Chromosome doubling can increase heat tolerance in *Lonicera japonica* as indicated by chlorophyll fluorescence imaging

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Abstract

Imaging fluorometry was applied to investigate the tolerance of Japanese honeysuckle (*Lonicera japonica* Thunb.) of different ploidy levels to heat stress. Seedlings of *L. japonica*, the diploid cv. Damaohua and the tetraploid cv. Jiufengyihao, were exposed to heat stress of 42 ºC for 6 h and a recovery for 10 h. Heat stress significantly decreased maximum photochemical efficiency, electron transport rate, effective quantum yield of photosystem 2, and photochemical quenching of both cultivars, but it decreased the non-photochemical quenching (NPQ) only in the tetraploid. Heat stress increased the content of total soluble sugars, proline, and malondialdehyde in both cultivars while it increased NPQ only in the diploid. Our findings suggest that the tetraploid showed to be more resistance to heat stress than the diploid of *L. japonica*, which was indicated by different chlorophyll fluorescence imaging techniques and metabolic changes. Moreover, the degree of recovery in the tetraploid was higher than that of the diploid. The tetraploid also possessed thicker epidermis (both upper and lower) and palisade tissue as well as denser pubescence.

Additional key words: diploid, Japanese honeysuckle, leaf anatomy, malondialdehyde, proline, soluble sugars, tetraploid.

Introduction

High temperature, which seriously inhibits plant growth, is a common stressor for plants (Boyer 1982). Heat stress can alter the organization of the thylakoid membranes and photosynthetic electron transport. The latter is particularly sensitive to high temperature dependent inhibition (Haldimann and Feller 2005). Photosystem 2 (PS 2) is a thermolabile component of the thylakoid membranes (Havaux and Tardy 1996). Heat not only damages the oxygen-evolving complex of PS 2 (Enami *et al*. 1994), but also impairs electron transfer within the PS 2 reaction centres (Kouřil *et al*. 2004) and downstream of PS 2 (Sinsawat *et al*. 2004). Chlorophyll fluorescence is a highly sensitive and reliable method for detection and quantification of temperature-induced changes in the photosynthetic apparatus (Haldimann and Feller 2005).

The development of chlorophyll fluorescence imaging systems has greatly increased the versatility of chlorophyll fluorometry as a non-invasive technique for studying photosynthesis, especially under environmental stresses (Oxborough 2004). Through imaging of chlorophyll fluorescence, it is possible to produce parameterized fluorescence images that estimate the operating quantum efficiency of PS 2 photochemistry. Although responses of a number of plants to heat stress have been investigated (Enami *et al*. 1994, Qiu and Lu 2003, Haldimann and Feller 2005, Xu and Zhou 2006), the mechanisms involved in chromosome doubling have never been clarified.

Compared to diploids, chromosome doubling (polyploidy) is often associated with increases in leaf thickness and area (Xu *et al*. 2010) as well as in numbers of shoots and flowers (Xiong *et al*. 2006, Li *et al*. 2009). These anatomical and morphological variations generally lead to different physiological traits and ecological adaptations, *e.g*., higher drought tolerance (Li *et al*. 1996, Xiong *et al*.

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Abbreviations: Chl - chlorophyll; ETR - electron transport rate; F_m - maximum fluorescence; F_v - variable fluorescence; MDA - malondialdehyde; NPQ - non-photochemical quenching; PPFD - photosynthetic photon flux density; PS - photosystem; qP - photochemical quenching; Φ_{PS2} - effective qualtum yield of PS 2.
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2006, Li *et al*. 2009). Chromosome doubling can increase drought resistance in *Lonicera japonica* in relation to leaf anatomy improvement (Li *et al*. 2009). However, there have been few studies on the response of polyploid organisms to high temperatures (Khanna-Chopra and Viswanathan 1999, Chinnusamy and Khanna-Chopra 2003).

Japanese honeysuckle (*Lonicera japonica* Thunb.) is commonly cultivated for more than 1 000 years as a highly valued medicinal plant in East Asia, particularly in China (Chai *et al*. 2005). We used colchicines to develop a tetraploid cultivar (Jiufengyihao) from the diploid one (Damaohua; Tan *et al*. 2005). Besides yielding significantly more flowers in the tetraploid, we also observed bigger leaves and improved capacity of stress resistance compared to the diploid (Li *et al*. 2009).

In this work, the tetraploid and diploid *L. japonica* were compared for different anatomical and physiological responses to heat stresses to investigate the relationship between chromosome doubling and heat-tolerance of *L. japonica* and its possible mechanisms.

Materials and methods

Stem cuttings of two Japanese honeysuckle (*Lonicera japonica* Thunb.) cvs. Damaohua (diploid) and Jiufengyihao (tetraploid) were rooted in pots containing a mixture of peat-moss and *Perlite* (4:6, v/v) on 10 July, 2004 and then transplanted into 35-cm pots filled with a mixture of *Vermiculite*, peat, and soil (1:3:6, v/v). After sprouting and growing for about two years, 30 healthy and uniform seedlings per cultivar were selected for experiment initiated on 5 July 2006.

Whole plants were exposed to high temperature stress (42 ºC) for 6 h under photosynthetically active radiation (PAR) of 300 μmol m^{-2} s^{-1} on the surface of the leaves. After heat stress, plants were moved to temperature of 26 ºC and recovery proceeded for 10 h. Chlorophyll fluorescence was measured by an imaging-PAM fluorometer (*Walz*, Effeltrich, Germany) at heat stresses lasting 0, 2, 4 and 6 h and recovery 10 h. Photosynthetic photon flux density (PPFD) incident on the leaf was 186 μmol m^{-2} s^{-1} in kinetics induction curve measurements and 0 to 701 μmol m^{-2} s^{-1} in rapid light curves.

Blue light-emitting diodes (LEDs; peak wavelength, 470 nm) were used for fluorescence excitation, actinic irradiance and saturating light pulses. The charge-coupled device (CCD) camera had a resolution of 1392 × 1040 pixels. Pixel value images of the fluorescence parameters were displayed with help of a false colour code ranging from black (0) through red, yellow, green, blue, to pink (ending at 1) (Berger *et al*. 2004). Mature leaves were kept in dark for 30 min prior to measurement. The minimum fluorescence (F_0) was obtained by under PPFD 0.5 μmol m^{-2} s^{-1}. The maximum fluorescence (F_m) was determined by applying a saturating blue pulse (2 800 μmol m^{-2} s^{-1}). Following this, actinic irradiance (204 μmol m^{-2} s^{-1}) was switched on and saturating pulses were applied at 20 s intervals for 5 min in order to determine the maximum fluorescence yield during the actinic illumination (F_m'), and the chlorophyll fluorescence yield during the actinic illumination (F_s). For each interval, saturation pulse images and values of various chlorophyll fluorescence parameters were captured. To correctly determine F_0', we switched off the actinic irradiance and quickly reoxidise the PS 2 acceptor side with the help of far-red radiation. However, this is not feasible with imaging-PAM since far-red radiation would penetrate the CCD-detector and cause serious disturbances to fluorescence images (Calatayud *et al*. 2006). The value of F_0' was estimated using the approximation of Oxborough and Baker (1997), $F_0' = F_0/(F_v/F_m + F_0/F_m')$. The following parameters were calculated using the key parameters mentioned above: maximum photochemical efficiency [$F_v/F_m = (F_m - F_0)/F_m$], the effective quantum yield of PS 2 [$\Phi_{PS2} = (F_m' - F_s)/F_m'$], electron transport rate [$ETR = \Phi_{PS2} \times PPFD \times 0.5 \times 0.84$], photochemical quenching [$q_p = (F_m' - F_s)/(F_m' - F_0')$] and non-photochemical quenching [$NPQ = (F_m - F_m')/F_m'$] (Schreiber *et al*. 1994).

The sampled leaves were harvested after the chlorophyll fluorescence measurements. After the midrib of each leaf was removed, leaf samples were immediately frozen in liquid nitrogen, lyophilized and kept at -80 ºC until analysed. Total soluble sugars were quantified using the anthrone method described by Souza *et al*. (2004).

Proline content was determined following the method of Bates *et al*. (1973). About 1.0 g of frozen leaf sample was homogenized with 10 cm^{-3} of 30 g dm^{-3} sulfosalicylic acid and boiled for 10 min. Following this, the homogenate were centrifuged at 3 000 *g* for 20 min and the supernatant was collected. The reaction mixture, which contained 1.2 cm^{-3} of the supernatant, 2 cm^{-3} of glacial acetic acid, and 3 cm^{-3} of 25 g dm^{-3} acid ninhydrin, was boiled for 40 min. After termination of the reaction in an ice bath, the reaction mixture was extracted by 5 cm^{-3} of toluene and the absorbance at 520 nm was determined by spectrophotometer (*Specord 200*, *Analytik Jena AG*, Jena, Germany).

Malondialdehyde (MDA) was determined according to the method of Heath and Packer (1968). Frozen tissue (0.5 g) was homogenized in 5 cm^{-3} of 10 g dm^{-3} trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 *g* for 10 min, and 4 cm^{-3} of 200 g dm^{-3} TCA containing 50 g dm^{-3} thiobarbituric acid (TBA) was mixed with 1 cm^{-3} of supernatant, heated in boiling water for 30 min, and then cooled to room temperature. The mixture was centrifuged at 15 000 *g* for 15 min and the absorbance of the supernatant was measured at 535 nm. The content of MDA was calculated based on the coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Statistical analyses were performed using the *SPSS* statistical package (*Ver. 13.0, SPSS*, Chicago, IL, USA). Chlorophyll fluorescence determinations were obtained from three fully developed leaves. Area of interest (AOI) was selected for the whole leaf, and an average value was calculated per leaf. Means were analyzed using the Student's *t*-test between the diploid and the tetraploid cultivars at each high temperature treatment and the differences were considered significant if $P < 0.05$.

Results

The maximum photochemical efficiency (F_v/F_m) in leaves of both cultivars decreased significantly as heat stress increased (Fig. 1). Compare to their controls, F_v/F_m of the diploid and the tetraploid decreased by 42.2 and 33.5 %, respectively (Table 1). Following recovery for 10 h after heat stress, F_v/F_m of the diploid and the tetraploid returned

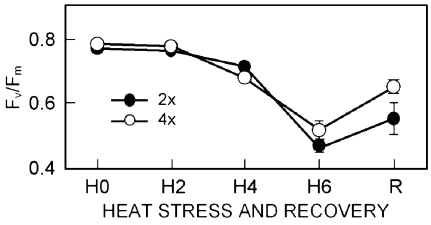

Fig. 1. The maximum efficiency of PS 2 photochemistry (F_v/F_m) in response to duration of heat stress from 0 to 6 h (H0 - H6) and to 10 h recovery (R) in the diploid (2x) and tetraploid (4x) *Lonicera japonica*. Mean ± SE, $n = 4$.

Table 1. Changes in chlorophyll fluorescence parameters and contents of metabolites [% of control] of the diploid and tetraploid *Lonicera japonica* at maximum heat stress (42 °C, 6 h) and recovery (10 h). Mean ± SE, $n = 4$. The asterisks *, ** and *** indicate significant differences between treatment and control at $P < 0.05$, 0.01, and 0.001, respectively.

	Maximum heat stress		Recovery	
	2x	4x	2x	4x
F_v/F_m	42.2±0.6***	33.5±3.8***	72.2±5.8**	84.2±2.6**
ETR	94.1±5.9***	50.8±4.2***	53.0±9.1**	80.1±4.2*
Φ_{PS2}	94.1±5.9***	50.9±4.1***	52.9±9.1**	80.0±4.2*
q_p	100.0±0.0***	21.6±2.4**	80.7±7.9*	99.8±2.8
NPQ	51.7±20.7	40.0±6.5*	91.0±14.5	81.8±9.2
TSS	32.1±7.4**	8.2±7.0	97.6±1.0	102.4±2.7
Proline	174.0±14.3***	113.0±13.3***	135.0±10.3*	84.1±3.9*
MDA	65.3±17.8**	31.8±5.9*	113.9±13.8	104.1±4.6

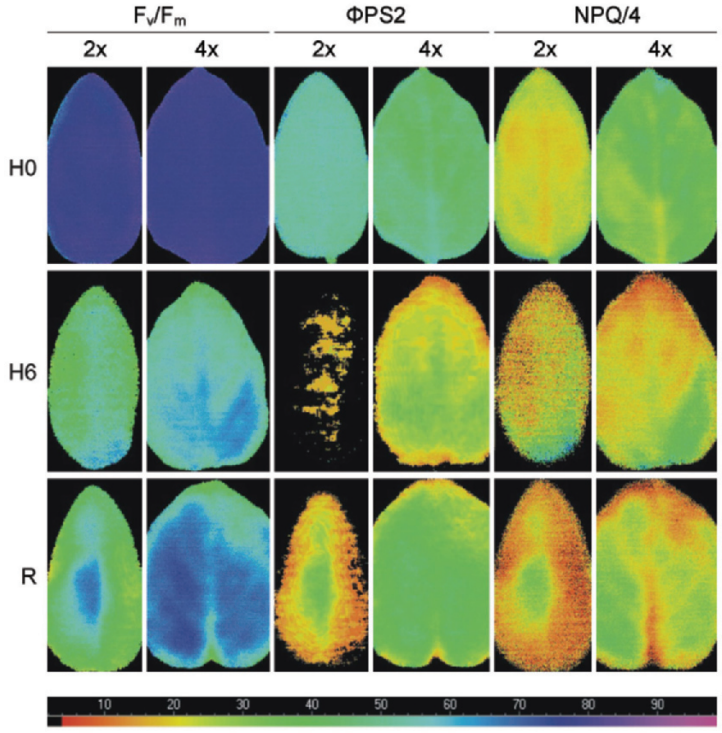

Fig. 2. The maximum efficiency of PS 2 photochemistry (F_v/F_m), actual PS 2 efficiency (Φ_{PS2}), and non-photochemical quenching (NPQ/4) in response to 6 h of heat stress and 10 h recovery after heat stress in the diploid and tetraploid *Lonicera japonica*. Φ_{PS2} and NPQ/4 reached a steady-state as indicated by the kinetics induction curve. Photosynthetic photon flux density (PPFD) on the leaf was 186 μmol m^{-2} s^{-1}.

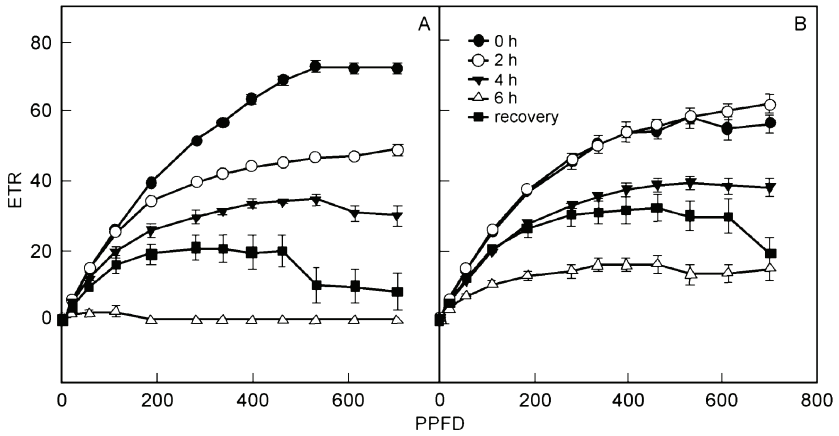

Fig. 3. The relationship between electron transport rate, ETR and PPFD [μmol m^{-2} s^{-1}] in different time of heat stress and recovery in the diploid (*A*) and tetraploid (*B*) *Lonicera japonica*. Mean ± SE, $n = 4$.

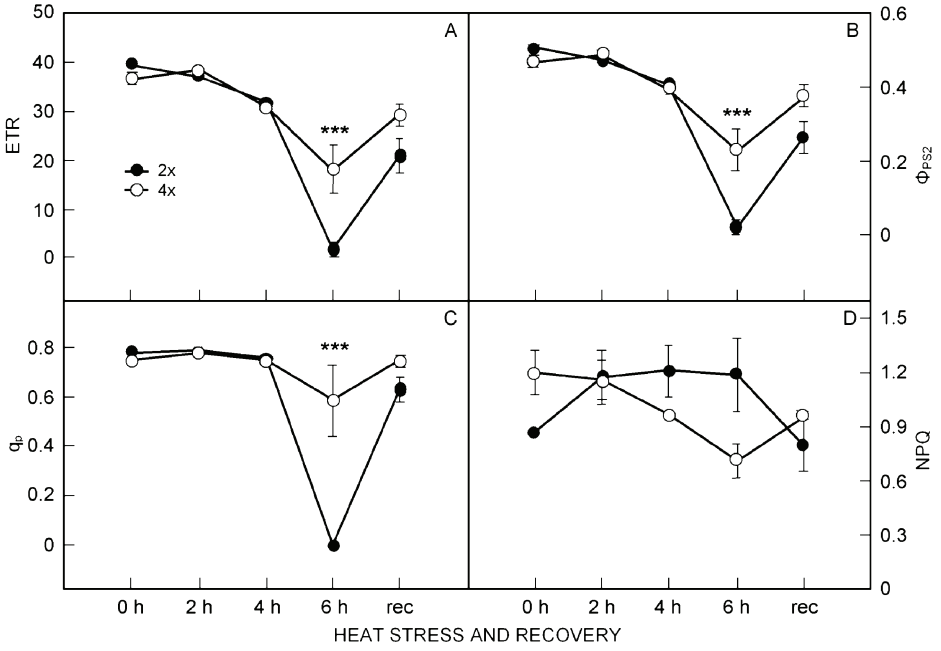

Fig. 4. Steady-state values of electron transport rate (ETR, *A*), actual PS 2 efficiency (Φ_{PS2}, *B*), photochemical quenching (q_p, *C*), and non-photochemical quenching (NPQ, *D*) in different times of heat stress and 10 h recovery after heat stress in the diploid and tetraploid *Lonicera japonica*. PPFD was 186 μmol m^{-2} s^{-1}. Mean ± SE, $n = 4$. The asterisks *** indicate significant differences between diploids and tetraploids at $P < 0.001$.

to 72.2 and 84.2 % of their controls, respectively (Table 1). Chlorophyll fluorescence imaging showed the difference in F_v/F_m between the diploid and the tetraploid in response to heat stress and recovery (Fig. 2).

Electron transport rate (ETR) gradually increased with an increase of PPFD (Fig. 3). Although severe heat stress caused almost cessation of ETR in the diploid, the tetraploid was still able to maintain some levels of ETR (Fig. 3). Meanwhile, the recovery in the tetraploid was better than that of the diploid (Fig. 3).

At PPFD 186 μmol m^{-2} s^{-1}, the steady values of ETR, Φ_{PS2}, and q_p gradually decreased with heat stress in both cultivars (Fig. 4). There were no significant differences in ETR, Φ_{PS2}, and q_p between the diploid and the tetraploid under mild heat stresses (4 h). However, ETR, Φ_{PS2} and q_p in the tetraploid were significantly higher than those in the diploid after 6-h heat stress (Fig. 4*A*,*B*,*C*). After 10-h recovery, ETR, Φ_{PS2}, and q_p recovered to 53.0, 52.9, and 80.7 %, in diploid while to 80.1, 80.0 and 99.8 % in teptaploid, respectively (Table 1). The imaging of chlorophyll fluorescence (Fig. 2) also showed the difference in Φ_{PS2} between the diploid and the tetraploid subjected to heat stress and recovery.

Non-photochemical quenching (NPQ) increased in the diploid, but decreased in the tetraploid with heat stress duration (Fig. 4*D*). At the maximal heat stress (6 h), NPQ

increased by 51.7 % for the diploid while decreased by 40.0 % for the tetraploid as compared to their controls. NPQ recovered to 91.0 and 81.8 % of the control in the diploid and the tetraploid, respectively (Table 1). The change of NPQ showed a similar trend as compared with the imaging of chlorophyll fluorescence (Fig. 2).

An increase in contents of total soluble sugars, proline and malondialdehyde (MDA) under heat stresses was observed in both cultivars. However, the diploid displayed a greater increase than the tetraploid (Fig. 5, Table 1). The contents of total soluble sugars, proline, and MDA in heat-stressed leaves of both cultivars decreased during the recovery almost to the control level (Table 1), with the exception of the proline content of the diploid, which remained was still higher than that of the control (Fig. 5).

Discussion

Changes of F_v/F_m, ETR, Φ_{PS2}, and q_p were slightly less in tetraploid than in diploid *L. japonica* after heat stress (Table 1, Figs. 1-4), indicating that the PS 2 reaction centre have been less affected in the tetraploid than in the diploid (Qiu and Lu 2003, Xu and Zhou 2006). Heat stress increased NPQ in the diploid but decreased NPQ in the tetraploid (Fig. 4*D*), indicating that non-radiative energy dissipation might be higher in the diploid than in the tetraploid (Schreiber *et al*. 1994). Polyploid cultivars are generally less sensitive to high temperature stresses of photosynthesis than diploid ones. For example, hexaploid *Triticum* species were less sensitive to heat than their diploid relatives as concern their production (Chinnusamy and Khanna-Chopra 2003, Khanna-Chopra and Viswanathan 1999). Moreover, the degree of recovery in the tetraploid was higher than that of the diploid (Table 1, Figs. 1 - 4). Consequently, it appears that this tetraploid was more adaptable to high temperature habitats than the diploid.

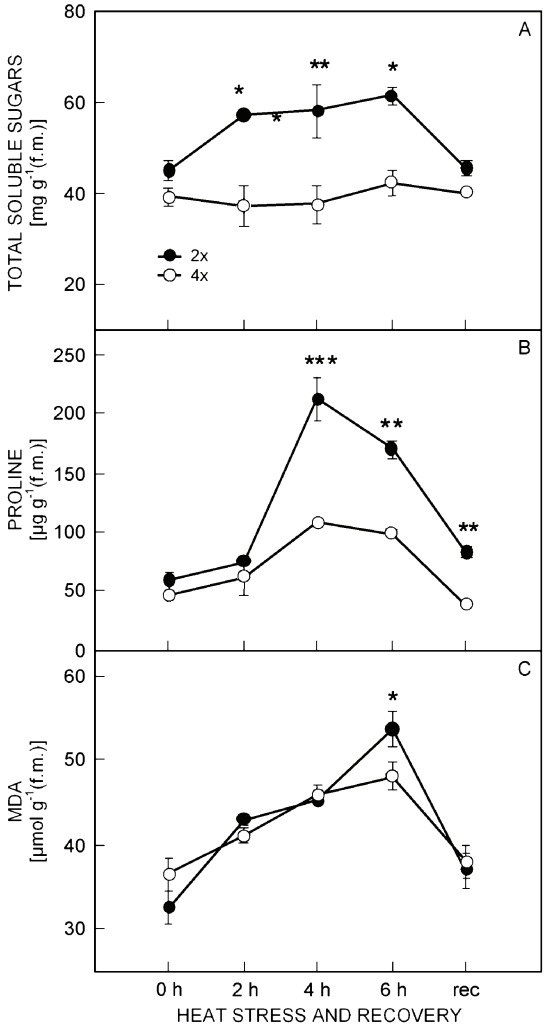

Fig. 5. The contents of total soluble sugars (*A*), proline (*B*), and malondialdehyde (MDA, *C*) in different times of heat stress and recovery 10 h after heat stress in the diploid and tetraploid *Lonicera japonica*. Mean ± SE, $n = 4$. The asterisks *, ** and *** indicate significant differences between diploids and tetraploids at $P < 0.05$, 0.01, and 0.001, respectively.

Anatomical features of the polyploids such as thicker epidermis (both low and upper) provide a structural basis for an increase in heat resistance (Li *et al*. 1996, 2009). We also reported that chromosome doubling of *L. japonica* increased drought resistance based on leaf anatomy improvement (Li *et al*. 2009).

Increases in the total soluble sugars and proline contents have been commonly observed in heat-stressed plants (Kocsy *et al*. 2005). Those compounds are known to function in osmotic adjustment, but they are often also considered as symptoms of tissue damage by drought (Souza *et al*. 2004). Another notable chemical change in leaf tissues during heat stresses in both the diploid and the tetraploid forms of *L. japonica* was the accumulation of MDA, a product of peroxidation of unsaturated fatty acids in phospholipids and indication of cell membrane damage (Xu *et al*. 2006). Significant increases in MDA have been found in other heat-stressed plants and the magnitude of increase was used to indicate the extent of tissue damage by heat (Xu and Zhou 2006). The significantly greater accumulation of total soluble sugars, proline, and MDA in the diploid form of *L. japonica* (Fig. 5) demonstrated that it sustained a higher degree of heat-related injury than the tetraploid.

In conclusion, the tetraploid showed to be more resistance to heat stress than the diploid of *L. japonica*, which was indicated by different chlorophyll fluorescence imaging techniques and metabolic changes.

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