Interspecific Variation in Heat Stress Tolerance and Oxidative Damage among 15 C₃ Species

Wagdi S. Soliman, and Shu-ichi Sugiyama

Abstract—The C₃ plants are frequently suffering from exposure to high temperature stress which limits the growth and yield of these plants. This study seeks to clarify the physiological mechanisms of heat tolerance in relation to oxidative stress in C₃ species. Fifteen C₃ species were exposed to prolonged moderately high temperature stress 36/30°C for 40 days in a growth chamber. Chlorophyll fluorescence (Fv/Fm) showed great difference among species at 40 days of the stress. The species showed decreases in Fv/Fm and increases in malondialdehyde (MDA) content under stress condition as well as negative correlation between Fv/Fm and MDA (r = -0.61*) at 40 days of the stress. Hydrogen peroxide (H₂O₂) content before and after stress in addition to its response under stress showed great differences among species. The results suggest that the difference in heat tolerance among C₃ species is closely associated with the ability to suppress oxidative damage but not with the content of reactive oxygen species (ROS) which is regulated by complex network.

Keywords—C₃ species, Fv/Fm, heat stress, oxidative stress.

I. INTRODUCTION

The plants from different habitats have different optimum growth temperature. The C₃ species adapt to temperate climates, while C₄ species can be tolerant to hot and drought conditions. The anticipated higher summer temperatures under climate warming are likely to cause serious damages to the growth and yield of C₃ crops [1-2]. Therefore, improving the tolerance of C₃ crops to heat stress is a major target for breeders [3-4]. However, the key traits that confer such tolerance in the field have not been clearly identified so far [5-7].

Plants exposed to temperature stress are suffering from the accumulation of reactive oxygen species (ROS) which cause oxidative stress. ROS is produced in leaves as a result of the imbalance between electron transfer rate and carboxylation capacity in photosynthetic process [8-10]. In previous study, we found that the sensitive cultivar of Lolium perenne to summer climates showed greater accumulation of hydrogen peroxide (H₂O₂) in leaves than that in the tolerant cultivars under prolonged moderately high temperature stress [11]. This result suggested that functional damage under summer high temperature is mainly caused by oxidative stress, which is derived from excess light energy generated under heat stress.

The ROS are generated by aerobic respiration in mitochondria, photosynthetic light reaction in chloroplasts, and photorespiration in peroxisomes [12-13]. The balance of ROS content regulates by its production and scavenging system which in turn is regulated by a redundant and complex biochemical network. Breakdown of gene expression in two major scavenging enzymes, namely ascorbate peroxidase (APX) and catalase, does not bring substantial changes in oxidative balance [14-15].

To understand the tolerance mechanism of plants to heat stress, it is important to make comparative studies both within species and among species which differ in their tolerance. So far, most studies that compared heat tolerance have been limited to comparison among a few numbers of cultivars [16-19] or a few numbers of species which are closely related [20-22]. Few studies have examined differentiation among large number of unrelated species under long-term heat stress. In this study, responses to heat stress were compared among fifteen C₃ grass species belonging to different genus with diverse genetic background with special reference to the relationship between heat tolerance and oxidative tolerance.

II. MATERIALS AND METHODS

A. Plant Materials

In this study, fifteen C₃ species were used including: Agrostis alba L., Agrostis tenuis Sibth., Anthoxanthum odoratum L., Bromus inermis Leyss., Dactylis glomerata L., Festuca arundinacea Schreb., Festuca ovina L., Festuca pratensis Huds., Festuca rubra L., Lolium multiflorum Lam., Lolium perenne L., Phalaris arundinacea L., Phleum pratense L., Poa annua L., and Poa pratensis L.

B. Growth and Heat Stress Conditions

Seeds of the 15 species were germinated on wet filter paper in Petri dishes, and the seedlings were transplanted into pots—one seedling in each pot—7.5 cm in diameter and 8 cm deep and filled with sandy loam containing 0.35 g of each of N, P₂O₅, and K₂O for every kilogram of soil. The plants were grown in a controlled growth chamber with day/night temperatures of 23/16 °C, a 16-h photoperiod (4:00 to 20:00 h) with photon flux of 250 µmol m⁻² s⁻¹, and relative humidity of 70% round the clock. Forty days after transplanting, the plants were exposed to 30 °C for 3 days for acclimation and then to 36/30 °C (day/night) for 40 days. The plants were watered daily to avoid water stress. The experiment was set up in a randomized block layout incorporating four replications.

C. Chlorophyll Fluorescence Measurement

The minimum (F₀) and maximal (Fₘ) levels of fluorescence were measured in leaves adapted to dark for 20 min with a
portable photosynthesis measuring system (LI-6400, Li-cor, Lincoln, Nebraska, USA). The maximal photochemical efficiency of photosystem II (PSII), the most heat-sensitive component in photosynthesis, was calculated as \( F_v/F_m = (F_m – F_o)/F_m \). Measurements were made before the acclimation (control) and at 10-day intervals during the period of exposure to high temperatures.

**D. Physiological Measurements**

Membrane lipid peroxidation (MDA) and hydrogen peroxide (H\(_2\)O\(_2\)) content were recorded twice, before the acclimation and at 40 days of stress exposure. Membrane lipid peroxidation was determined by malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method as described before [11, 23]. Fresh leaves (50 mg samples) were ground in 1.5 mL of 0.1% solution of trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 rpm at 3 °C for 5 min, and 1 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. After heating the mixture for 20 min in boiling water and cooling it quickly in an ice bath, the supernatant was used for spectrophotometric determination of MDA. Absorbance at 532 nm was recorded and corrected for non-specific absorbance at 600 nm. Concentrations of MDA were calculated on fresh weight (FW) basis by the following formula with an extinction coefficient of 155 mmol\(^{-1}\) cm\(^{-1}\).

A modified version of the ferrous ammonium sulphate/xylene orange (eFOX) method was used to measure H\(_2\)O\(_2\) content of leaves following the methods of [24-25]. Leaf extracts were prepared by grinding 50 mg leaf samples in 500 \( \mu \)L of 0.1 M potassium phosphate buffer (pH 6.5) containing 5 mM Na \( _3 \) as an inhibitor of peroxidase activity. The extracts were centrifuged at 10 000 rpm at 5 °C for 5 min. The supernatant (200 \( \mu \)L) was added to 5 mL of the assay solution containing 250 \( \mu \)M ferrous ammonium sulphate, 100 \( \mu \)M sorbitol, 100 \( \mu \)M xylene orange, 1% ethanol, and 25 mM H\(_2\)SO\(_4\), which had been deoxygenated with gaseous nitrogen to prevent artefact production in hydrogen peroxide during the reaction. The spectrophotometric assay was conducted by measuring the difference in absorbance between 550 nm and 800 nm after 15 min of the reaction. H\(_2\)O\(_2\) content was calculated by a standard curve using a series of diluted solutions of commercial, high-grade 30% H\(_2\)O\(_2\).

**E. Statistical Analysis**

Analysis of variance (ANOVA) was used to test the significance of differences among the species for each measurement. The statistical analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

III. RESULTS

Chlorophyll fluorescence (Fv/Fm) showed no significant differences among 15 species before the exposure to heat stress with overall mean value of 0.779±0.001. Fv/Fm significantly decreased at 40 days of heat stress (0.636±0.032). The differences among species began to appear at 10 days of the stress and the differences became two-folds at 40 days of the stress (Fig. 1). The species were divided into three categories according to the degree of damage: (1) high tolerant species (seven species) which maintained more than 85% of Fv/Fm at 40 days of the stress, (2) medium tolerant species (six species) which maintained 75 ~ 85% of Fv/Fm and (3) sensitive species (two species) with less than 50% of Fv/Fm (Fig. 1).

Lipid peroxidation of membrane (malondialdehyde, MDA) and hydrogen peroxide (H\(_2\)O\(_2\)) showed highly significant differences among species before and after exposure to heat stress (Table I). MDA showed significantly negative correlation with Fv/Fm at 40 days of the stress (Fig. 2). The MDA content differed by eightfold before exposure to stress and by threefold after exposure to stress (Table I). *Bromus inermis* and *Festuca rubra* had the highest values of MDA content both before and after exposure to the stress. After exposure to the stress, MDA content increased significantly in all species except *Phalaris arundinacea* (Table II). H\(_2\)O\(_2\) content showed the same response to MDA except for the significant decrease of H\(_2\)O\(_2\) content in *Dactyles glomerata* and *Poa annua* (Table II). The H\(_2\)O\(_2\) content differed by fifteen-folds and six-folds before and at 40 days of the stress, respectively (Table I). The highest values of H\(_2\)O\(_2\) content both before and after exposure to stress were in *Festuca rubra* and *Festuca ovina*, respectively.

**Table I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>40-day</th>
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<tbody>
<tr>
<td></td>
<td>Rang</td>
<td>F value</td>
</tr>
<tr>
<td>MDA</td>
<td>6.3 – 25.5</td>
<td>50.2*</td>
</tr>
<tr>
<td>H(_2)O(_2)</td>
<td>0.16 – 2.38</td>
<td>188.5*</td>
</tr>
</tbody>
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*The value represents significance at probability level of \( p > 0.001 \)
Chlorophyll fluorescence (Fv/Fm) is used widely as an indicator of physiological damage to abiotic stress [26]. In this study, the decreases in Fv/Fm varied greatly among species, ranging from less than 10% to more than 50% at 40 days of exposure to heat stress. The decreases in Fv/Fm varied significantly even within the same genus (Fig. 1). This indicates that there are great differences among the C₃ species in tolerance to heat stress (Fig. 1). The decline of Fv/Fm represents that the reaction centre of PSII was damaged and inactivated by the stress [27].

Reactive oxygen species (ROS) plays the two opposite roles in processes of heat stress responses: a toxic molecule and a signal transduction molecule [28-30]. Levels of hydrogen peroxide (H₂O₂) vary greatly among species under natural conditions [24-25]. In this study, the species showed great differences in H₂O₂ content even under unstressed conditions. The great differences in H₂O₂ suggest that the species have different strategy to utilize H₂O₂ in regulating molecular and physiological networks. The significant increases in MDA content and decreases in Fv/Fm under stress condition as well as the significant correlation between them at 40 days of the stress (r = -0.61*) suggest that the difference in heat tolerance is closely associated with the ability to suppress oxidative stress. This is consistent with our previous studies within Loli um perenne cultivars [11, 23]. The differences in Fv/Fm and MDA after the stress were not associated with H₂O₂ content, this may be due to that the species used in this study had wide genetic background and roles of H₂O₂ in stress response cascade differed with each other as exemplified by the two species, Poa annua and Dactylis glomerata, which showed great sensitivity to stress and H₂O₂ content significantly decreased after the stress exposure (Fig. 1 and Table II).

Plants develop several defense mechanisms against toxic reactive oxygen molecules. These mechanisms include suppressing ROS production, scavenging the produced ROS and repairing the damage caused by ROS [31]. The results of this study suggest that the differentiation among species in heat stress tolerance is mainly associated with the ability to suppress the producing of ROS species. The great variation among species in H₂O₂ content even under unstressed condition is due to the wide genetic background among them. This wide genetic background led to difficulty of determining the role of antioxidants, not included, in heat stress tolerance among the species.

**IV. DISCUSSION**

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**REFERENCES**


