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Classification and characteristics of heat tolerance in *Ageratina adenophora* populations using fast chlorophyll a fluorescence rise O-J-I-P

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ABSTRACT

Croftonweed originated from Mexico is a worldwide notorious invasive weed. The objectives of this study were to screen heat tolerance in different croftonweed populations, determine the effect of heat stress on two photosystems and probe the mechanism of acquired heat tolerance. According to conventional tests of plant injury and fast chlorophyll fluorescence rise kinetics, four different croftonweed populations collected from South China were successfully classified into three categories by exposing whole plants to heat treatment at 40 °C: sensitive, intermediate, tolerant. Evidence from the JIP-test indicated that inhibition of the oxygen evolution complexes (OEC) and inactivation of PSII reaction centers (RCs) were the primary cause of heat damage. In mild heat stress (<40 °C), slightly damaged the OEC without creating a visible K-step in the fluorescence rise OJIP curve. In moderate heat stress or stronger (≥40 °C), a pronounced K-step due to irreversible severe damage on the OEC occurred. Additionally, inactivation of PSII RCs, down-regulation of energetic connectivity of PSII units, destruction of PSII antenna architecture, losing overall photosynthetic activity of PSII, increase of PSI activity also took place. Furthermore, the tolerant population had lesser damage degree on photosynthetic capacity relative to intermediate and sensitive populations. Finally, a reliable model, based on the most sensitive parameter PI_{ABS} and V_K as a characteristic parameter for heat stress, is presented for ranking and identifying heat tolerance in different croftonweed populations. The heat sensitivity index (HSI) is also introduced as an indicator of plant heat sensitivity. The smaller the HSI value is, the higher the level of tolerance to heat stress is. We also found that the tolerance degree of four croftonweed populations to heat stress is significantly correlated to the extreme high temperature. This indicates that acquired heat tolerance in croftonweed populations results from plant adaptation to ambient high temperatures. Acquirement of heat tolerance confers a possible risk for croftonweed to spread further to currently hotter areas.

1. Introduction

Ageratina adenophora (Spreng.) King & H. Rob. (croftonweed), originated from Mexico, is an alien invasive weed in more than 30 countries and regions worldwide (Xie et al., 2001). It is mainly distributed in the tropical and subtropical areas at latitudes between 37 degrees north and 35 degrees south, with annual average temperatures of 10–22 °C. In the 1940 s, croftonweed was introduced into Yunnan China via Myanmar, and then diffused to Guangxi, Guizhou and Sichuan and Chongqing in southwest China, currently spreading further northward and southward. Its invasion has caused serious local ecosystem destruction and economic loss (Wan et al., 2010; Yu et al., 2014). Temperature and humidity are two major ecological limiting factors that impact croftonweed spread and colonization. Building an expeditious method for screening heat/chilling tolerant plants among different croftonweed populations and probing their tolerance mechanism would allow the identification of those populations most likely to colonize hotter or colder areas. It would be a helpful tool in the risk assessment of croftonweed further spread in China.

In the last two decades, in vivo fast chlorophyll a fluorescence rise kinetics OJIP and JIP-test analysis, based on the so-called "Theory of Energy Fluxes in Biomembranes", has been widely and successfully used as a powerful tool in the investigation of plant stress physiological states due to its nondestructive, precise and quick characteristic (Strasser et al., 1995, 2004). The fluorescence

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rise kinetics OJIP is extremely sensitive to different environmental changes, such as light stress (Krüger et al., 1997; Lazár, 2003; Kalaji et al., 2012), chemical influences (Srivastava et al., 1995, 1998; Schansker et al., 2005; Tóth et al., 2005a; Chen et al., 2007, 2011; Xiang et al., 2013), heat (Strasser, 1997; Srivastava et al., 1997; Lu and Zhang, 1999; Tóth et al., 2005b, 2007; Mathur et al., 2011), chilling or cold (van Heerden et al., 2003; Strauss et al., 2006, 2007; Gururani et al., 2015), drought (Oukarroum et al., 2007, 2009; Strasser et al., 2010; Goltsev et al., 2012), heavy metal or salt stress (Ouzounidou et al., 1997; Susplugas et al., 2000; Appenroth et al., 2001; Misra et al., 2001; Rivera-Becerril et al., 2002; Xia et al., 2004; Demetriou et al., 2007; Roccotiello et al., 2010; Li and Zhang, 2015), malnutrition (Hermans et al., 2004; Ceppi et al., 2012; Yadavalli et al., 2012; Kalaji et al., 2014), atmospheric CO_2 or ozone elevation (Meinander et al., 1996; Clark et al., 2000; Nussbaum et al., 2001; Bussotti et al., 2007; Pollastrini et al., 2014; Sekhar et al., 2014), and disease (Tsimilli-Michael et al., 2000; Christen et al., 2007). Plants exhibit a specific fluorescence rise OJIP curve shape with different peaks after each different stress-treatment (Strasser et al., 2004). A typical fast chlorophyll fluorescence rise kinetics shows a sequence of phases from the initial (F_O) to the maximal (F_M) fluorescence value, which have been labeled step O (20 μs, all RCs open), J (~2 ms), I (~30 ms), and P (equal to F_M when all RCs are closed) (Strasser and Strasser 1995). Besides the basic O-J-I-P steps, others also appear in certain conditions, such as the L-step (reflecting the energetic connectivity of the PSII units), the K-step (relating to the inactivation of the oxygen-evolving complex, OEC), or the H- and G-steps in corals and foraminifers (Tsimilli-Michael et al., 1999; Strasser et al., 2004). For example, an additional rapid step, denoted as K-step, appears at about 200 to 300 μs if the samples suffer heat or drought stress. Nitrogen deficiency was also found to result in the appearance of the K-step, the H- and G-steps (Strasser et al., 2004). On the other hand, one to two of the basic O-J-I-P steps will disappear in some stress situations. In PSII-herbicide (e.g., diuron, tenuazonic acid) treated samples, the J-step increases quickly equal to the P level and the IP phase disappears, which contributes to the large accumulation of Q_A^- (primary plastoquinone acceptor) in PSII RCs due to the blocking of the electron transport from Q_A to Q_B (secondary plastoquinone acceptor) by herbicides (Strasser et al., 2004; Tóth et al., 2005a; Chen et al., 2007). Under strong heat stress (above 44 °C), the J- and I-steps disappear with a concomitant appearance of the K peak as a predominant step in fluorescence rise kinetics

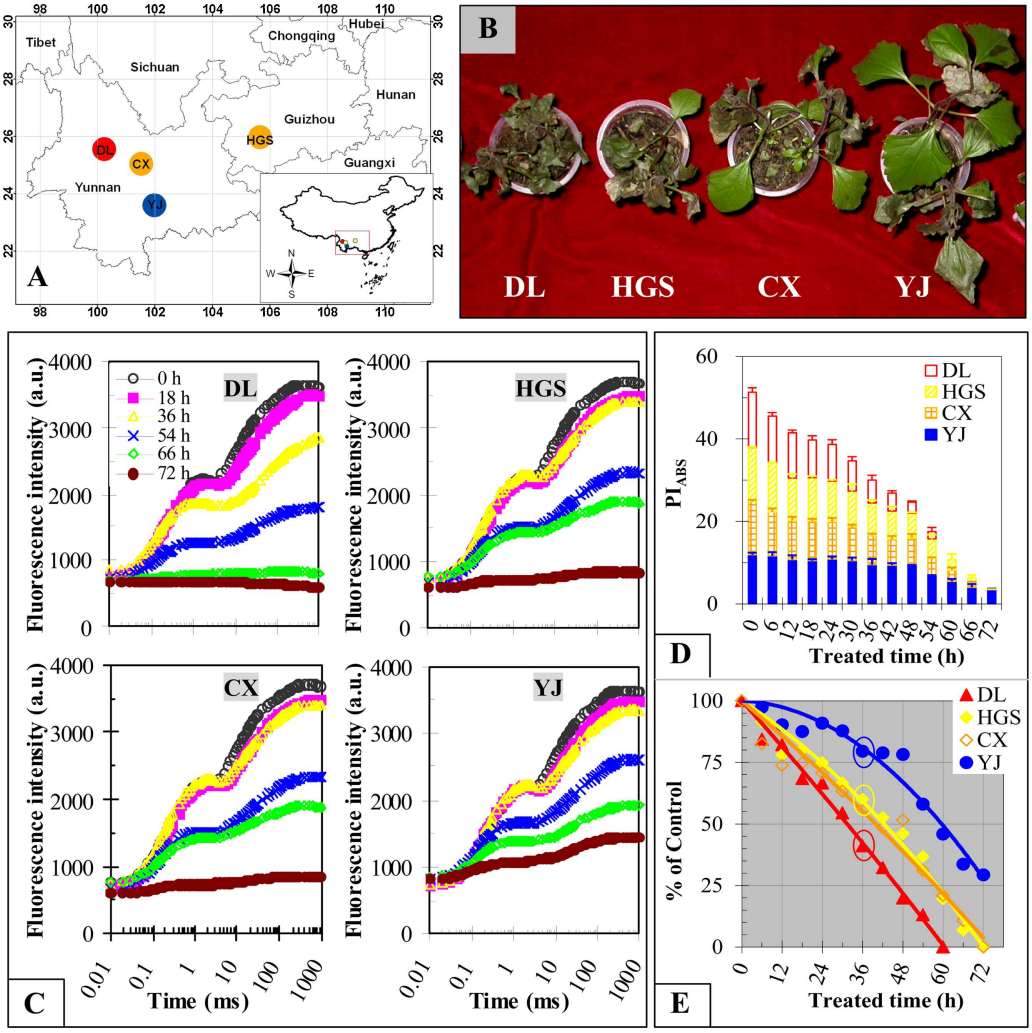

Fig. 1. Effect of heat treatment on four populations of croftonweed plants in vivo. (A) Sites of croftonweed seeds collected in South China. Dali City (DL), Huangguoshu (HGS), Chuxiong City (CX) and Yuanjiang (YJ). (B) Lesion in croftonweed whole plant treated by the temperature 40 °C for 72 h. (C) Chl a fluorescence rise kinetics OJIP of croftonweed leaves. Four populations of croftonweed whole plants were treated with the treatment 40 °C for indicated times. (D) The performance index PI_{ABS} of four populations of croftonweed leaves after heat treatment (40 °C). (E) The percentage of performance index PI_{ABS} relative to control (100% of control). The position of the half treated-time (36 h) is marked with an ellipse (yellow and brown for intermediate, blue and red for tolerance and sensitive, respectively). Data were derived from Figure 1D. Each value is the average of about 20 measurements.

because the OEC has been damaged completely (Strasser et al., 2004).

The shape change of the fluorescence rise kinetics OJIP under different environmental cases is highly dependent on the physiological conditions. A quantitative analysis of the OJIP transient has been developed, named as "JIP-test". The JIP-test translates the shape changes of the OJIP transient to quantitative changes of a constellation of structural, conformational and functional parameters quantifying the behavior of the photosynthetic organisms (for review, see Strasser et al., 2004; reviewed under a fully different nomenclature by Stirbet and Govindjee, 2011). Hence, the JIP-test provides a good access to in vivo vitality screenings, used e.g., to analyze environmental effect on the photosynthetic physiological process, or to screen aimed materials (Strasser et al., 2000, 2004).

The present study was performed to establish a standard routine to evaluate heat tolerance in different croftonweed populations using fast chlorophyll fluorescence rise kinentics technique. Chlorophyll fluorescence rise kinetics and JIP-test parameters are frequently used to assess plant physiological responses to heat stress. However, an easy systemic analysis method on JIP-test for high-throughput screening for heat tolerance in plants has never been built. Thus, we aimed to test two hypotheses as following.

Firstly, PI_{ABS} and the heat sensitivity index (HSI), a parameter calculated from PI_{ABS} and V_K, would be sensitive enough to evaluate the response of different croftonweed populations to heat stress and to rank them according to heat stress tolerance degree.

Secondly, the high photosynthesis, especially high activity of the OEC and PSII RCs, maintained after heat stress is necessary to heat tolerance in croftonweed populations.

Here, we determined chlorophyll fluorescence rise kinetics OJIP of heat-treated whole plants of four croftonweed populations and checked the capacity of this technique for screening heat tolerance. A further analysis was carried out to identify, localise and quantify effects of heat stress on two photosystems, and investigate the correlation between the effects on photosynthetic process and the level of heat stress. Finally, a model was developed for evaluating plant heat tolerance based on two characteristic JIP-test parameters for heat stress. In this model, the absolute value of the slope (K) of the linear relationship between fluorescence parameters $\log PI_{ABS}$ and V_K is confirmed as an indicator of heat tolerance level. We also found that heat tolerance of different croftonweed

Table 1
Formulae and explanation the technical data of the OJIP curves and the selected JIP-test parameters used in this study.[a]

Technical fluorescence parameters	
F_t	Fluorescence at time t after onset of actinic illumination
$F_O \cong F_{20\mu s}$ or $F_{50\mu s}$	Minimal fluorescence, when all PSII RCs are open
$F_L \equiv F_{150\ \mu s}$	Fluorescence intensity at the L-step (150 μs) of OJIP
$F_K \equiv F_{300\ \mu s}$	Fluorescence intensity at the K-step (300 μs) of OJIP
$F_J \equiv F_{2ms}$	Fluorescence intensity at the J-step (2 ms) of OJIP
$F_I \equiv F_{30ms}$	Fluorescence intensity at the I-step (30 ms) of OJIP
F_P (= F_M)	Maximal recorded fluorescence intensity, at the peak P of OJIP
$F_v \equiv F_t - F_O$	Variable fluorescence at time t
$F_v \equiv F_M - F_O$	Maximal variable fluorescence
t_{FM}	Time (in ms) to reach the maximal fluorescence intensity F_M
$V_t \equiv (F_t - F_O)/(F_M - F_O)$	Relative variable fluorescence at time t
$V_K = (F_K - F_O)/(F_M - F_O)$	Relative variable fluorescence at the K-step
$V_J = (F_J - F_O)/(F_M - F_O)$	Relative variable fluorescence at the J-step
$W_t \equiv (F_t - F_O)/(F_J - F_O)$	Relative variable fluorescence F_v to the amplitude $F_J - F_O$
$W_L = W_{150\mu s} = (F_{150\mu s} - F_O)/(F_J - F_O)$	Relative variable fluorescence at the L-step to the amplitude $F_J - F_O$
$W_K = W_{300\mu s} = (F_{300\mu s} - F_O)/(F_J - F_O)$	Relative variable fluorescence at the K-step to the amplitude $F_J - F_O$
$M_0 \equiv 4(F_{300\mu s} - F_O)/(F_M - F_O)$	Approximated initial slope (in ms^{-1}) of the fluorescence transient normalized on the maximal variable fluorescence F_V
$W_{OK} = (F_t - F_O)/(F_K - F_O)$	Ratio of variable fluorescence $F_t - F_O$ to the amplitude $F_K - F_O$
$W_{OJ} = (F_t - F_O)/(F_J - F_O)$	Ratio of variable fluorescence $F_t - F_O$ to the amplitude $F_J - F_O$
$W_{OI} = (F_t - F_O)/(F_I - F_O)$	Ratio of variable fluorescence $F_t - F_O$ to the amplitude $F_I - F_O$
Quantum efficiencies or flux ratios	
$\varphi_{Pt} = PHI(P_t) = TR_t/ABS = 1 - F_t/F_M$	Quantum yield for primary photochemistry at any time t, according to the general equation of Paillotin (1976)
$\varphi_{Po} = PHI(P_0) = TR_0/ABS = 1 - F_O/F_M$	Maximum quantum yield for primary photochemistry
$\psi_{Eo} = PSI_0 = ET_0/TR_0 = (1 - V_J)$	Probability that an electron moves further than Q_{A^-}
$\varphi_{Eo} = PHI(E_0) = ET_0/ABS = (1 - F_O/F_M)\ (1 - V_J)$	Quantum yield for electron transport (ET)
$\varphi_{Do} = PHI(D_0) = 1 - \varphi_{Po} = F_O/F_M$	Quantum yield (at $t = 0$) of energy dissipation
$\varphi_{Ro} = RE_0/ABS = \varphi_{Po} \times \psi_{Eo} \times \delta_{Ro} = \varphi_{Po} \times (1 - V_I)$	Quantum yield for reduction of the end electron acceptors at the PSI acceptor side (RE)
$\delta_{Ro} = RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Probability that an electron is transported from the reduced intersystem electron acceptors to the final electron acceptors of PSI (RE)
$\gamma_{RC} = Chl_{RC}/Chl_{total} = RC/(ABS + RC)$	Probability that a PSII Chl molecule functions as RC
Phenomenological energy fluxes (per excited leaf cross-section-CS)	
$ABS/CS = Chl/CS$	Absorption flux per CS
$TR_0/CS = \varphi_{Po} \times (ABS/CS)$	Trapped energy flux per CS
Density of RCs	
$RC/CS = \varphi_{Po} \times (V_J/M_0) \times (ABS/CS)$	Q_A-reducing RCs per CS
OEC centers = $[1 - (V_K/V_J)]_{treatment}/[1 - (V_K/V_J)]_{control}$	The fraction of Oxygen Evolving Complexes (OEC) centers
Performance indexes	
$PI_{ABS} \equiv \frac{\gamma_{RC}}{1-\gamma_{RC}} \times \frac{\varphi_{Po}}{1-\varphi_{Po}} \times \frac{\psi_{Eo}}{1-\psi_{Eo}}$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors

[a]Subscript "0" (or "o" when written after another subscript) indicates that the parameter refers to the onset of illumination, when all RCs are assumed to be open.

populations was developed due to the heat adaptation to local climate conditions.

2. Materials and methods

2.1. Sample collection and plant culture

Croftonweed seeds were collected in 2003–2004 at four locations (Latitude N/ Longitude E) in two provinces in South China: Dali city (DL, 25°33′/100°14′), Chuxiong city (CX, 25°02′/101°31′), Yuanjiang city (YJ, 23°36′/101°59′) of Yunnan province and Huangguoshu city (HGS, 25°58′/105°39′) of Guizhou province (Fig. 1A). Seeds from the four geographically distinct populations were planted in plastic cups containing a mixture of peat, vermiculite and perlite (3:1: 0.5). Seedlings were grown under approximate 300 μmol m^{-2} s^{-1} white light (day/night, 12 h/12 h) and relative humidity (about 70%) at 20–25 °C in a greenhouse. Healthy 45-day-old plants were used furthering the experiments.

2.2. Heat treatments

2.2.1. Heat treatment of whole plants in vivo

Plants of the four croftonweed populations (DL, HGS, CX and YJ) were dark-adapted for 1 h at room temperature (about 25 °C before being subjected to heat 40 °C in the growth chamber (E-36HO, Percival Scientific Inc., U.S.A). After heat treatment of 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h in complete darkness, plants were removed from the growth chamber and kept for 5 min at room temperature before measurements. Chlorophyll fluorescence measurements were performed on the second and third pairs of leaves counted from the apex of each plant. After measurements, plants were returned to the growth chamber and continued heat treatment. Five plants from each population were used and four measurements per plant were taken.

After heat treatment of 72 h, visible lesion of the whole plants was also recorded using a digital camera (Nikon, COOLPIX 4500, Japan).

2.2.2. Treatment of leaf discs

Four populations of croftonweed plants (DL, CX, HGS and YJ) were dark-adapted for 1 h, and then the second and third pairs of leaves (numbered as before) were collected to prepare leaf discs of 7-mm diameter. The leaf discs were immersed in a water bath (KW-1000DC, JIERUIER Co., Ltd., China) for 15 min at various temperatures of 25 °C, 30 °C, 35 °C, 40 °C, 42 °C, 45 °C and 50 °C in the dark, and then acclimated for 2 min at room temperature before chlorophyll fluorescence measurements. Ten different leaf discs were measured for each temperature treatment. Three independent experiments were conducted.

2.3. Measurement of chlorophyll fluorescence rise kinetics

Chlorophyll fluorescence rise kinetics was measured at room temperature with a Handy-PEA fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) as described as Strasser and Govindjee (1992). Fluorescence rise OJIP curves were induced by 2 s pulses of red light (650 nm, 3500 μmol m^{-2} s^{-1}). O refers to the initial fluorescence level, K (300 μs), J (~2 to 3 ms) and I (~30 ms) are intermediate level, and P (500 ms–1 s) is the peak level. The sequential events are reflected in the fluorescence polyphasic rise proceed. The OJ phase is largely driven by primary photochemistry, where only single-turnover Q_A reduction events are carried out. The JP phase is dominated by the biochemical reaction, where multiple-turnover Q_A reduction events are performed. The JI phase is suggested to mainly reflect the reduction of the intersystem electron carriers i.e., $>Q_B$, plastoquinone PQ, cytochrome Cyt and plastocyanin PC. The IP phase reflects the reduction of PSI electron acceptor i.e., ferredoxin-Fd, other intermediates, and NADP (Strasser et al., 2004; Yusuf et al., 2010).

The fluorescence OJIP transients can be analyzed using the JIP-test (Strasser et al., 2004; Stirbet and Govindjee, 2011). The JIP-test defines the maximal (subscript "o") energy fluxes in the energy cascade for the events Absorption (ABS), Trapping (TR_0), Electron Transport (ET_0), Dissipation (DI_0), Reduction of End acceptors of PSI (RE_0), excited leaf Cross-Section (CS). In this paper the following original data were utilized: the initial fluorescence F_O, at this time all reaction centers (RCs) are open; F_K, the fluorescence intensity at the K-step (300 μs); F_J, the fluorescence intensity at J-step (2 ms); F_I, the fluorescence intensity at I-step (30 ms); the maximal fluorescence intensity F_M (all reaction centers were closed). Table 1 summarizes the formulae and explanation of the technical data of the OJIP curves, as well as the selected JIP-test parameters used in this study (Strasser et al., 2004).

The relative variable fluorescence at time t, V_t, is defined as $(F_t - F_O)/(F_M - F_O)$; thus, the relative fluorescence at the K-step $V_K = (F_K - F_O)/(F_M - F_O)$, the relative fluorescence at the J-step $V_J = (F_J - F_O)/(F_M - F_O)$ and the relative fluorescence at the I-step $V_I = (F_I - F_O)/(F_M - F_O)$. The active OEC centers can be quantified by utilizing the value of V_K and V_J. The fraction of OEC centers can be calculated in comparison with the control sample as:

$$\text{Fraction of OEC centers} = \left(1 - \frac{V_K}{V_J}\right)_{\text{treatment}} / \left(1 - \frac{V_K}{V_J}\right)_{\text{control}}$$

The maximum quantum yield of PSII primary photochemistry, φ_{Po}, is defined as $TR_0/ABS = 1 - (F_O/F_M)$. The probability that an electron moves further than Q_A^- is defined as $\psi_{Eo} = ET_0/TR_0 = (1 - V_J)$. The quantum yield for the reduction of the end electron acceptors at the PSI acceptor side (φ_{Ro}) is given as $\varphi_{Ro} = RE_0/ABS = \varphi_{Po} \times \psi_{Eo} - \delta_{Ro} = \varphi_{Po} - (1 - V_I)$. Here, $\delta_{Ro} = RE_0/ET_0 = (1 - V_I)/(1 - V_J)$, which expresses the probability that an electron is transported from the reduced intersystem electron acceptors to final electron acceptors of PSI.

The performance index on an absorption basis, PI_{ABS}, was introduced as a term expressing the overall photosynthetic activity of PSII:

$$PI_{ABS} = \frac{\gamma_{RC}}{1 - \gamma_{RC}} \times \frac{\varphi_{Po}}{1 - \varphi_{Po}} \times \frac{\psi_{Eo}}{1 - \psi_{Eo}}$$

where γ is the fraction of reaction center chlorophyll (Chl_{RC}) per total chlorophyll ($Chl_{RC} + Chl_{Antenna}$), therefore $\gamma_{RC}/(1 - \gamma_{RC}) = Chl_{RC}/Chl_{Antenna} = RC/ABS$.

2.4. Statistical analysis

One-way ANOVA was carried out and means were separated by Duncan's least significant ranges (LSR) at 95% using Statgraphics Plus software Ver.2.1 (Manugistics, Rockville, MD, USA).

3. Results

3.1. Identification of heat tolerance of four croftonweed populations

The four croftonweed populations (Fig. 1A) could be grouped into heat tolerant, intermediate and sensitive based on the whole-plant damage level after being exposed to 40 °C for 72 h (Fig. 1B). DL plants exhibited highly sensitive to heat treatment because whole plants already died totally. Conversely, YJ plants only developed slight damage on some leaves and are therefore considered tolerant to heat stress. HGS and CX were intermediately tolerant to heat for some leaves keeping alive. To further confirm this result,

we monitored simultaneously plant vitality by the measurement of chlorophyll fluorescence rise kinetics in vivo during these four croftonweed populations were treated for 72 h at 40 °C. The fluorescence rise OJIP curves of DL plants showed higher sensitivity to heat than that of HGS, CX and YJ plants (Fig. 1C). For heat-treated DL plants, a significant change of fluorescence rise OJIP curve already started to occur after 36 h, and the fast induction fluorescence rise lost completely the O-J-I-P polyphasic transient and became a smoothly straight line after 66 h. A same result was observed in the fluorescence rise OJIP curve of both HGS and CX plants after 72 h of heat treatment. This means that at this time point the photosynthetic activity was completely inhibited by heat treatment. However, the fluorescence rise of YJ plants still kept a whole O-J-I-P polyphasic transient curve at 72 h. Obviously, the response to heat stress among these four croftonweed populations is remarkable different.

To quantify the heat sensitivity of plants exposed to 40 °C, the PSII performance index on absorption basis (PI_{ABS}) was used as an indicator of plant vitality that allowed ranking croftonweed populations based on their levels of heat tolerance. Average of PI_{ABS} quickly declined when plants of croftonweed populations DL, HGS and CX were treated at 40 °C for different durations, however, the PI_{ABS} of heat-tolerant YJ plants slowly decreased during heat treatment and just had a remarkable decrease after 36 h (Fig. 1D). From the data in Fig. 1E, on the position at 36 h (half time of the whole treatment process) the PI_{ABS} value of DL, CX, HGS and YJ plants is about 41%, 56%, 60% and 79% of the control (0 h), respectively. The treatment time producing 50% inhibition of PI_{ABS} in DL plants (about 30 h) is earlier than CX and HGS (about 40 h), and JY (about 60 h) plants. The three categories of heat response of the four croftonweed populations was clearly distinguished on the basis of PI_{ABS}: sensitive DL, intermediate HGS and CX, tolerant JY.

3.2. Effect of different heat stress degree on fluorescence rise kinetics OJIP

To more in-depth investigate the effect of heat stress on photosynthesis, DL and CX and YJ three croftonweed populations with different sensitivity were selected. Their leaf discs were subjected to relatively mild heat stress (30 and 35 °C), moderate heat stress (40 and 42 °C) and strong heat stress (45 and 50 °C) for 15 min. Heat stress clearly induced several changes in the fluorescence rise kinetics without any normalization (Fig. 2A–C). The fluorescence rise kinetics of control plant samples of the three populations (25 °C) depicted a typical O-J-I-P shape. The fluorescence rise kinetics in leaves exposed to mild heat-stress did not differ from that of the respective controls. With an increase of heat-stress temperatures (from 40 to 50 °C), the fluorescence rise kinetics O-J-I-P of the three croftonweed populations exhibited a gradual transformation into an O-K-J-I-P fluorescence rise kinetics with a new intermediate "K" step appearing at about 300 μS. Along with the induction of the K-step, a sharp decrease in the variable chlorophyll fluorescence intensity and in the P fluorescence level was also observed when the leaf discs were exposed to the moderate and strong heat stress (Fig. 2A–C). It is clear that a correlation exists between changes in the fluorescence rise kinetics OJIP and the fluorescence intensity versus temperature.

For sensitive DL plants, the new specific "K-step" became dominant with a concomitant J- and I-step disappearing in the fluorescence rise kinetics under moderate heat stress (42 °C), moreover, a more pronounced emerging of the "K-step" was

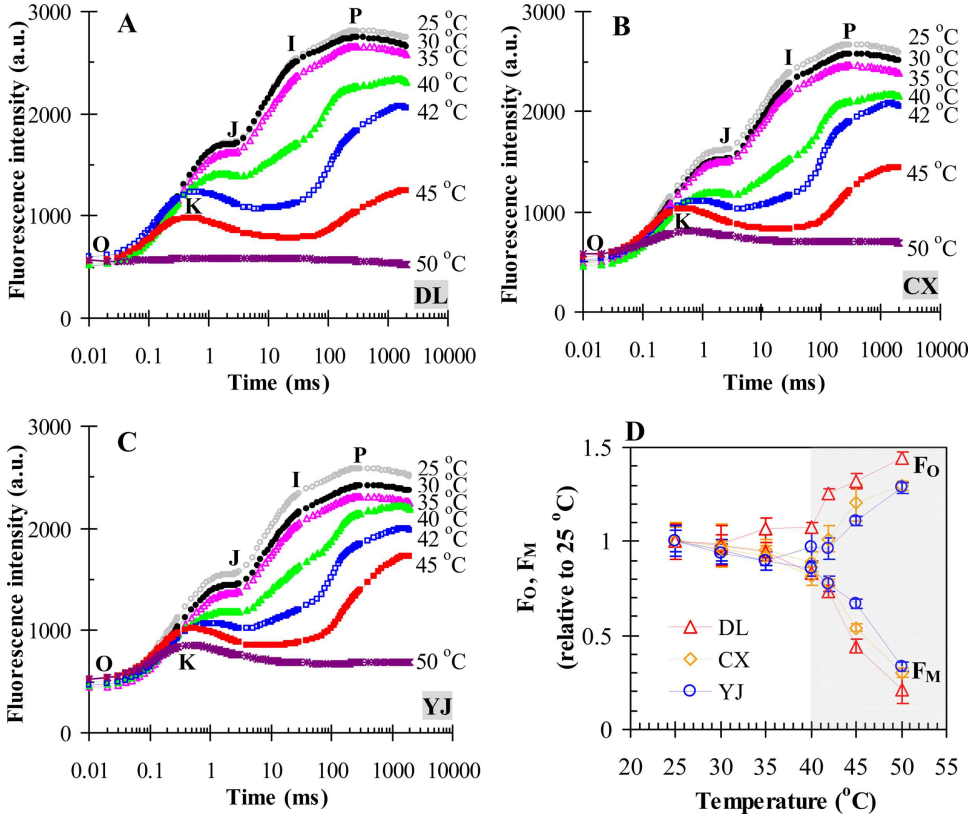

Fig. 2. Changes in Chl *a* fluorescence rise kinetics OJIP of croftonweed leaves after heat treatment. Leaf discs of three populations of croftonweed plants were treated with indicated temperature for 15 min before measurements. (A) DL, (B) CX, (C) YJ. The figure shows the original data without any normalization on logarithmic time scale. Each curve represents the average of three independent experiments about 30 repetitions. (D) Effect of heat treatment on the initial fluorescence F_O and the maximal fluorescence F_M of three populations of croftonweed leaves. The values are derived from the transients in (A–C).

induced under strong heat stress (45 °C). At higher temperature (50 °C), the fluorescence polyphasic rise kinetics of DL leaves lost all steps O-K-J-I-P and became an aclinic straight line (Fig. 2A). In contrast, when tolerant YJ leaves were heat-stressed at 42 °C or 45 °C, the variable chlorophyll fluorescence was much less reduced and the K-step was also less pronounced. In the case of the strongest heat treatment (50 °C), the K-step became a dominant peak in the fluorescence rise kinetics of YJ leaves followed by a large dip thus departing from a straight line (Fig. 2C). For CX plants with intermediate response, the change level of chlorophyll fluorescence rise kinetics was just between DL and YJ plants after heating leaf discs at different temperatures (Fig. 2B). Additionally, an increase in F_O and a decrease in F_M (P peak) also were observed when croftonweed leaves were heated for 15 min at 40 °C and up to 50 °C. The value of F_O and F_M only slightly changed in YJ relative to that in DL and CX (Fig. 2D). These results corroborate that heat stress differentially injured leaves of the three croftonweed populations with different heat tolerance.

To further study the effect of heat-stress on chlorophyll fluorescence rise kinetics OJIP properties, the fluorescence curves of three populations were normalized between F_O (50 μs) and F_M (P peak) and presented as relative variable fluorescence $V_t = (F_t - F_O)/(F_M - F_O)$ and $\Delta V_t = V_{t(\text{treated})} - V_{t(\text{control})}$ vs logarithmic time scale (Fig. S1, the "control" is the samples treated at 25 °C). This allows revealing more bands and richer information that is usually hidden in the actual fluorescence rise kinetics curve. The V_t and ΔV_t showed that the main change in the fluorescence rise kinetics of three populations was the appearance of the positive K peak and the negative I peak, dependent on heat-stress temperatures (Figs. S1A-F). Comparison of the V_t and ΔV_t curves of three populations leaves heated at 45 °C, showed that the heat sensitive DL population had a well pronounced higher K and ΔK peak (300 μS) than the intermediate CX and tolerant YJ populations (see Fig. S1G).

To compare heated croftonweed leaves for the events, reflected in the OK, OJ, OI and IP phase, other normalizations and corresponding subtractions (difference kinetics) of the fluorescence rise kinetics were also done (see Figs. 3–5). In Fig. 3A–C, the fluorescence rise kinetics of the three croftonweed populations was normalized between the O-step (50 μs) and K-step (300 μs), as $W_{OK} = (F_t - F_O)/(F_K - F_O)$ kinetics (top), and plotted with the different kinetics between heat stress and control (25 °C) $\Delta W_{OK} = W_{OK(\text{treated})} - W_{OK(\text{control})}$ (bottom) in the linear time scale from 50–300 μs. An additional step about 150 μs, L-band hidden between the steps O and K, can be observed by such a subtraction. The L-band is an indicator of the energetic connectivity or grouping of the PSII units, being higher when connectivity or grouping probability is lower (Strasser et al., 2004). Therefore, Fig. 3A demonstrated that, in sensitive DL croftonweed, the heat stress even mild stress (30 and 35 °C) caused a distinct temperature-dependent decrease of the energetic connectivity based on the positive L-bands. Under the same heat-stress temperature, DL plants had much lower connectivity than intermediate CX and tolerant YJ plants (Fig. 3A–C). The L-band can be further quantified using the relative fluorescence at the L-step to the amplitude $F_J - F_O$, as $W_L = (F_{150\ \mu s} - F_O)/(F_J - F_O)$. In Fig. 3D, the difference (ΔW_L) between the three croftonweed populations reveled that the amplitude of L-bands of DL plants increased much faster and bigger after heat treatment compared to CX and YJ plants. Thus, DL plants that lose more PSII energetic connectivity are more sensitive to heat stress.

In Fig. 4, the fluorescence data were double normalized by F_O (50 μs) and F_J (2 ms) for single turnover events, as $W_{OJ} = (F_t - F_O)/(F_J - F_O)$, and different kinetics $\Delta W_{OJ} = W_{OJ(\text{treated})} - W_{OJ(\text{control})}$ (the insert) in the 50–2 μs time range. The major plots of Fig. 4A–C demonstrated that the step-K in fluorescence rise kinetics could be elicited under moderate (40 and 42 °C) or strong (45 and 50 °C) but not mild (30 and 35 °C) heat stress. In the insert, a clearer positive

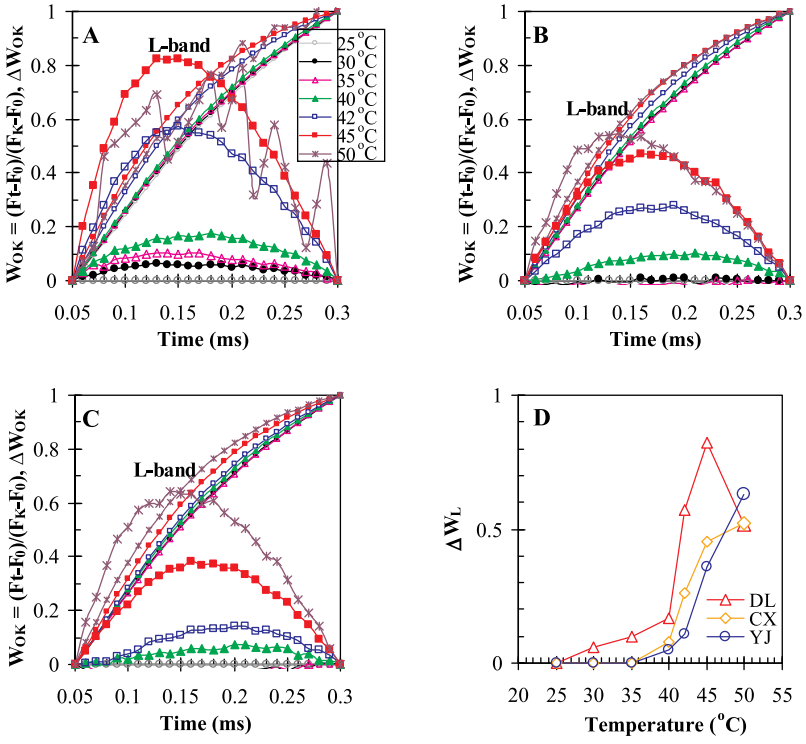

Fig. 3. Responses of the L-band of three populations of croftonweed leaves to heat treatment. The fluorescence rise kinetics normalized by F_O and F_K as $W_{OK} = (F_t - F_O)/(F_K - F_O)$ (top), and the difference kinetics $\Delta W_{OK} = W_{OK(\text{treated})} - W_{OK(\text{control})}$ (bottom) were represented. (A) Sensitive DL, (B) intermediate CX, (C) tolerant YJ. (D) The difference in the relative variable fluorescence at the L-step to the amplitude $F_J - F_O$, $\Delta W_L = W_{L(\text{treated})} - W_{L(\text{control})}$. Each value is the averages of 3 independent measurements with about 30 repetitions.

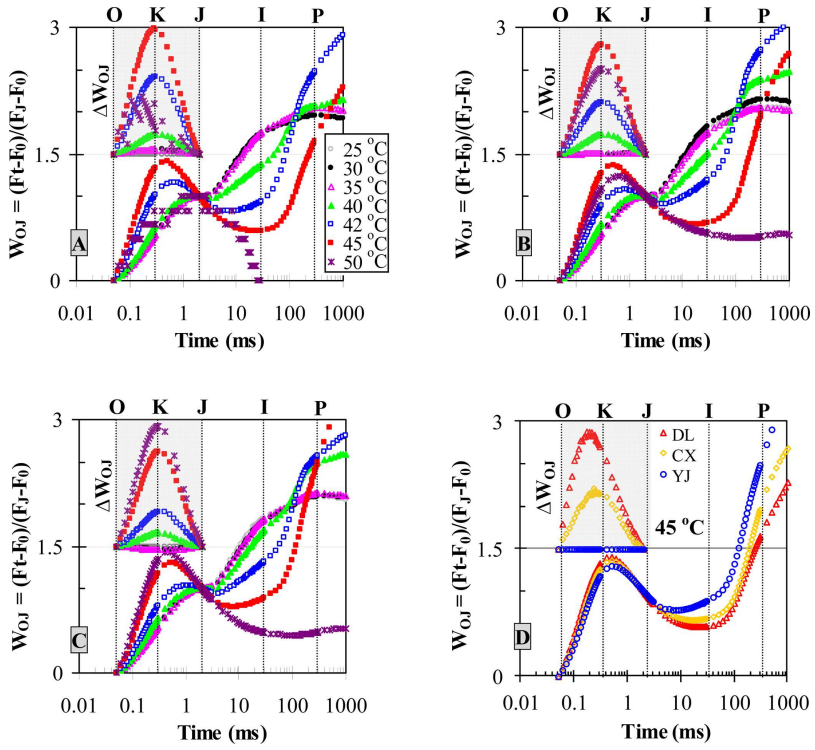

Fig. 4. Responses of the K-band of three populations of croftonweed leaves to heat treatment. The fluorescence rise kinetics normalized by F_O and F_J were expressed as $W_{OK} = (F_t - F_O)/(F_J - F_O)$. The insert shows the difference kinetics $\Delta W_{OJ} = W_{OJ(treated)} - W_{OJ(control)}$. (A) Sensitive DL, (B) intermediate CX, (C) tolerant YJ. (D) Changes in the K-band of three populations of croftonweed leaves heated at 45 °C. Each value is the averages of 3 independent measurements with about 30 repetitions.

K-band was exhibited in the different kinetics ΔW_{OJ}, which was temperature dependent. The trend was the same as that in Fig. 3A–C. Different heat stress resulted in a higher intensity of the *K* peak in sensitive DL plants than intermediate CX and tolerant YJ plants. Moreover, under the strongest heat stress at 50 °C, the K-step of DL plants became again unnoticeable attributed to the rise kinetics with very low fluorescence intensity (a straight line) (Fig. 4A). Comparing different kinetics ΔW_{OJ} between DL, CX and YJ plants at 45 °C further proved that heat provoked a positive K peak that was large for sensitive, medium for intermediate and small for tolerant populations (Fig. 4D). An increase of the K-step or K peak for ΔW_{OJ} indicates the inactivation of the OEC centers.

To evaluate the effect of heat stress on the IP phase, two different normalization procedures were performed (Fig. 5). As shown in Fig. 5A–C, the fluorescence rise kinetics with double normalization between the O (50 μs) and I (30 ms), as $W_{OI} = (F_t - F_O)/(F_I - F_O)$ (only $W_{OI} \geq 1$ was shown), was plotted in the linear 30-830 ms time range. For each W_{OI} curve, the maximal amplitude of the fluorescence rise reflects the size of the pool of the end electron acceptors at PSI acceptor side. There was a marked temperature-dependent increase in the pool size after plants were heated at 40–45 °C. Mild heat stress (30 and 35 °C) could not cause a distinct change in the pool size compared to the control (25 °C). Additionally, the amplitude size of the W_{OI} was lower at 50 °C than 25 °C (control)) because of the total breakdown of the photosynthetic apparatus in the heated-leaves. However, no significant difference in the pool size was observed between the three croftonweed populations heated at the same temperature (Fig. 5A–C). In Fig. 5D–F, the fluorescence rise kinetics of the three croftonweed populations also were normalized between the I- (30 ms) and P- (peak) step, as $W_{IP} = (F_t - F_I)/(F_P - F_I)$, and plotted on a linear time scale from 30 ms to 830 ms. This interpretation allows the evaluation of the reduction rates of PSI end electron acceptors' pool in various samples. A lower (or higher) conduction rate of samples is reflected by a greater (or lower) value of the half-time, corresponding to the time point at $W_{IP} = 0.5$ (half rise-time of the curves). As compared to 25 °C (control), 40 and 42 °C (moderate) as well as 45 °C (strong) heat stress resulted in a gradual decrease of the conduction rate for a temperature-dependent increase of the half-times in all samples. Heat-stress at 50 °C is too strong for all populations to calculate the half-times. The half-times for the three populations of croftonweed heated with mild temperatures (30 and 35 °C) or 25 °C (control) were 80 ms (Fig. 5D–F). This suggests that 40 °C or higher treatment temperatures can quickly decrease the rate of the reduction of the end electron acceptors on PSI. However, the conduction rate did not respond to mild heat stress. By comparison with the W_{IP} kinetics of the three croftonweed populations, we also observed that their half-times at same heat stress temperatures are only slight different. For example, the half-time of DL, CX and YJ plants heated at 45 °C is 250, 240 and 230 ms, respectively (Fig. 5D-F). Therefore the conduction rate (inverse of the half-time) could not be used as an indicator to discriminate croftonweed populations having different heat tolerance level.

3.3. Effect of different heat stress degree on PSII and PSI by JIP-test

To assess the effect of heat stress on the two photosystems of the three croftonweed populations, some structural and functional parameters quantifying the photosynthetic behavior were deduced by the JIP-test. The variable fluorescence at the K-step at 300 μs as $V_K = (F_K - F_O)/(F_M - F_O)$ became significantly higher at 45 °C and further increased at higher heat stress temperatures (Fig. 6A). On the other hand, an increase in the variable fluorescence at the J-step at 2 ms as $V_J = F_J - F_O)/(F_M - F_O)$ was observed just up to 50 °C while at 40 and 42 and 45 °C, it slightly decreased (see the insert). This indicates that K and J are two independent steps of chlorophyll fluorescence rise kinetics. To remove the possible effect of the J-step on the K-step, the relative

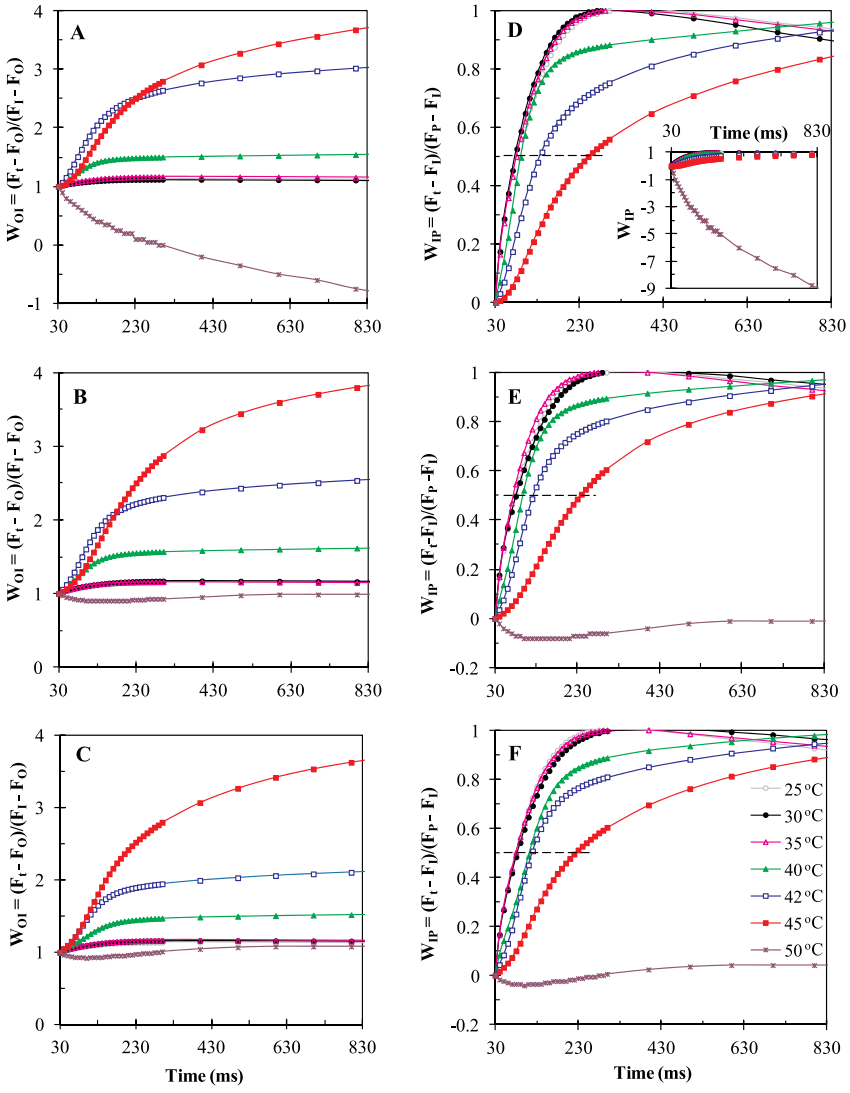

Fig. 5. Changes in the I-P phase of Chl a fluorescence rise kinetics OJIP of three populations of croftonweed leaves after heat treatment. The fluorescence rise kinetics normalized by F_O and F_I as $W_{OI} = (F_t - F_O)/(F_I - F_O)$ ($W_{OI} \geq 1$, A–C), and the fluorescence rise kinetics normalized by F_I and F_P as $W_{IP} = (F_t - F_I)/(F_P - F_I)$ were plotted in the 30–300 ms time range (linear scale) (D–F). The insert of Fig. 8D mainly shows the W_{IP} of DL croftonweed leaves treated with 50 °C. The half-times are shown by the crossing of the curves with the horizontal dashed line drawn at $W_{IP} = 0.5$ (half rise). A, D: Sensitive DL; B, E: intermediate CX; C, F: tolerant YJ. Each value is the averages of 3 independent measurements with about 30 repetitions.

variable fluorescence at the K-step to the amplitude $F_J - F_O$ ($W_K = (F_K - F_O)/(F_J - F_O)$) and the difference of W_K between heat stress and 25 °C (control) croftonweed samples ($\Delta W_K = W_{K(treated)} - W_{K(control)}$) also were calculated (Fig. 6B). By increasing heat stress temperature from 30 to 45 °C, a visible temperature-dependent increase in the level of W_K and ΔW_K had started early at 40 °C. Moreover, sensitive DL plants had a faster increase in the K level (W_K and ΔW_K) relative to intermediate CX and tolerant YJ plants. At the highest temperature (50 °C), the rate of increase in the K level of YJ plants started to fall, however, the K level of CX and DL plants decreased significantly. Obviously, the level of the specific K-step for heat stress is very sensitive to high temperature. The occurrence of the K-step under natural conditions is specifically attributed to damage on the OEC at the PSII donor side (Strasser et al., 2004). The fraction of the active OEC centers after heat treatment of the three croftonweed populations at different temperatures was shown in Fig. 6C. DL leaves heated at 30 °C already had an evident decrease in the fraction of the active OEC centers and then the fraction became zero up to 42 °C. In the case of YJ leaves heated at 42 °C, the fraction of the active OEC centers just began to fall clearly and the fraction reduced into zero at 45 °C. For CX leaves heated with different temperatures, the decrease rate in the OEC centers was less than DL but was more than YJ. Like the OEC centers, the density of the active RCs per excited leaf cross-section (RC/CS) also had a similar decrease tendency after leaves were heated at the temperature plus from 30 to 50 °C (Fig. 6D). This indicates that inactivation of PSII RCs is also a result of moderate and strong even mild heat stress.

ABS/CS expresses the absorption flux per excited leaf cross-section that can be taken as a measure for an average antenna size or chlorophyll concentration. TR_0/CS refers to the trapped energy flux per excited leaf cross-section, which reflects the specific rate of the exciton trapped by open RCs per excited leaf cross-section. As shown in Fig. 6E and F, exposure of leaves to temperatures of 25–35 °C only slightly affected the antenna chlorophyll concentration and trapped energy flux per excited leaf cross-section, but if the temperature was raised to 40 °C and up to 50 °C, a gradual decrease in both variables was observed. Moreover, the ABS/CS and TR_0/CS of sensitive DL plants decreased more compared to intermediate CX and tolerant YJ plants under the moderate and strong heat stress.

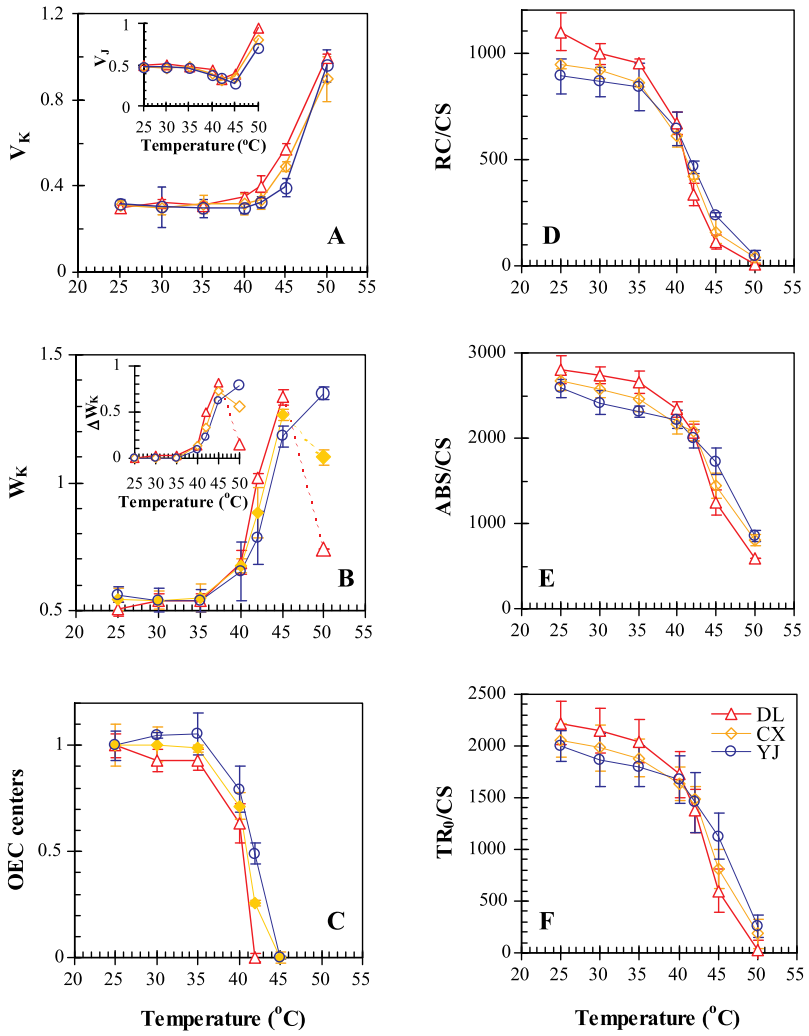

Fig. 6. Effect of heat treatment on some selected JIP-test parameters of three populations of croftonweed leaves. (A) The relative variable fluorescence at the K-step V_K and the relative variable fluorescence at the J-step V_J in the insert. (B) The relative variable fluorescence at the K-step to the amplitude $F_J - F_O$, $W_K = (F_{300\mu s} - F_O)/(F_J - F_O)$, $\Delta W_K = W_{K\,(treated)} - W_{K(control)}$ in the insert. (C) The fraction of Oxygen Evolving Complex (OEC) centers. (D) The Q_A-reducing reaction centers per excited leaf cross-section (RC/CS). (E) The chlorophyll concentration per excited leaf cross-section (ABS/CS). (F) Trapping energy flux per excited leaf cross-section (TR_0/CS). Each value represents the mean ± SD of about 30 repetitions.

The performance index PI_{ABS} is the most sensitive JIP-test parameter expressing the overall photosynthetic activity of PSII. PI_{ABS} decreased sharply when sensitive DL leaves were exposed to temperatures above 30 °C (Fig. 7A). However, for intermediate YJ and tolerant CX, a distinct decrease of PI_{ABS} only occurred when leaves were heated at 42 °C or above. At 42 °C, PI_{ABS} of DL already decreased by around 56% compared to the control (25 °C). At 45 °C, the PI_{ABS} value of the three croftonweed populations declined to 9% (DL), 20% (CX) and 49% (YJ) of control, respectively. Up to 50 °C, no marked difference in PI_{ABS} between the three populations was found because all leaves lost the PSII activity. Thus, PI_{ABS} can be used as a proxy of heat-induced plant damage.

The maximum quantum yield of primary photochemistry φ_{Po} and the quantum yield of energy dissipation φ_{Do} are shown in Fig. 7B. The φ_{Po} and φ_{Do} of the three croftonweed populations remained constant 0.77 ± 0.02 and 0.23 ± 0.02, respectively, under mild (30 and 35 °C) and moderate (40 °C) heat stress. Above 40 °C, a significant decrease in φ_{Po} and a similar strong increase in φ_{Do} were observed. Furthermore, φ_{Po} and φ_{Do} of DL plants was more sensitive to high temperatures above 40 °C than that of CX and YJ plants. From the data in Fig. 7C and D, no visible effect on the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A (ψ_{Eo}) and the quantum yield of PSII electron transport (φ_{Eo}) was seen when three populations leaves were exposed to mild heat stress. Under moderate heat stress (40 and 42 °C) conditions, there was a slight increase in ψ_{Eo} and φ_{Eo}. Up to 50 °C, ψ_{Eo} and φ_{Eo} decreased drastically, especially ψ_{Eo} and φ_{Eo} of sensitive DL plants almost declined to zero.

The parameter δ_{Ro} expresses the probability that an electron is transported from the reduced intersystem electron acceptors to final electron acceptors of PSI, which became visible bigger at 40 °C and further lifted with increasing of heat stress temperatures up to 50 °C (Fig. 7E). The quantum yield for the reduction of the end electron acceptors at the PSI acceptor side (φ_{Ro}) also became significantly higher at 40 and 42 °C. Many investigators have found that moderate heat stress increases indeed PSI activity (Havanx et al., 1991; Oukarroum et al., 2009; Huther et al., 2013). However, the φ_{Ro} rise of DL and CX plants was reversed at 45 °C and it further declined up to 50 °C. At the highest temperature, φ_{Ro} of YJ plants also has a prominent decrease (Fig. 7F). Additionally, mild heat stress (30 and 35 °C) did not result in a visible change in δ_{Ro} and φ_{Ro}. Only in the case of leaves heated at 50 °C, a significant difference in δ_{Ro} and φ_{Ro} between the three croftonweed populations was observed (Fig. 7E and F). These results are consistent with the

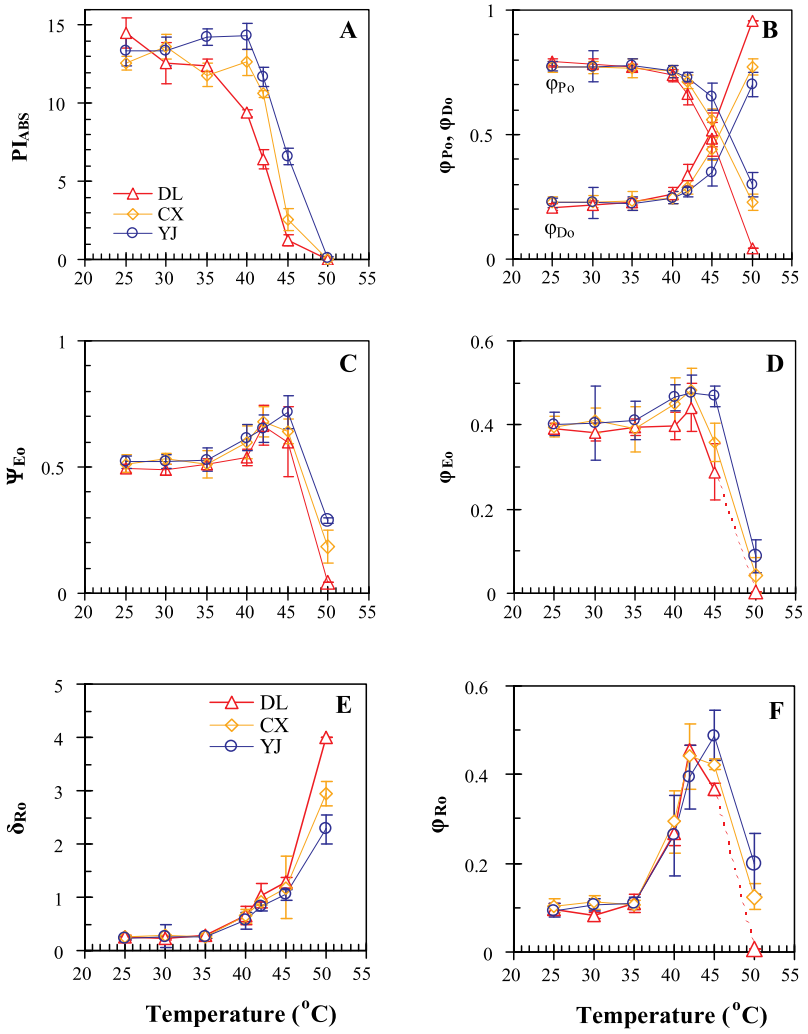

Fig. 7. Effect of heat treatment on (A) the performance index PI_{ABS}, (B) maximum quantum yield for primary photochemistry (φ_{Po}) and quantum yield of energy dissipation (φ_{Do}), (C) probability that an electron moves further than Q_A^- (ψ_{Eo}), (D) quantum yield for PSII electron transport (φ_{Eo}), (E) probability that an electron is transported from the reduced intersystem electron acceptors to final electron acceptors of PSI (δ_{Ro}) and (F) quantum yield for reduction of end electron acceptors at the PSI acceptor side (φ_{Ro}) of three populations of croftonweed leaves. Each value represents the mean ± SD of about 30 repetitions.

previous analysis of W_{OI} and W_{IP}. It is concluded that the increase of PSI activity should not be the real reason plants develop tolerance to heat stress.

3.4. A suggested model

According to above results, the five parameters V_K, W_K, RC/CS, OEC centers and PI_{ABS} correlated well with heat stress. Nevertheless, from mild to moderate and strong heat stress level, only PI_{ABS} distinguished between croftonweed populations with different heat tolerances. To further assess the heat tolerance among different croftonweed populations, a model was developed based on the parameter PI_{ABS} and the characteristic parameter for heat stress V_K (Fig. 8). The logarithm of PI_{ABS} ($logPI_{ABS}$) is defined as the total driving force for photosynthesis (Strasser et al., 2004). In this model, the $logPI_{ABS}$ values decreased linearly as the K-step level (V_K) increased at incremental heat stress temperatures from 30 to 50 °C. This linear relationship indicates that the most important determinant of the PSII loss of function is the damage of OEC centers. The slope of the straight line could be obtained from the corresponding equation from the plot of $logPI_{ABS}$ versus V_K. The absolute value of the slope (K) of the relationship between $logPI_{ABS}$ and V_K quantifies plant tolerance to high temperature, here being

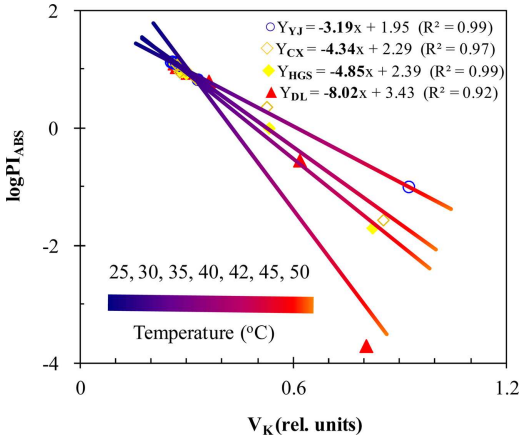

Fig. 8. A proposed model of sensitivity to heat based on both JIP-test parameters $logPI_{ABS}$ and the relative variable fluorescence at the K-step V_K. The linear corresponding equation were obtained: sensitive DL (▲, $y = -8.02x + 3.43$, $R^2 = 0.92$), intermediate HGS (◆, $y = -4.85x + 2.39$, $R^2 = 0.99$) and CX (◇, $y = -4.34x + 2.29$, $R^2 = 0.97$), tolerant YJ (○, $y = -3.19x + 1.95$, $R^2 = 0.99$). Here, in equation, $y = logPI_{ABS}$, $x = V_K$. Each value is the average of three independent experiments with about 30 repetitions.

named as Heat Sensitivity Index (HSI). The HSI of four croftonweed populations is 3.19 (tolerant YJ plants), 4.34 (intermediate CX plants) and 4.85 (intermediate HGS plants), and 8.02 (sensitive DL plants), respectively. The HSI in sensitive DL plants is about 2.5 times as much as tolerant YJ plants, and is 1.85 and 1.65 times relative to intermediate CX and HGS plants. It is clear that a lower HSI means a stronger tolerance to heat stress.

3.5. Effect of ambient temperature on acquired heat tolerance in different croftonweed populations

In nature, it is common for plants to be forced suffering environmental temperature changes. Consequently, plants have evolved mechanisms to survive temperature stress. The heat tolerance is acquired as mechanism due to hot adaptation in growth environment, which can be a short or long, moderate or strong high temperature treatment (Larkindale and Vierling, 2008). The geographical information showed that the tolerant YJ plants grew in a lower altitude (395 m) and latitude area (23°36′) relative to intermediate (HGS and CX) and sensitive DL plants growing in altitude above 1000 m and latitude above 25° (Table 2). These four croftonweed populations came from three different climate zones, DL in northern subtropics, HGS and CX in center subtropics, and YJ in north tropics, respectively. Therefore, a visible difference in meteorological conditions, especially temperature changes, was observed at the four sample sites where croftonweed populations were collected (Table 2). Based on recent 30 year (1971–2000) meteorological data (data before 1970 is unavailable), YJ plants from the north tropical area having much higher annual average temperature (23.7 °C) experienced a much stronger extreme maximum temperature over 42 °C compared to the other three populations HGS, CX and DL (Table 2). In fact, annual average extreme maximun temperature (38.4 °C) in Yuanjiang area has almost reached to the level of moderate high temperature stress.

To further probe the reason that different heat susceptibility was formed in four croftonweed populations, the correlation between the heat sensitivity index (K value) and latitude and ambient temperature was analyzed (Fig. 9). There was a good positive correlation between the K value and the latitude ($P < 0.05$), indicating that plants from the lower latitude are more tolerant to heat stress. A significant negative correlation between the K value and annual average temperature ($P < 0.05$) or extreme maximum temperature ($P < 0.01$) was also observed. However, we found that the correlation between the K value and extreme maximum temperature is highest ($r = 0.65$, $P < 0.01$). So, it is concluded that the heat tolerance of croftonweed populations should be attributed to plants adaptation of ambient extreme maximum temperature.

4. Discussion

4.1. High temperature affects on PSII and PSI

High temperature affects a broad spectrum of cellular components and metabolic processes (Sung et al., 2003). Photosynthesis is among the most sensitive physiological processes to high temperature stress, and maintenance of high photosynthetic activity is important for plant tolerance to heat stress (Liu and Huang, 2008). Extensive studies demonstrate that heat treatment can cause inactivation of OEC, inhibition of electron transport, and decrease in PSII photochemical efficiency (De Ronde et al., 2004; Wahid et al., 2007). Su (2005) proved that heat treatment at 40 °C led to a significant decrease in net photosynthetic rate of different croftonweed geographical populations. Our experiments show that appearance of the K-step is the major change in fast chlorophyll fluorescence rise kinetics of croftonweed leaves exposed to high temperature (Fig. 2A–C and S1). The phenomenological appearance of the K-step is a typical characteristic of the fluorescence rise kinetics in heated-samples, which is specifically attributed to the OEC destruction by release of the manganese cluster (Strasser et al., 2004). The manganese cluster of PSII has been identified as the most heat sensitive component of the photosynthetic transfer chain (Oukarroum et al., 2013). According to the model of De Ronde et al. (2004), while heat stress leads to the dissociation of the OEC causing an imbalance between the electron flow from the OEC to the RC and toward PSII acceptor side, the alternative internal electron donor such as proline can donate electrons to PSII instead of H_2O. This will result in a short-lived increase in the reduced $Pheo^-/Q_A^-$ concentration, creating a K-peak appearing at about 300 μs. Hence, the increasing amplitude of the K-step or ΔK peak is associated with the OEC injury degree (Strasser, 1997). In fact, the OEC with manganese cluster is very sensitive to heat stress. The OEC activity was completely lost in sensitive DL plants heated at 42 °C and even in the case of very mild heat treatment (30 or 35 °C) the fraction of active OEC centers exhibited a visible decrease by over 10% (Fig. 6C). However, the appearance of a conspicuous K peak requires higher temperature intensities (above 40 °C) (Fig. 2A–C) or long heat duration in moderate temperature at 40 °C (Fig. 1C). Just above 40 °C high temperatures, a significant increase of the level of K-step (V_K or W_K) or ΔK peak (ΔW_K) was observed. Moreover, tolerant YJ plants had less damage to their OEC activity and a less increase of the ΔK peak than sensitive DL plants at different heat stress level (Fig. 4 and 6A–C). Consequently, the OEC damage is one of the earliest events affected by heat stress.

Beside a well pronounced K-step, the fluorescence rise kinetics of croftonweed leaves treated by high temperatures (>40 °C) also showed a large dip after the K-step, a drastically decreased F_M and an increase F_O (Fig. 2). This indicates that heat stress probably affects components of photosynthetic apparatus other than the OEC (Strasser, 1997). The possible explanation for the dip after the K-step is the reopening of PSII RCs by electron transfer from Q_A^- to Q_B, and eventually by a subsequent accumulation of P_{680}^+centers with a low fluorescence yield (Strasser, 1997; Tóth et al., 2007). The F_O increase attributes to the physical separation of the PSII RCs from their associated pigment antennae resulting in blocked energy transfer to the PSII traps (Fig. 2D, Srivastava et al., 1997). Thus, heat inactivation of PSII may be accompanied by the aggregation and subsequent dissociation of the light-harvesting

Table 2
The geographical and meteorological information of four sites where croftonweed seeds were collected.[b]

Sample No.	Dali(DL)	Huangguoshu(HGS)	Chuxiong(CX)	Yuanjiang(YJ)
Altitude(m)	2047	1060	1823	395
Latitude (N) andLongitude (E)	25°33′/100°14′	25°58′/105°39′	25°02′/101°31′	23°36′/101°59′
Climate zone	Northern subtropicalzone	Central subtropicalzone	Central subtropicalzone	North tropicalzone
Annual average temperature (°C)	14.8	14.1	16.0	23.7
Extreme max. temperature (°C)	31.6	33.4	33.0	42.2
Annual average Extreme max. temperature (°C)	27.4	29.0	28.9	38.4

[b] The geographical data were measured by Handy GPS. The meteorological data (1971–2000) were obtained from Chinese meteorological Yearbook.

complex (Li et al., 2009). A significant decrease in RC/CS, ABS/CS and TR_0/CS further demonstrates that high temperatures (>40 °C) indeed inactivated PSII RCs, reduced the function antenna size, and declined the specific rate of the exciton trapped by open RCs (Fig. 6D–F, Mathur et al., 2011). We also notice that partial inactivation of PSII RCs in DL plants already starts in mild heat stress at 30 °C, moreover, complete inactivation of PSII RCs happens at 50 °C severe elevated temperature (Fig. 6D). It is proposed that the decrease of TR_0/CS is mainly attributed to heat inactivation of RCs due to the dissociation of the manganese-stabilizing extrinsic 33 kDa protein from the PSII reaction center complex (Enami et al., 1994). The previous analysis reveals that the primary main effect of heat stress lies in the RC not in the chlorophyll antennae. This is in agreement with previous observation that the loss of grana stacking following dissociation of PSII peripheral light-harvesting complex from its core complex is one of the earliest events caused by heat stress (Gounaris et al., 1984). The structural and functional aspects of PSII are interrelated. Under heat stress, the damage to the photosynthetic machinery will greatly affect the energetic cooperativity between the PSII units. This assumption is supported by the positive L-band and a temperature-increase in ΔW_L observed when heat treatment causes a decrease in the energetic connectivity of the samples. A lower connectivity results in a poor utilization of the excitation energy and lower stability of the PSII units (Strasser et al., 2004). Loosing cooperativity also indicates that the fraction of active RCs has also decrease.

Our data showing a temperature-dependent linear decrease in the performance index PI_{ABS} of DL plants suggests that heat treatment results in a significant decrease of the overall photosynthetic activity (Fig. 7A). In contrast, a higher vitality is maintained in tolerant YJ plants heated by increased high temperatures (Fig. 7A) or prolonged time (Figs. 1D and E). PI_{ABS} indeed can be regarded as a standard to successfully identify heat sensitivity of different croftonweed populations since it is the most sensitive experimentally derived parameter to various stress conditions 7 A, Strasser et al., 2004). PI_{ABS} is produced by the three independent components RC/ABS (the density of active RCs per chlorophyll absorption) and φ_{Po} as well as ψ_{Eo} (Appenroth et al., 2001; Strasser et al., 2004; Demetriou et al., 2007). Fig. 7B shows that the maximum quantum yield for primary photochemistry φ_{Po} and quantum yield for energy dissipation φ_{Do} do not occur distinct change in lower heat stress temperatures (<40 °C), but with further increasing treatment temperature a drastic φ_{Po} decrease and a remarkable φ_{Do} increase arise. Briantais et al. (1996) suggested that heat stress declines the quantum efficiency of PSII photochemistry due to several reasons including a decrease of the rate of primary charge separation, a reduction of the stabilization of charge separation, an increase in the recombination rate constant of the radical pair of the RC, and the disconnection of some minor antenna from the PSII RCs. A decrease in the efficiency of light photochemical reactions (φ_{Po}) certainly causes a rise of energy dissipation as heat, fluorescence and energy transfer to other systems, which will result in a conversion of PSII active RCs into heat sink centers. In Fig. 7C, a slight increase in the probability that an electron moves further than Q_A^- (ψ_{Eo}) by increasing temperature treatment is found, while at 50 °C, ψ_{Eo} declined dramatically (Fig. 7C). This result is also supported by the data V_J (insert of Fig. 6A). The V_J level is linked to the peak concentrations of $Q_A^-Q_B$ and $Q_A^-Q_B^-$ formed by electron transport from Q_A to Q_B (Strasser et al., 2004). The quantum yield for PSII electron transport φ_{Eo} also exhibits a similar change (Fig. 7D). So, a dramatic lowering of the overall photosynthetic activity of PSII (PI_{ABS}) should be attributed to inactivation of RCs (RC/CS) and inhibition of light reactions (φ_{Po}). Havaux et al. (1991) reported that heat stress inhibits PSII activity but at the same time stimulates PSI activity. From our experimental evidence, high temperature treatment (≥40 °C) actually increases the probability that an electron is transported from the reduced intersystem electron acceptors to the final electron acceptors of PSI (δ_{Ro}) (Fig. 7E), and increases the quantum yield for reduction of the end electron acceptors at the PSI acceptor side (φ_{Ro}) while at 50 °C the value of φ_{Ro} is reversed (Fig. 7F). This is in agreement with the observation of Oukarroum et al. (2013). A higher PSI electron transport activity can result in a bigger pool size of the end electron acceptors at the PSI, which might be due to a thermal activation of the dark reactions (Fig. 5A–C). However, the overall rate constant for the reduction of the end electron acceptor pool shows a gradual decrease (Fig. 5D–F), revealing an independent regulation compared to the pool size (Yusuf et al., 2010).

Heat response mechanism of croftonweed plants thus can be summarized as follows. Mild heat stress (<40 °C) partially inhibits both the OEC, without creating a visible K-step, and the PSII RCs, followed by a decrease in the energetic connectivity of PSII units and the overall photosynthetic activity of PSII, without significantly affecting on the architecture of antenna complexes and primary light reactions and energy dissipation and the electron transfer activity of two potosystems. Thus, it is proposed that inhibition of the OEC and PSII RCs are the initial cause of heat damage. Moderate heat stress (40–45 °C) produces an irreversible severe damage of the OEC with a K-step appearance and inactivates a great deal of PSII RCs. It further down-regulates PSII energetic connectivity and overall photosynthetic activity, additionally declining the antenna size. Moreover, an increase in energy dissipation and the electron transfer activity of two potosystems and a decrease in the quantum yield for primary photochemistry are observed. Strong heat stress (≥45 °C) destroyed entirely the OEC with a pronounced K-step and almost inactivated completely PSII RCs, and the overall photosynthetic activity of PSII decreases to near zero due to damage on the whole PSII structure and function. Especially at 50 °C incubation, sensitive DL plants loss totally vitality because extremely high temperature harms seriously PSII and PSI activity. Tolerant YJ plants show lesser damage degree on photosynthetic organisms compared to sensitive DL and intermediate CX plants in different heat stress level. This support the idea that higher photosynthetic activity is necessary for croftonweed plants to acquire heat tolerance.

4.2. model for evaluating heat tolerance in croftonweed populations

Growing studies have demonstrated that fast chlorophyll fluorescence rise kinetics OJIP combining the JIP-test analysis is a useful and practical method for screening and assessing plant stress tolerance. This technique has successfully been applied to evaluate salinity sensitivity in mung bean and *Brassica* seedlings (Misra et al., 2001). The fluorescence transients OLKJIP and the JIP-test, especially the changes in the L- and K-band, have predictive value with respect to the leaf vitality and the tolerance of the barley varieties to drought stress (Oukarroum et al., 2007). The fluorescence kinetics and a drought factor index (DFI) based on JIP-test parameter PI_{ABS} were used to screen for drought tolerance in mutant germplasm of sesame (Boureima et al., 2012). Silvestre et al. (2014) suggested that PI_{ABS} is the best proxy to screen and identify elite genotypes of *Lathyrus* genus with improved drought resistance. The JIP-test has also been reported to be cost-effective for the selection of the freezing tolerance in wheat genotypes (Rapacz and Wozniczka, 2009) and can be used to characterize and evaluate response of chilling stress in tomato leaf and fruit (Zushi et al., 2012). In addition, a novel parameter chill factor index (CFI) produced by PI_{ABS} is introduced as a sensitive indicator to rank chilling tolerance in large numbers of soybean genotypes (Strauss et al., 2006).

Recently, some approaches based on chlorophyll fluorescence rise kinetics has been established to determine and estimate heat tolerance in several plant species. First, the initial fluorescence F_O can be regarded as an index to determine the heat tolerance of leaves (Oukarroum et al., 2009). However, the method only monitors an effect of heat stress on the PSII acceptor side, furthermore, the F_O does not starts to go up till heat treatment temperatures excess 40 °C (Fig. 2D). Second, the parameter F_V/F_M has been used to select for heat or chilling tolerance. However, a growing body of evidence indicates that F_V/F_M is often insensitive to stress and is not always a suitable parameter for screening stress tolerance (Strauss et al., 2006). A third approach is to develop the F_K/F_J ratio as an excellent parameter for monitoring heat stress effect because F_K/F_J is an indication for limitation of electron donation on the donor side of PSII and also reflects electron transfer status of the PSII acceptor side (Oukarroum et al., 2009, 2013). However, F_K/F_J fails to distinguish croftonweed populations with different sensitivity to heat stress (data not shown) although there is a linear correlation between the parameter F_K/F_J and stress temperatures. Finally, it has been demonstrated that a multi-parametric expression PI_{ABS} is the most sensitive and best reliable JIP-test parameter for screening heat stress tolerance (Figs. 1 D–F and 7 A, Strasser et al., 2004). An advantage of the present approach relative to the three methods previously described is that PI_{ABS} takes into consideration the three main functional steps of the entire photosynthetic activity of PSII. However, the single parameter, PI_{ABS}, cannot distinguish well one stress treatment from other stresses since it is highly sensitive to any stress. A number of JIP-test parameters not only a single parameter should be choose to evaluate stress tolerance. For this consideration, earlier we introduced a model based on both PI_{ABS} and a characteristic parameter V_J for classification of the susceptibility in croftonweed, arabidopsis, common crabgrass, tobacco and cotton plants to PSII inhibitor tenuazonic acid (Chen et al., 2015).

The appearance of the K-step is a specific indicator of injury of the OEC on the donor side of PSII, which has already evidenced for drought and nitrogen deficiency, as well as for heat stress (Strasser et al., 2004). However, so far it is found that only in the case of severe high temperature stress the pronounced K-step appears directly in the actual fluorescence rise kinetics. Hence, the most sensitive parameter PI_{ABS} and V_K as a characteristic parameter for heat stress are selected among many JIP-test parameters to establish a model for ranking and distinction of four croftonweed populations into heat sensitive (DL), intermediate (HGS and CX) and tolerant (YJ) (Fig. 8). This approach allows us to discriminate well the heat sensitivity in four croftonweed populations. Its reliability could be confirmed by our earlier studies showing a good classification of heat tolerance in seventeen croftonweed populations by heat injury index (Su, 2005). In this model, the sensitivity level of four populations to heat stress is clearly represented by K (also called heat sensitivity index HSI) (Fig. 8). The smaller the HSI value is, the higher the level of tolerance to heat stress is. This methodology based on leaf discs system is a very simple, expeditious and non-destructive. Therefore, we proposed that it could be used as a reliable tool to screen heat tolerance in not only croftonweed plants but also other plant species.

For an effective model, it is necessary to find the suitable intensity, duration and rate of increase in high temperature since the sensitivity of heat response is different among plant species or in experimental systems. First of all, the selection of high temperature span should include all mild (<40 °C), moderate (40–44 °C) and strong (>44 °C) heat treatment, whatever in *vivo* whole plant or in vitro detached-leaf system. It is also suggested to avoid extreme heat treatment temperature above 50 °C, which is too strong for detached-leaves to obtain the typical fluorescence kinetics OKJIP. Secondly, the duration of heat treatment is also important to build a good linear correlation between JIP-test parameters and temperatures, and finally exhibit clearly heat sensitivity difference in samples. However, how to choose the suitable duration for each research mainly depends on the plant species and experimental systems.

4.3. Acquired heat tolerance in croftonweed populations due to prior ambient high temperature adaptation

Plants have the inherent basal heat tolerance capacity to survive temperatures over the optimal growth temperature without any prior acclimation and an ability to acquire tolerance to otherwise lethal heat temperatures (Mueller et al., 2015). The former may strongly vary depending on the plant species. The latter acquired heat tolerance is a plant cell autonomous phenomenon and results from prior exposure to short sublethal high temperatures or moderate heat treatment (Larkindale and Vierling, 2008). In the past three decades (1971–2000), croftonweed populations (DL, HGS, CX and YJ) from three different climatic zones underwent different temperature changes and ultimately colonized successfully in four areas of South China. In fact, the extreme high temperatures (>30 °C) four croftonweed populations suffered already come up to the standard of mild even moderate heat stress, which is around twice as high as the annual average temperature (Table 2). In general, a transient elevation in temperature, usually 10–15 °C above ambient, is considered heat stress (Wahid et al., 2007). Because plants are sessile organisms that cannot escape heat stress, they are forced to invest valuable resources in modifying their cellular components and metabolism to prevent damage caused by heat stress, in a process generally referred to as acclimation (Mittler et al., 2012). Wherein, the changes in photosynthetic capacity may be one of the adaptive mechanisms to cope with high temperature stress (Figs. 2–7; Mathur et al., 2011). It had been stated that heat stress differentially affects the stability of various proteins, membranes and photosynthetic pigments, and alters the activity of enzymes and even the morphological structure of cells during croftonweed plants adapted to ambient high temperature (Su, 2005; Yang, 2008). Our results show that YJ population possesses higher photosynthetic activity than other three populations (HGS, CX and DL) when plants growing under optimal controlled conditions are subjected to an episode of severe heat stress (Figs. 1–7). According to photosynthetic response to heat treatment, these four croftonweed populations are classified into three categories: tolerant (YJ), intermediate (HGS and CX) and sensitive (DL). This means that there has been much genetic variability within croftonweed populations as far as the adaptation of the photosynthetic apparatus to the growth temperature. Obviously, croftonweed plants are capable of acquiring heat tolerance by exposing gradually to mild or moderate temperatures, which enhances plant tolerance to subsequent exposure to strong heat stress.

However, the magnitude of heat tolerance a given plant acquired depends on plant types as well as rate of temperature increase, intensity and duration (Sung et al., 2003). For four croftonweed populations, YJ plants growing in tropical region have faced more chances of suffering severe high temperatures above 40 °C than HGS and CX in center subtropical climate and DL in northern subtropical climate, where the extreme high temperatures are 31–34 °C (Table 2). Prior exposure of severe high temperatures certainly results in stronger heat tolerance in YJ population than HGS, CX and DL populations with experience of mild heat stress. The tolerance degree of four croftonweed populations to heat stress is significantly correlated to the latitude and annual average temperature, especially extreme high temperature (Fig. 9). Thus, the development of croftonweed populations

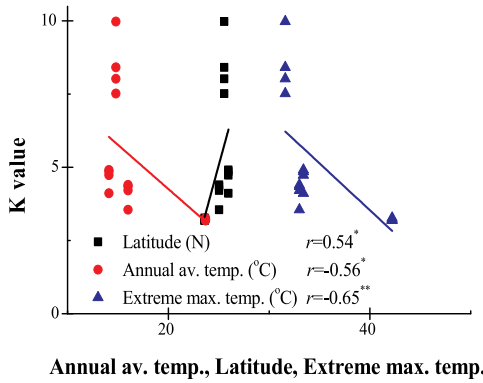

Fig. 9. The relationship between heat sensitivity index (the *K* value) and latitude, annual average temperature and extreme maximum temperature. The *K* values were obtained from Fig. 8, and the latitude and temperature data were obtained from Table 2. * and ** indicates the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

with enhanced heat tolerance is a result of plants adapting the changes of extreme high temperatures. The acquired heat tolerance also confers a potential risk for invasive alien plant croftonweed to spread further to currently hotter areas.

Author contributions

S.G. Chen and S. Qiang designed research; S.G. Chen and J. Yang performed experiments; S.G. Chen, M.S. Zhang and R.J. Strasser analyzed data and wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2015.09.011.

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