthi *et al.* 2019). Moreover, OsDRZ1, which is C2H2 zinc finger protein was reported to be the transcriptional repressor and regulate drought tolerance (Yuan *et al.* 2018). Based on transcriptome analysis of the *OsDRZ1* overexpression line, 317 genes were downregulated and approximately 50% of them were also downregulated by drought stress, suggesting that OsDRZ1 may have the transcriptional repression activity to drought responsive genes. *OsGLP1* (*LOC_Os08g35760*), encoding cupin protein, was shown to be one of the target gene of OsDRZ1 (Yuan *et al.* 2018).

In conclusion, our results indicate that CSSL4 plants possess adaptive mechanisms related to the maintenance of photosynthetic activities and PSII efficiency that provide drought stress tolerance. These were supported by the responses of Chl *a* fluorescence parameters detected during drought stress. Additionally, the analyzed regions located on chromosome 1 suggested the association with photosynthetic processes, especially related to the light reaction potentially by the regulation of C2H2 zinc finger protein *via* Ca^{2+} signaling. Therefore, CSSL4 plants represent a valuable genetic resource useful for rice breeding programs, and provide new insights into plant responses to drought stress. Future studies on the effects of rewatering on leaf gas exchange, chlorophyll fluorescence, and rice yields in drought-stressed CSSL4 plants are warranted.

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