Chapter 5

INTERSPECIVIC VARIATION IN HEAT TOLERANCE AND SUPPRESION OF OXIDATIVE STRESS

5.1 COMPARISON AMONG 15 C_3 SPECIES

INTRODUCTION

The plants from different habitats have different optimum growth temperature. The C_3 species adapt to temperate climates, while C_4 species can 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 C_3 crops (Lobell and Asner 2003; Lobell and Field 2007). Therefore, improving the tolerance of C_3 crops to heat stress is a major target for breeders. 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 number of cultivars (Huang et al. 2001; Larkindale and Huang 2004; Almeselmani et al. 2006; He and Huang 2007) or a few number of species which are closely related (Xu et al. 2006; Xu and Huang 2008, 2010) and few studies have examined number of unrelated species under long-term heat stress. In this chapter, responses to heat stress were compared among fifteen C_3 grass species belonging to different genus with diverse genetic background with special reference to the relationship between heat tolerance and oxidative tolerance.

MATERIALS AND METHODS

Plant materials

In this study, fifteen C_3 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., *Poa pratensis* L.

Growth and heat stress conditions

The growth and stress conditions were described in detail on the *L. perenne* cultivars experiment (Chapter 2.1 and Chapter 3). After transplanting the seedlings in the pots, four replicates of each species were maintained under control conditions 23/16°C for 40 days, acclimation for 3 days at 30°C and then exposed to stress conditions (36/30°C) for 40 days.

Measurement methods

Chlorophyll fluorescence (Fv/Fm) was measured at 10-day intervals. Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase enzyme activity (APX) were measured twice, before and at 40 day of the exposure to stress as describes in details in the previous chapters (Chapter 2.1 and Chapter 3). The data of leaf traits were obtained from (Sugiyama 2005a, b).

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).

RESULTS

Chlorophyll fluorescence (Fv/Fm) showed no significant differences among 15 species before the exposure to stress with overall mean value of 0.779±0.001. Fv/Fm significantly decreased at 40 day of heat stress (0.636±0.032). The differences among species began to appear at 10 day of the exposure to stress and the differences became two-folds at 40 day of the stress (Figure 5.1.1). 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 day 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 (Figure 5.1.1).

Lipid peroxidation of membrane (malondialdehyde, MDA), hydrogen peroxide

(H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX) showed highly significant differences among species before and after exposure to heat stress (Table 5.1.1). MDA showed a significantly negative correlation with Fv/Fm at 40 day of the stress (Figure 5.1.2). The MDA content differed by eightfold before the exposure to stress and by threefold after the exposure to stress (Table 5.1.1). *Bromus inermis* and *Festuca rubra* had the highest values of MDA content both before and after the exposure to stress. After exposure to heat stress, MDA content increased significantly in all species except no significant change in *Phalaris arundinacea* (Table 5.1.2). H_2O_2 content showed the same response to MDA except for the significant decrease of H_2O_2 content in *Dactyles glomerata* and *Poa annua* (Table 5.1.2). The H_2O_2 content differed by fifteen-folds and six-folds before and at 40 day of the stress, respectively (Table 5.1.1). The highest values of H_2O_2 content both before and after the exposure to stress were in *Festuca rubra* and *Festuca ovina*, respectively. The species showed different responses after the stress in term of ETR, q_N, AsA and APX (Table 5.1.2).

The functional leaf traits showed significant differences among species (Table 5.1.3). However, there were no significant correlations between leaf traits and physiological damages, Fv/Fm and MDA. In *L. perenne* cultivars (Chapter 3), H_2O_2 content after the stress showed a negative correlation with leaf water content (LWC) and a positive correlation with leaf dry matter content. In this study, H_2O_2 content showed significantly correlations with both traits either before or after the stress (Figure 5.1.3).

DISCUSSION

Chlorophyll fluorescence (Fv/Fm) is used widely as an indicator of physiological damage to abiotic stress (Maxwell and Johnson 2000). In this study, the decreases in Fv/Fm varied greatly among species, ranging from less than 10 % to more than 50 % at 40 day of exposure to heat stress. The decreases in Fv/Fm varied significantly even within the same genus (Figure 5.1.1). This indicates that there are great differences among the C_3 species in tolerance to heat stress (Figure 5.1.1). The decline of Fv/Fm represents that the reaction centre of PSII was damaged and inactivated by the stress (Long et al. 1994).

Reactive oxygen species (ROS) plays the two opposite roles in processes of heat

stress responses: a toxic molecule and a signal transduction molecule (Foyer and Noctor 2005; Suzuki and Mittler 2006; Jaspers and Kangasjarvi 2010; Miller et al. 2010). Levels of hydrogen peroxide (H_2O_2) vary greatly among species under natural conditions (Cheeseman 2006; Queval et al. 2008). In this study, the species showed great differences in H_2O_2 content even under unstressed conditions. The great differences in H_2O_2 suggest that the species have different strategy to utilize H_2O_2 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 day of the stress ($r = -0.61^*$) suggest that the difference in heat tolerance is closely associated with the ability to suppress oxidative stress. In contrast to *L. perenne* cultivars, the differences in Fv/Fm and MDA after the stress were not associated with H_2O_2 content. This may be due to that, compared with the cultivars with narrow genetic background, the species used in this study had a wide genetic background and roles of H_2O_2 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 H_2O_2 content significantly decreased after the exposure to stress (Figure 5.1.1 and Table 5.1.2).

The changes in antioxidant activity (AsA content and APX) under the stress conditions varied among the species, but it should be noted that the species of the same genus had similar responses with varying degrees (Table 5.1.2). Antioxidant activity did not show any clear correlations with H_2O_2 content, Fv/Fm and MDA. However, these do not imply that the effects of antioxidants on heat tolerance are negligible because the complex networks of the antioxidant system exits in plants as suggested previously (chapter 3). Although leaf traits showed significant contribution to heat tolerance of *Lolium perenne* cultivars (Chapter 3), no clear contribution of leaf traits was observed among C_3 species. Although H_2O_2 content correlated significantly with leaf water content and leaf density, this is not necessarily evidence of the importance of structural leaf traits because (1) the same level of correlation was observed before the stress, and (2) both leaf traits and H_2O_2 content did not show correlations with physiological damage, Fv/Fm and MDA.

Table 5.1.1 Minimum and maximum values as well as the F value of variation among the 15-species of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX).

	Control		40-day	
	Rang	F value	Rang	F value
MDA (μmol g^{-1} FW)	6.3 ~ 52.5	50.2***	20.9 ~ 72.9	32.9***
H_2O_2 (μmol mg^{-1} FW)	0.16 ~ 2.38	188.5***	0.51 ~ 3.12	110.7***
ETR (μmol g^{-1} FW s^{-1})	0.42 ~ 1.02	12.9***	0.18 ~ 1.00	7.6***
q_N	0.825 ~ 0.897	4.4***	0.812 ~ 0.953	5.4***
AsA (μmol mg^{-1} FW)	22.6 ~ 171.9	109.2***	57.8 ~ 136.1	21.4***
APX (μmol mg^{-1} FW)	28.7 ~ 322.0	71.0***	18.1 ~ 83.1	10.6***

The value represents significance at probability level of $p > 0.001$

Table 5.1.2 The relative values of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), ascorbic acid (AsA) and ascorbate peroxidase (APX). The relative values were calculated from the equation (the value at 40 days of stress / the value before stress * 100), and the probability of significant (*, **, *** at 0.05, 0.01, and 0.001, respectively) represent the significance under stress condition compare to before stress.

Species	MDA	H_2O_2	ETR	q_N	AsA	APX
1. *Agrostis alba*	317.8***	268.6***	65.3*	101.8	84.1**	84.6
2. *Agrostis tenuis*	238.2***	132.1*	109.9	97.1	79.9**	101.7
3. *Anthoxanthum odoratum*	407.9***	325.0***	44.0***	107.1*	98.1	15.9***
4. *Bromus inermis*	138.9*	175.0**	57.1	107.8**	79.2***	59.7*
5. *Dactylis glomerata*	417.7***	67.3**	83.3	98.3	111.9	106.1
6. *Festuca arundinacea*	135.2***	145.7**	100.0	97.7	45.7***	95.3
7. *Festuca ovina*	156.1*	142.0**	55.1***	104.2*	86.3**	111.6
8. *Festuca pratensis*	241.0***	157.1***	59.0*	103.1	54.4***	145.1
9. *Festuca rubra*	130.4**	131.1***	78.2*	104.3*	83.6*	112.6
10. *Lolium multiflorum*	133.5***	303.8***	29.0***	111.5**	326.0***	183.4***
11. *Lolium perenne*	143.9***	241.2**	80.4	100.2	191.6***	170.9**
12. *Phalaris arundinacea*	124.2	116.2	86.5	100.8	82.7**	25.8***
13. *Phleum pratense*	475.2***	244.4***	44.9*	101.6	107.7	64.5**
14. *Poa annua*	284.3***	52.2***	26.5***	106.8	77.6**	149.6***
15. *Poa pratensis*	339.6***	159.5*	67.6*	98.0	73.6**	121.8*

*, **, ***, significant difference at 5, 1 and 0.1% levels, respectively

Table 5.1.3 Specific leaf area (SLA, cm^2 mg^{-1}), leaf water content (LWC, %), leaf area (LA, cm^2), leaf density (LD, mg cm^{-3}) and leaf thickness (LT, μm) of the eighteen species before exposure to heat stress.

Species	SLA	LWC	LA	LD	LT
1. *Agrostis alba*	0.426	79.3	8.59	191	123
2. *Agrostis tenuis*	0.561	81.9	3.91	163	111
3. *Anthoxanthum odoratum*	0.494	79.6	12.44	167	123
4. *Bromus inermis*	0.425	80.6	18.25	176	136
5. *Dactylis glomerata*	0.433	80.6	13.54	153	152
6. *Festuca arundinacea*	0.389	83.5	16.4	153	170
7. *Festuca ovina*	0.283	76.1	1.81	222	167
8. *Festuca pratensis*	0.460	83.6	15.03	133	167
9. *Festuca rubra*	0.309	74.4	2.48	277	126
10. *Lolium multiflorum*	0.475	87.0	7.88	122	177
11. *Lolium perenne*	0.490	83.9	11.99	131	160
12. *Phalaris arundinacea*	0.605	83.5	14.28	147	116
13. *Phleum pratense*	0.496	81.7	17.14	131	155
14. *Poa annua*	0.623	81.1	4.06	188	86
15. *Poa pratensis*	0.453	80.2	8.53	155	149

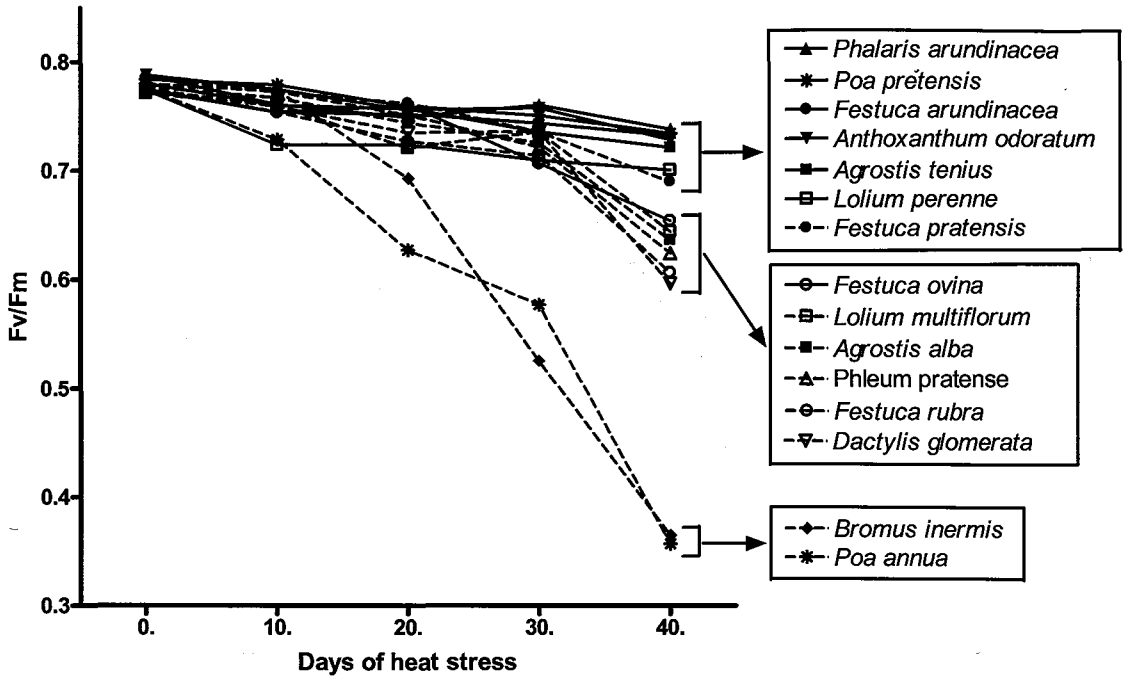

Figure 5.1.1 Response pattern of chlorophyll fluorescence in 15 C_3 species at different durations (days) of continuous exposure to heat stress. The top group maintained more than 85%, the middle group maintained 75 ~ 85%, and the bottom group maintained less than 50% of Fv/Fm at 40 day of the exposure to stress.

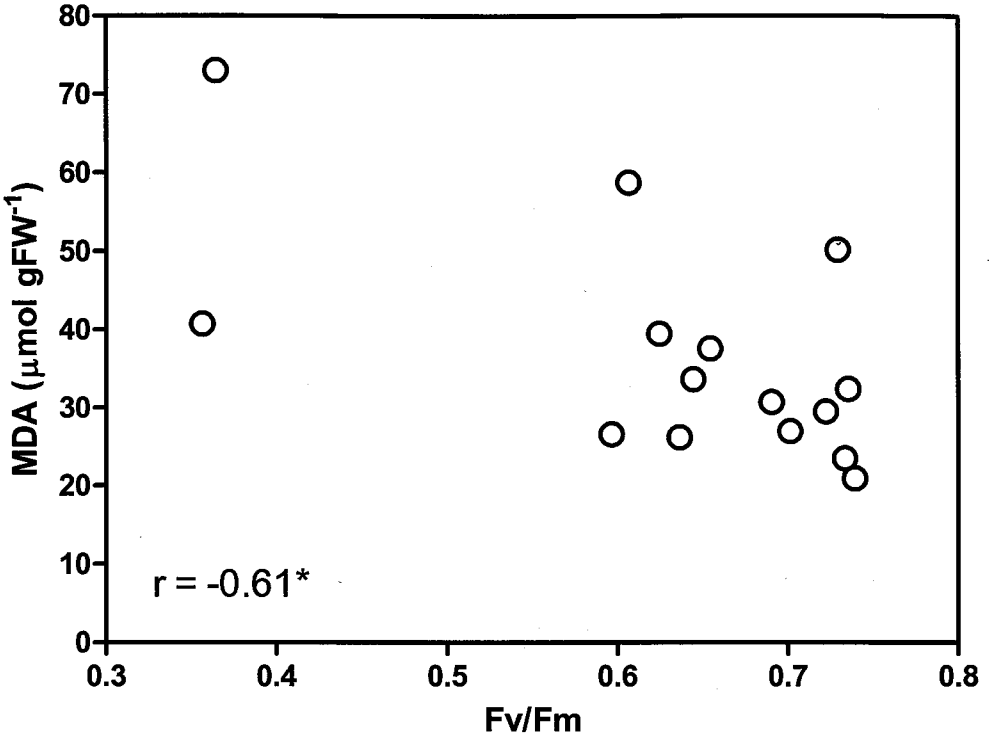

Figure 5.1.2 The correlation between chlorophyll fluorescence (Fv/Fm) and malondialdehyde content (MDA, μmol g^{-1} FW) at 40 day of exposure to heat stress.

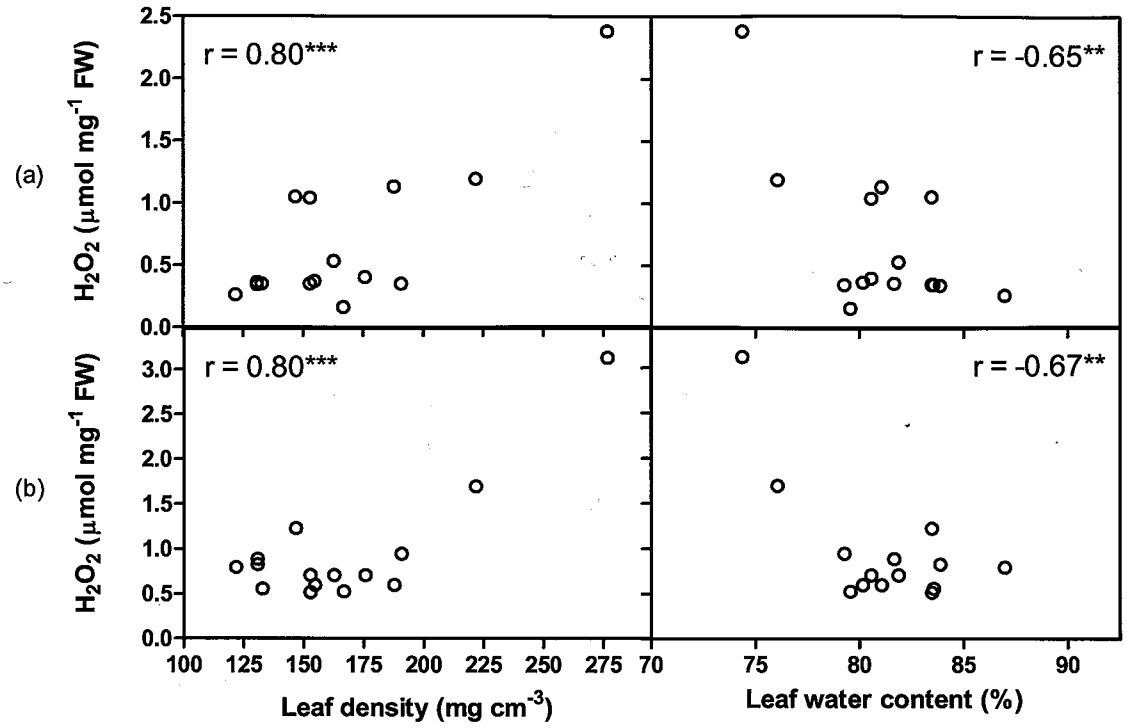

Figure 5.1.3 The correlation of leaf water content (LWC) and leaf density with hydrogen peroxide contents (H_2O_2) before the exposure to stress (a, the above part) and at 40 day of the stress (b, the bottom part).

5.2 COMPARISON BETWEEN C_3 AND C_4 SPECIES

INTRODUCTION

The plants have C_3 and C_4 types of photosynthetic systems. The C_4 species have higher ability to minimize the extent of photorespiration than C_3 species under stress conditions by boosting the CO_2 concentrations in the bundle-sheath cells. There are many studies which focused on the difference between C_3 and C_4 species in terms of photosynthesis thermal stability under heat stress (Salvucci and Crafts-Brandner 2004; Sage and Kubien 2007; Hamilton et al. 2008). However, as far as I know, no studies focused on the difference between C_3 and C_4 species in terms of oxidative damage under prolonged temperature stress. In this chapter, three C_3 species and three C_4 species were exposed to prolonged moderately high temperature (36/30°C) for 40 days. The objectives of this study were (1) to clarify the difference in the physiological response among species under stress conditions, (2) to examine if the C_3 species suffer more oxidative damage than C_4 species does, and (3) to clarify the association between the functional leaf properties and oxidative damage.

MATERIALS AND METHODS

Plant materials, growth conditions and heat treatment

In this study, six species were used including three C_3 species, *Agrostis tenuis* Sibth., *Festuca arundinacea* Schreb., *Lolium perenne* L. and three C_4 species, *Chloris gayana* K., *Paspalum notatum* Flugge., *Zoysia japonica* Steud.

Seeds of the six species were germinated and then the seedlings were transplanted into pots as the same conditions as described in Chapter (2.1). The plants were grown in a controlled growth chamber with day/night temperatures of 23/16°C for the C_3 species and 28/24°C for the C_4 species, a photoperiod of 16–h with 250 μmol m^{-2} s^{-1} photon flux, and 70% relative humidity during the whole day. The plants were maintained in the controlled condition for 40 days after transplanting for all species except for *Zoysia japonica* which was maintained for two months because of its low growth. All the plants were exposed to 30°C for three days for acclimation followed by exposure to 36/30°C for 40 days. Water was supplied daily to avoid water stress. The experiment was set up

in a randomized block layout incorporating four replications.

Physiological measurements and leaf traits

Chlorophyll fluorescence (Fv/Fm), membrane lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA), ascorbate peroxidase (APX) and leaf traits were recorded in the same measurement times as *Lolium perenne* cultivars experiment (Chapter 3) using the methods described previously (Chapter 2.1 and Chapter 3).

Statistical analysis

The statistical analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA). The significant difference among the species was tested by analysis of variance (ANOVA) for each measurement time. The difference between C_3 and C_4 species was also tested by *t*-test.

RESULTS

There were no significant difference in the maximum PSII activity (Fv/Fm) among the species before the stress treatment, but differences were found at all measurement times after the exposure to stress (Table 5.2.1). Fv/Fm gradually decreased with the exposure to stress and the decreases became significant at 40 day of the stress against the control for all species except for *Paspalum notatum*. *Lolium perenne* had a larger decline at 10 day after the stress and had the lowest Fv/Fm value at 40 day of the stress (Figure 5.2.1). Although the significant difference was not found between C_3 and C_4 species before the treatment, the C_4 species had significantly higher Fv/Fm values after the stress (Table 5.2.1). Fv/Fm was significantly decreased after the stress at all measurement times for C_3 species and at 40 day only for C_4 species (Figure 5.2.1, inserted).

Lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX) showed significant differences among species before and after the exposure to stress (Table 5.2.2). Both MDA and H_2O_2 contents significantly increased for all C_3 species, while the C_4 species showed no significant changes under the stress except for *Zoysia*

japonica which showed significant decreases in both traits (Figures 5.2.2a and 5.2.2b). *Z. japonica* had the highest MDA and H_2O_2 contents before the stress and the highest H_2O_2 content after the stress, whilst the C_3 species showed higher MDA content after stress treatment. Both ETR and q_N showed no significant changes under the stress for all species except the significant increase in q_N for *Z. japonica* (Figures 5.2.2c and 5.2.2d). The species showed different responses in term of scavenging system, AsA and APX (Figures 5.2.2e and 5.2.2f). Surprisingly, both AsA and APX showed highly negative correlations with Fv/Fm ($r = -0.94$ and -0.89) as well as a positive correlation between APX and MDA ($r = 0.96$) at 40 day of the stress. Significant differences between C_3 and C_4 were found only in H_2O_2 and ETR before the exposure to stress, while at 40 day of the stress significant differences were found in all traits except H_2O_2 (Table 5.2.2).

All functional leaf traits showed significant differences among the species. Furthermore, significant differences were shown between C_3 and C_4 species except for leaf water content and leaf area (Table 5.2.3). The C_4 species had lower specific leaf area (SLA), higher leaf density and thicker leaves than C_3 species. There were negative correlations of SLA with Fv/Fm and MDA at 40 day of the stress (Figure 5.2.4). SLA and leaf thickness also showed significant correlations with ETR (Figure 5.2.5).

DISCUSSION

Photosynthesis in most plans is limited by the lack of CO_2, but the limitation is substantially less in C_4 species than C_3 species because of its CO_2-concentrations mechanisms (Taiz and Zeiger 2002). In this study, the maximal PSII efficiency (Fv/Fm) decreased with duration of heat stress. All C_4 species maintained higher Fv/Fm value at 40 day of stress treatment than C_3 species (Figure 5.2.1). The result of this study clearly demonstrates that C_4 species have much tolerance to prolonged heat stress than C_3 species.

Both lipid peroxidation (MDA) and H_2O_2 content significantly increased in the C_3 species. In contrast, the C_4 species showed no significant changes except for *Z. japonica*. This suggests that oxidative stress generated under prolonged heat stress is a major cause for damage in C_3 species and that C_4 species have high ability to suppress the production of ROS under stress conditions. This is consistent with our previous findings that heat tolerance is associated with the ability to suppress oxidative stress (Chapter

2-4).

In this study, SLA showed a significantly negative correlation with Fv/Fm and a significantly positive correlation with MDA at 40 day of the stress ($r = -0.87$ and 0.88, respectively). Although leaf traits did not show a significant correlation with H_2O_2, SLA and leaf thickness showed significant correlations with ETR which showed a significant contribution to ROS accumulation in *L. perenne* (Chapter 3). These results suggest that leaf properties play an important role to cope with oxidative damage.

Temperature plays an important role in the plant distribution and productivity. It is well known that there is a differentiation between C_3 and C_4 plants in the optimal growth temperature. The C_4 species is more abundant in warm, dry climates than C_3 species because of its ability to photosynthesize more efficiency under such condition (Taiz and Zeiger 2002). In this study, although there were no significant differences in H_2O_2 content between C_3 and C_4 species at 40 day of heat stress treatment, C_4 species had higher Fv/Fm and lower lipid peroxidation (MDA) than C_3 species. Also, the relative change of H_2O_2 (the percentage of H_2O_2 content at 40 days of the stress to before the stress) for C_4 species was lower (102 %) than that for C_3 species (173%). These results suggest that that C_4 species has more tolerance to heat stress than C_3 species by having the ability to suppress the production of ROS and thus reduced the oxidative damage.

Table 5.2.1 One-way ANOVA among six species and between C_3 and C_4 species of chlorophyll fluorescence (Fv/Fm) at different durations of continuous exposure to heat stress.

Days of exposure	F value	
	Species	Type
0	0.54^{ns}	1.67^{ns}
10	14.03***	17.57***
20	4.89**	12.93**
30	7.84***	21.76***
40	4.64**	16.27***

*, **, ***, significant difference at 5, 1 and 0.1% levels, respectively

Table 5.2.2 One-way ANOVA among six species and between C_3 and C_4 species of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX) before and after exposure to heat stress.

	F value			
	Before		After	
	Species	Type	Species	Type
MDA (μmol g^{-1} FW)	63.2***	0.5	28.7***	40.3***
H_2O_2 (μmol mg^{-1} FW)	222.1***	6.0*	14.7***	0.9
ETR (μmol g^{-1} FW s^{-1})	27.2***	10.6**	11.5***	11.6**
q_N	8.7***	0.2	4.9**	6.2*
AsA (μmol mg^{-1} FW)	17.5***	2.7	50.9***	33.6***
APX (μmol mg^{-1} FW)	62.5***	7.1*	26.2***	53.9***

*, **, ***, significant difference at 5, 1 and 0.1% levels, respectively

Table 5.2.3 Mean values of specific leaf area (SLA), leaf area (LA), leaf water content (LWC), leaf density (LD) and leaf thickness (LT) for each species before the exposure to stress as well as statistical differences among species and between C_3 and C_4 species.

	SLA ($mm^2\ mg^{-1}$)	LA (cm^2)	LWC (%)	LD ($mg\ cm^{-3}$)	LT (μm)
C_3 species					
Agrostis tenuis	56.1	3.91	72.4	163	111
Festuca arundinacea	38.9	16.4	69.2	153	170
Lolium perenne	49.0	11.99	78.2	131	160
C_4 species					
Chloris gayana	22.6	18.09	74.0	260	175
Paspalum notatum	25.9	11.93	77.3	195	199
Zoysia japonica	24.6	5.61	69.6	256	163
Statistical differences					
Species	***	***	*	***	***
Type	***	ns	ns	***	**

*, **, ***, significant difference at 5, 1 and 0.1% levels, respectively

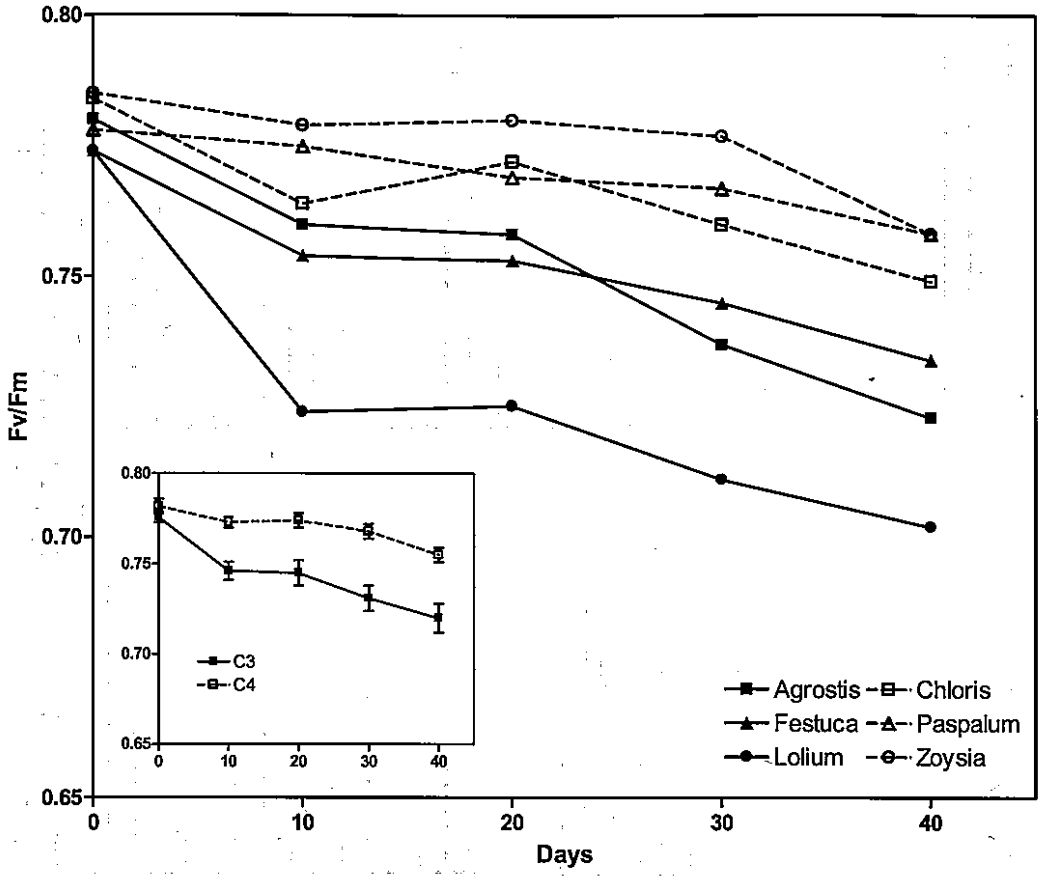

Figure 5.2.1 Response of chlorophyll fluorescence (Fv/Fm) to prolonged heat stress in three C_3 species (■ *Agrostis tenuis*, ▲ *Festuca arundinacea*, ● *Lolium perenne*) and three C_4 species (□ *Chloris gayana*, Δ *Paspalum notatum*, ○ *Zoysia japonica*). The inserted figure compares the overall Fv/Fm mean values of C_3 (■) and C_4 species (□).

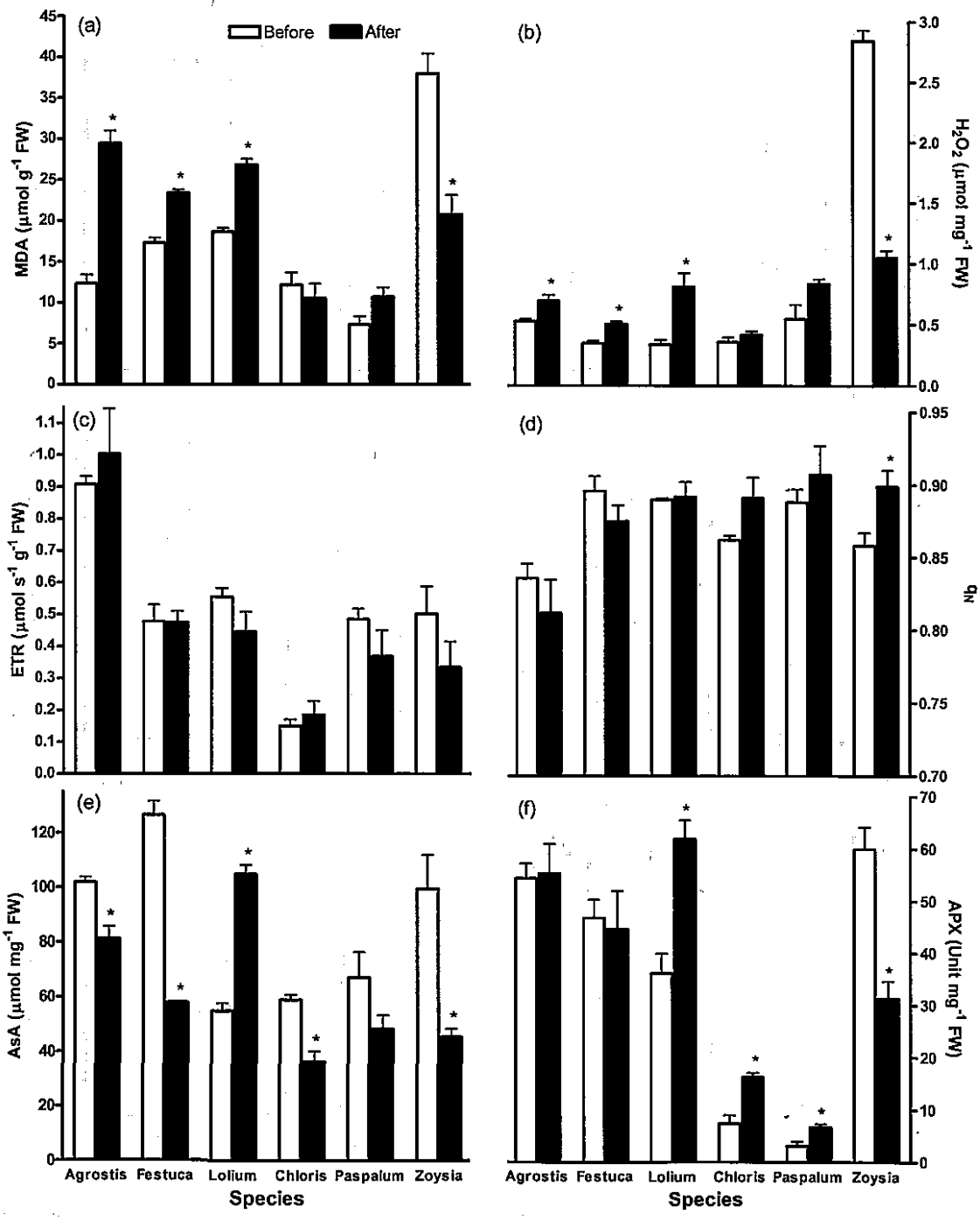

Figure 5.2.2 Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX) before and after exposure to heat stress for the three C_3 species (*Agrostis tenuis*, *Festuca arundinacea*, *Lolium perenne*) and the three C_4 species (*Chloris gayana*, *Paspalum notatum*, *Zoysia japonica*). The values represent the mean ± standard error. * represent the significant difference between after and before the stress.

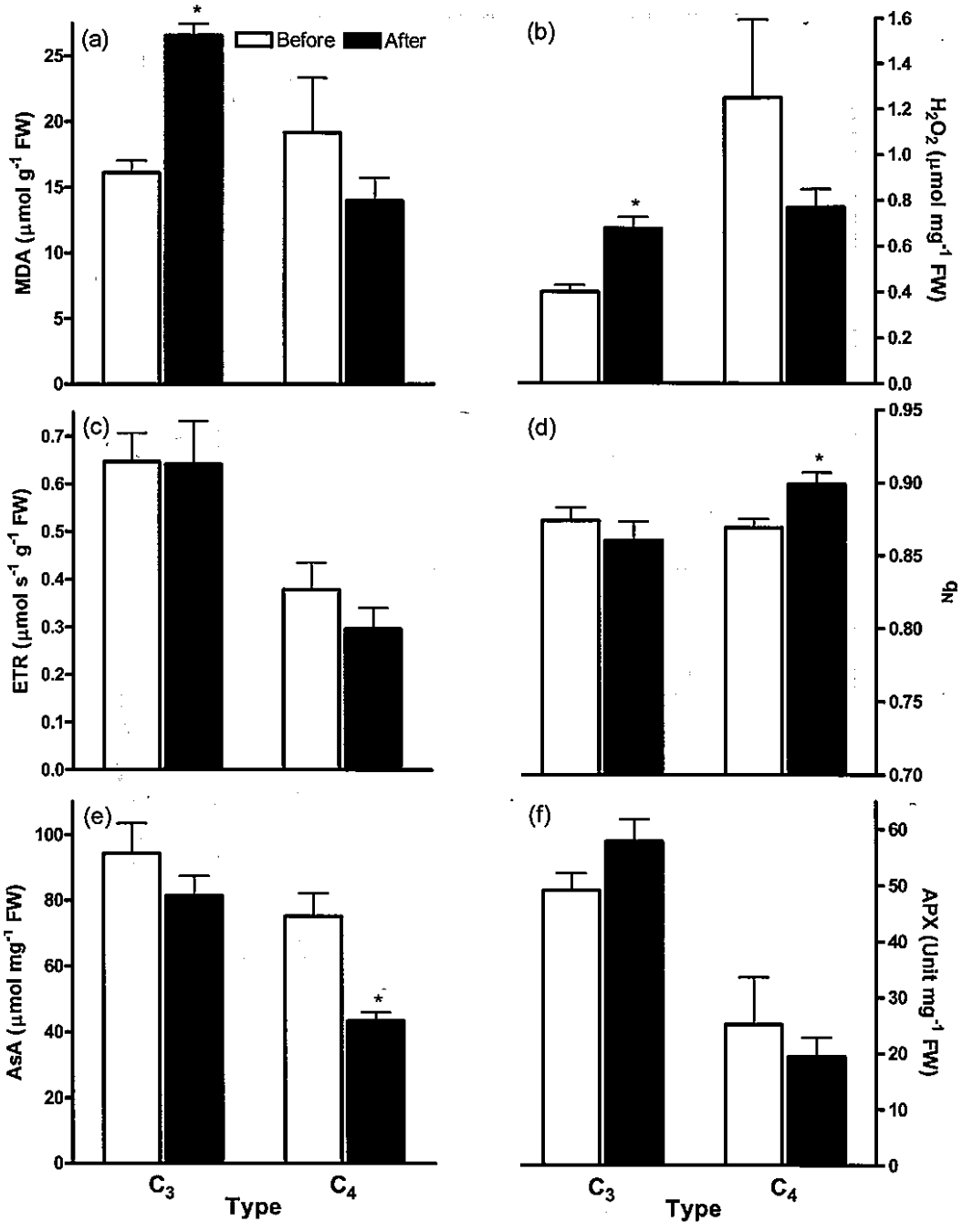

Figure 5.2.3 Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA) and ascorbate peroxidase (APX) before and after exposure to heat stress. The values represent the overall mean values of each C_3 and C_4 species ± standard error.

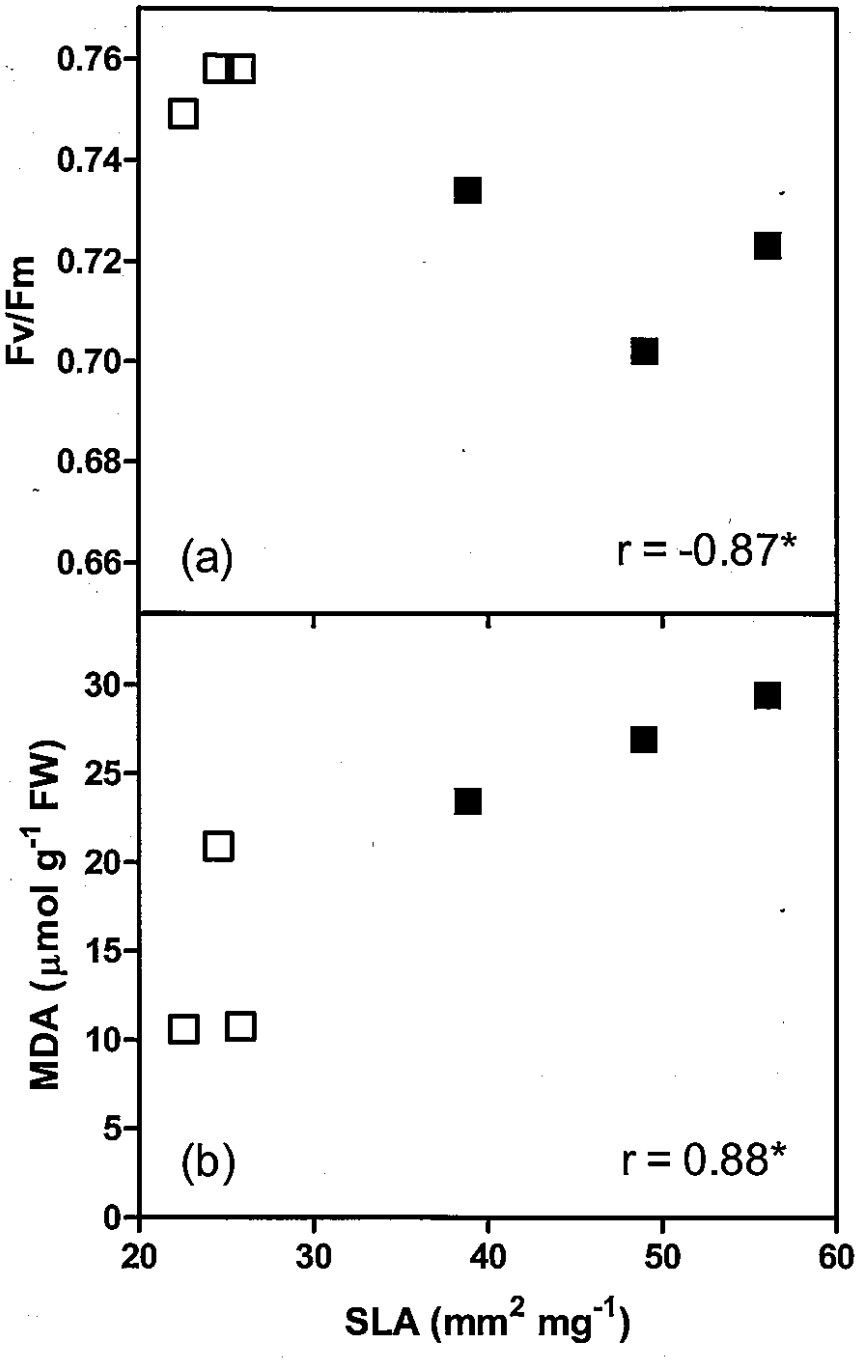

Figure 5.2.4 Correlation of (a) chlorophyll fluorescence (Fv/Fm) and (b) lipid peroxidation (MDA) at 40 day of stress treatment with specific leaf area (SLA). The closed squares (■) and the open squares (□) represent the C_3 species and C_4 species, respectively.

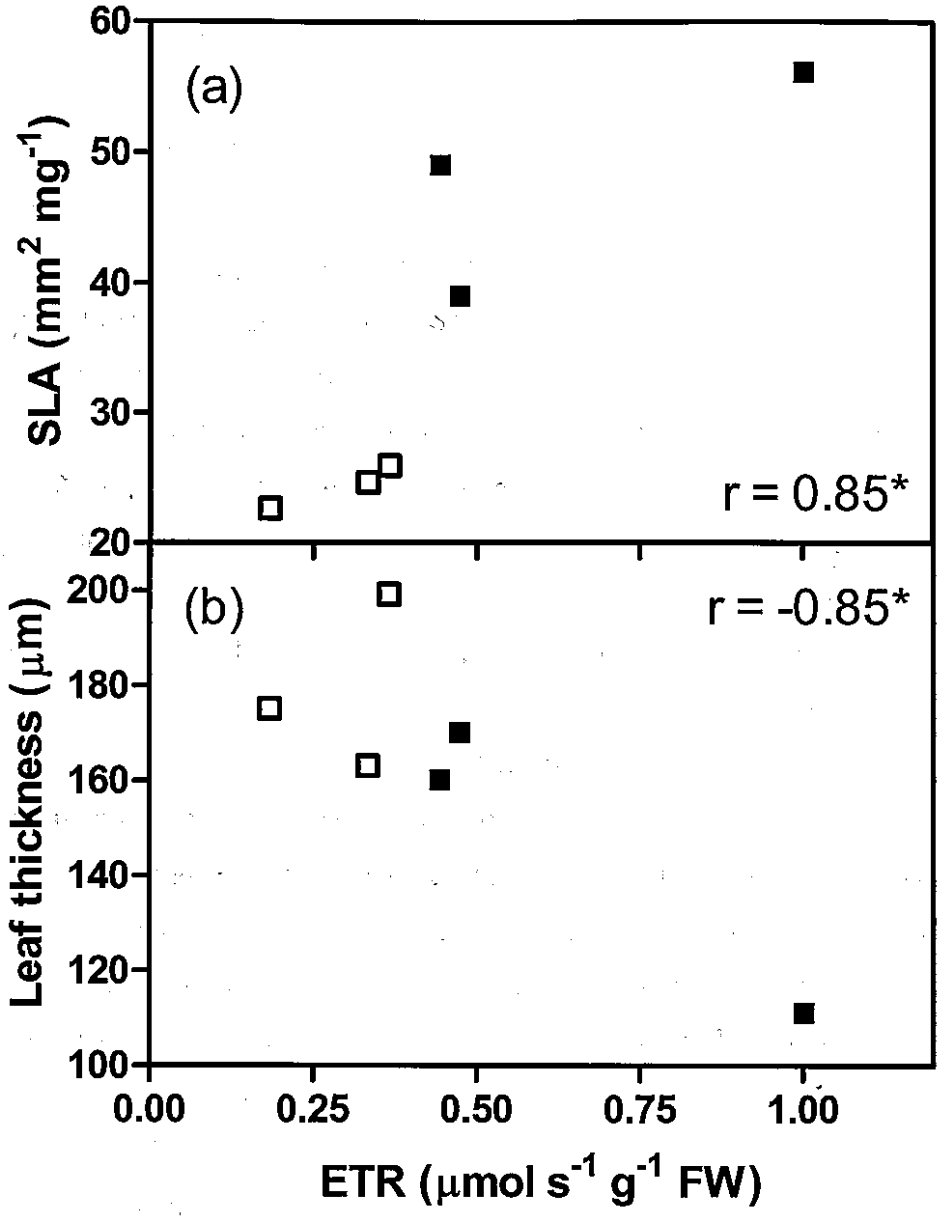

Figure 5.2.5 Correlation of (a) specific leaf area (SLA) and (b) leaf thickness with electron transport rate (ETR) at 40 days of heat stress. The closed squares (■) and the open squares (□) represent the C_3 species and C_4 species, respectively.

Chapter 6
GENERAL DISCUSSION

Heat stress and oxidative stress

High temperature stress (heat stress) is considered as one of the major worldwide limiting factors to plant growth and thus the yield. However, understanding the key traits responsible for regulating heat tolerance remains one of the formidable challenges to plant science researchers. This study aimed to enhance our understanding of the physiological mechanisms causing differences in heat tolerance within single species as well as between species in herbage and turfgrass species.

Many studies have shown that photosynthetic rate is reduced under heat stress conditions as a result of either decreased activity of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) or increasing electron leakage from thylakoids (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004; Schrader et al. 2004; Kubien and Sage 2008). In chapter 2.1, this study showed that *Lolium perenne* can maintain photosynthetic rate even after 10 days of short-term exposure to moderate heat stress. Furthermore, Rubisco activity was unchanged and $V_{c,max}$ and J_{max} values increased as well as membrane was protected against peroxidation of lipids. However, the two *L. perenne* cultivars, Norlea and Yatugadake-24, did not show significant difference in MDA content until 10 day after exposure of moderately high temperature stress, 36/30°C (Figure 2.1.4b). A large significant difference was evident at 30 days of the stress not only between the two *L. perenne* cultivars (Figure 2.2.1) but also among 25 cultivars of *L. perenne* (Table 3.3) and among species (Table 5.1.1 and Table 5.2.2). Significant differences also clearly found after continued exposure during 30 days either between diploids and tetraploids of *L. perenne* or between C_3 and C_4 species (Table 3.3 and 5.2.2, respectively). These results indicate that more than 30 days of continuous exposure of moderate heat stress is required to photosynthetic damage of plants.

The photochemical efficiency of photosystem II (PSII), represented by chlorophyll fluorescence (Fv/Fm), is the most sensitive component in photosynthesis and it is used commonly to evaluate heat tolerance in plants (Maxwell and Johnson 2000). In this study, Fv/Fm decreased significantly in all plants in response to high temperature stress. This decrease in Fv/Fm seems to indicate the occurrence of photo-inhibitory damage.

Photoinhibition, inactivation of and damage to the reaction center of PSII, is frequently observed under high photon intensity when light energy absorbed by leaves is in excess of that required for CO_2 assimilation (Long et al. 1994). Under abiotic sources of stress such as low temperatures, photo-inhibition is more likely to occur even under moderate light intensity (Hodges 2001; Zhou et al. 2006). The differences in Fv/Fm response to heat stress were clearly significant among *Lolium perenne* cultivars (Figures 2.1.4a, 2.2.1a, 2.2.2a and 3.1), among progenies between tolerant and sensitive genotypes (Table 4.1 and Figure 4.1) and among species (Figures 5.1.1 and 5.2.1). In addition, significant differences were observed between diploids and tetraploids in *L. perenne* (Table 3.2) and between C_3 and C_4 species (Table 5.2.1 and Figure 5.2.1inserted). The high tolerant plants in all comparisons maintained higher Fv/Fm value than sensitive ones. These results indicate that the tolerance to heat stress varies not only among species but also within the same species.

The reactive oxygen species (ROS) are produced through specific metabolic pathways such as photosynthesis and photorespiration (Queval et al. 2008). Under elevated high temperature, the generation of ROS resulting from disrupted balance between photochemical and biochemical reactions inhibits the photosynthesis process (Wahid et al. 2007). Reduction in photosynthetic rate was shown both after short-term exposure to sever heat stress (Liu and Huang 2008) and after prolonged exposure to moderate high temperature (Nagai and Makino 2009). The decrease in Fv/Fm was mostly associated with increase in lipid peroxidation of membrane as represented by malondialdehyde (MDA) content as well as increases of hydrogen peroxide content (H_2O_2) in leaves. Xu et al. (2006) reported that lipid peroxidation of membrane, which is an important damage to cell membrane, is a result of ROS accumulation in leaves. Significant correlations of Fv/Fm and MDA content with H_2O_2 content in leaves were observed in *L. perenne* (Figures 2.2.3 and 3.2). In addition, significantly negative correlations were shown either between Fv/Fm and MDA content at 40 day of the stress in *L. perenne* cultivars and C_3 species (Figures 3.2 and 5.1.2, respectively) or between Fv/Fm and H_2O_2 content at 40 day of the stress among *L. perenne* progenies (Figure 4.4). These results suggest that generation of ROS in leaves and resulting oxidative stress is the main cause of physiological damage seen under prolonged exposure to moderately high temperature.

Heat tolerance mechanisms

Plants under summer stress are frequently exposed to oxidative stress. In order to inhibit oxidative damage under stress condition, several defense systems against toxic ROS are involved. These include the suppression of ROS production, antioxidant scavenging systems and repairing systems (Asada 1999). In this study, the difference in physiological damage, Fv/Fm and MDA, among population of *L. perenne* was closely associated with H_2O_2 content (Chapter 2 and 3). The high tolerant populations showed lower physiological damage and lower H_2O_2 content. This result demonstrates that the difference in heat tolerance is closely associated with the ability to suppress ROS production and thus oxidative stress. On the other hand, although antioxidant activity, ascorbate peroxidase (APX) and ascorbic acid (AsA), did not show clear correlation with H_2O_2 content, both antioxidants showed significant contributions in field tolerance as well as a significant contribution of AsA in Fv/Fm (Figure 3.4) in *L. perenne*. These results suggest scavenging system also seems to contribute to heat tolerance. In contrast, antioxidant did not show significant correlations with H_2O_2 content, Fv/Fm and MDA among C_3 species (Chapter 5.1). Antioxidants were much higher in the C_3 species compared to C_4 species (Chapter 5.2). This inconsistent contribution suggests the complex network of the antioxidant in plants.

Heat-shock proteins have been shown to play important role to repair the damage induce by oxidative stress in many C_3 grasses under stress conditions (Park et al. 1996; Queitsch et al. 2000). Heckathorn et al. (1998) showed that small chloroplast heat-shock protein improves heat tolerance by protecting photosystem II and the electron transport chain. It is also reported that the difference in heat tolerance between populations of *Agrostis palustris* (Park et al. 1996; Zhang et al. 2006), between *A. scabra and A. stolonifera* (Xu and Huang 2010) and also among those of *Poa pratensis* (He and Huang 2007) were due to the difference in the expression of heat-shock protein. Although expression of heat-shock protein was not examined in this study, the difference in physiological damage between populations might also be influenced by the ability to repair the damaged protein.

In addition to the previous mechanisms of heat tolerance, the role of functional leaf traits cannot be neglected. Functional leaf traits play important roles in plant acclimation to environmental stress (Ackerly et al. 2002; Bussotti 2008; Atkinson et al.

2010; Terashima et al. 2011). Different leaf traits may play different roles in plant acclimation to environmental stress. Plants with high tissue density maintain higher photosynthesis rate per unit leaf mass which in turn supports detoxification of ROS. Plant with thicker leaves revealed higher Rubisco and mesophyll surface per unit leaf area and thus higher photosynthetic rate (Reich et al. 1997; Niinemets 1999; Bussotti 2008; Terashima et al. 2006). In addition, structural leaf traits contribute to maintain higher CO_2 concentration in the chloroplast (Terashima et al. 2011). In this study, leaf thickness had great contribution in variation in H_2O_2 content among populations of *L. perenne* (Figure 3.4). H_2O_2 content after the exposure to stress showed highly positive correlations with leaf density both in *L. perenne* and in C_3 species (Chapter 3 and 4.1). In contrast, leaf traits did not show clear contributions in *L. perenne* progenies (Chapter 4) probably due to the narrow variation in leaf traits between the two parents. It is worth mentioning that the higher tolerance of tetraploid populations of *L. perenne* than diploid ones is a result of their thicker leaves rather than genetic effects of chromosome doubling (Chapter 3). The C_4 specie had thicker leaves, higher leaf density and lower SLA than that in the C_3 species which may play important roles in their heat tolerance. These results suggest the important role of leaf traits in tolerance to oxidative stress.

Genetic basis of heat tolerance in perennial ryegrass

Improving heat tolerance of turfgrasses is a major breeding goal. However, compared to major crops, the molecular and genetic basis of heat tolerance in forage and turfgrass is relatively limited (Zhang et al. 2006). The genetic distribution of *L. perenne* progenies derived from a cross between tolerant and sensitive genotypes gave evidence that the Fv/Fm value, as a good indicator of physiological damage and heat tolerance, and H_2O_2 content in leaves, as a cause of oxidative damage, are genetically controlled (Chapter 4). Further studies that combine the physiological experiments with genetic approaches to identify and map genes conferring thermotolerance are highly recommended. Such studies will not only facilitate marker-assisted breeding for heat tolerance but also pave the way for cloning and characterization of underlying genetic factors which could be useful for engineering plant with improved heat tolerance.

Overall, this study showed that heat tolerance is controlled by complex array of mechanisms such as suppression of ROS, scavenging of toxic ROS, and leaf functions. However, the study provides evidence that the oxidative stress is the main cause of damages seen under heat stress.

SUMMARY

Improving tolerance to heat stress is a major challenge in many crops, especially C_3 crops, given the threat of recent global warming. While significant achievements have been made on understanding the molecular and physiological mechanisms to short–term exposure to high temperature, the physiological mechanisms of summer tolerance in fields, which are characterized by prolonged exposure to high temperature stress, are not still well understood. Turf grasses as perennial crops are expected to suffer from summer damage under anticipated climate warming and thus genetic improvement of tolerance to summer high temperature is an important target. In this study, six experiments were conducted to clarify the physiological mechanisms of tolerance to high temperature (heat) stress by comparing between genotypes, populations and different species including C_3 and C_4 photosynthetic types.

Firstly, two cultivars of perennial ryegrass *Lolium perenne* L. which are tolerant and sensitive to summer stress in the field were exposed to moderately high temperature stress (36/30°C) for 10 days. The response of photosynthesis and its parameters was monitored as well as chlorophyll fluorescence (Fv/Fm), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content. Photosynthesis rate and Rubisco activity were unchanged after the stress, while maximum capacity of Rubisco ($V_{c,max}$) and maximum capacity of RuBP regeneration (J_{max}) increased significantly after the stress. On the other hand, Fv/Fm decreased and H_2O_2 content increased significantly at 10 days of the stress. The MDA content showed no significant change after the stress. These results indicate that 10 days of continuous exposure of moderate heat stress do not largely inhibit photosynthesis rate in *L. perenne*.

The same populations were exposed to moderately high temperature stress (36/30°C) for 60 days or high temperature stress (40/36°C) for 14 days. The tolerant population showed significantly lower degree of physiological damage (higher Fv/Fm and lower MDA content and ion leakage) than the sensitive one only at moderate levels of stress (36/30°C); the tolerant population had significantly lower amounts of H_2O_2 in leaves. The accumulated H_2O_2 content showed a linear relationship with the extent of physiological damage. The results suggest that population difference in heat tolerance is associated with tolerance to oxidative stress and the difference in sensitivity is due to

accumulation of H_2O_2 rather than tolerance to H_2O_2. Plants exposed to the 40/36°C treatment showed physiological damage on the seventh day of exposure to the stress. However, leaf temperatures under field conditions rarely remain at 40°C for long. These results demonstrate that summer damage under field conditions can be simulated by the prolonged exposure to moderately high temperature rather than short-term extremely high temperature.

Accordingly, the third experiment was conducted to clarify the physiological mechanisms of heat tolerance in relation to oxidative stress in this species. Twenty-five cultivars of *L. perenne* with different degrees of field tolerance to high summer temperatures were exposed to moderately high temperatures (36/30°C) for 40 days in a growth chamber. Cultivars with low field tolerance had higher H_2O_2 content and higher degree of lipid peroxidation in leaves after 40 days of exposure to stress than those with high tolerance. The H_2O_2 content was positively correlated with electron transport rate and negatively correlated with leaf thickness. Tetraploid cultivars, because of their thicker leaves, had higher field tolerance and lower H_2O_2 content than diploid cultivars. Structural equation modeling showed that both reactive oxygen species (ROS) production and ROS scavenging contribute to field summer tolerance. These results demonstrate that heat tolerance under field conditions is mainly determined by the ability to suppress the production of ROS and using the system of defence to protect against oxidative damages.

The fourth experiment was conducted to understand the genetic basis of heat tolerance in the same species. In this study, 72 genotypes derived from a cross between tolerant and sensitive cultivars of *L. perenne* were used. The plants exposed to moderately heat stress (36/30°C) for 40 days in a growth chamber. Fv/Fm decreased significantly, while H_2O_2 content increased after the stress and a significant correlation was found between Fv/Fm and H_2O_2 content at 40 days of the stress. These results in agreement with the cultivar experiment suggest that the difference in heat tolerance shown by progenies of *L. perenne* is closely associated with the ability to suppress oxidative stress. The value of Fv/Fm and H_2O_2 content for the vast majority of the progenies were in the range between the two parents. This distribution of progenies indicates that the heat tolerance is under strong genetic control. In contrast, the genetic distribution of leaf traits was wider among the progenies than that between the two

parents. Leaf traits did not show significant correlation with Fv/Fm and H_2O_2 content at 40 days of the stress. This inconsistent pattern seems to result from the difference in genetic variability in leaf traits between the two parents and cultivars used in the previous experiment.

In addition to the previous experiments, responses to heat stress (36/30°C for 40 days) were compared among fifteen C_3 grass species belonging to different genus (the fifth experiment) and between C_3 and C_4 species (the last experiment) with special reference to the relationship between heat tolerance and oxidative tolerance. The fifteen C_3 species showed significantly decreases in Fv/Fm and increases in MDA content at 40 days of the stress, suggesting that the difference in heat tolerance is closely associated with the ability to suppress oxidative stress. In contrast, H_2O_2 content showed significant differences among species even under unstressed condition and H_2O_2 did not show any significant correlations with Fv/Fm and MDA content at 40 days of the stress. In contrary to other C_3 species, *Dactyles glomerata* and *Poa annua*, which showed sensitivity to heat stress, showed significant decreases in H_2O_2 content after the stress. These results suggest that different species have different strategy to utilize H_2O_2 to deal with oxidative stress in complex physiological and molecular networks.

On the other hand, C_4 species had much tolerance to prolonged heat stress than C_3 species. Both MDA and H_2O_2 content increased significantly in the C_3 species, but not clear change in the C_4 species. This is consistent with our previous findings that heat tolerance is associated with the ability to suppress oxidative stress. Specific leaf area (SLA) showed a significantly negative correlation with Fv/Fm and a significantly positive correlation with MDA at 40 days of the stress. The C_4 species had thicker leaves with higher leaf density and lower SLA than that in the C_3 species. These results suggest that leaf properties play an important role in the difference between C_3 and C_4 species in terms of coping with oxidative damage.

Overall, although the complexity of heat tolerance was revealed by the involvement of various mechanisms such as suppression of ROS, scavenging of toxic ROS, and leaf functions, this study definitively showed that the oxidative stress resulting from the excessive production of ROS is the main cause of damages under heat stress.

要約

近年の気候温暖化は,作物の生育や収量などに様々な影響を与えることが予想される。特に，芝草のような多年生作物は暑さによる夏枯れの影響を受けやすく，温暖化に耐性をもつ品種の育成が求められている。本研究は，このような観点から，芝草として広範に利用されているペレニアルライグラス(Lolium perenne)を中心に，夏の高温に由来するストレス（熱ストレス）により生理的障害が引き起こされるメカニズムを解明しようと試みたものである。本論文は，Introduction（1章）とGeneral Discussion(6章)，野外での夏枯れ被害を再現する適切な熱ストレス条件を見いだすための研究(2章)，野外での耐暑性の異なるペレニアルライスの品種間差異をもたらす生理的なメカニズムに関する研究（3章)，熱ストレスの高い遺伝子型と低い遺伝子型の雑種集団における熱ストレス耐性の変異の解析に関する研究(4章)，C3型とC4型のイネ科草の熱ストレス耐性の種間差異に関する研究（5章）からなる。得られた結果は，下記の通りである。

(1)野外での耐暑性の異なるペレニアルライグラス2品種を用い，グロースチャンバーで昼温と夜温が36℃/30℃で10日間処理し，光合成活性の変化を調べたところ，両品種とも10日程度の熱ストレス条件では光合成活性に変化がないことが分かった。次に36℃/30℃で60日間処理と40℃/40℃で14日間処理を行い，クロロフィル蛍光によるPSII活性(Fv/Fm)を調べたところ，36℃/30℃では40日後に明確な品種間差異が見られた。このPSII活性の低下は葉の過酸化水素濃度や脂質の過酸化度と関連しており，酸化ストレスに起因する障害であることが示された。40℃処理区でのPSII活性は10日間高い値を維持できることから，高温自体はペレニアルライグラスの夏枯れ障害とは関係していないことが推察された。

(2)野外での耐暑性の異なる25品種を用いて40日間36℃/30℃処理し，PSII活性と葉のH2O2濃度，脂質の過酸化度，アスコルビン酸濃度，アスコルビン酸パーオキシダーゼ活性などを測定し，耐暑性の品種間差異がどのような特性に起因しているかを調査した。その結果，野外で測定した耐暑性と実験室内での36℃処理40日目のPSII活性が高い相関関係を示し，熱ストレス耐性の指標として使えることが確認できた。PSII活性の低下は過酸化濃度と密接に関係しており，酸化ストレスがPSII活性の低下の原因であることが示された。

また，4倍体品種が有意に高い耐暑性を示したが，これは，4倍体品種では葉が厚くなることで，面積当たりに当たる光に対してルビスコなどの CO2 還元機能が相対的に高くなり，ストレス下での明反応と暗反応のバランスを維持し，活性酸素の生成による酸化ストレスによる障害を軽減するためと考えられた。

(3)熱ストレス耐性がどれくらい遺伝的に決まっているかを明らかにするために，耐性の高い遺伝子型と低い遺伝子型を交配した雑種集団の熱ストレス耐性を調査した。多くの雑種個体の PSII 活性は両親の中間に分布し，熱ストレス耐性が遺伝的に支配されていることが示唆された。また，PSII 活性は葉の二酸化炭素濃度と有意な負の相関関係に有り，耐性の高い個体は過酸化水素の発生を抑える傾向にあった。

(4)C3 型イネ科草 15 種の熱ストレス耐性を評価したところ，PSII 活性は脂質の過酸化度と高い相関関係を示し，酸化ストレスが種間の熱ストレス耐性に関係していることが示された。しかし，葉の過酸化水素濃度と PSII 活性には明確な相関は見られず，高温処理後にかえって過酸化水素濃度が減少する種も見られた。このことは，種間では過酸化水素は酸化ストレスの原因物質以外にストレスに対するシグナル物質としても作用することを示しており，種間には過酸化水素の役割に大きな差があることを示している。また，C3 型と C4 型の種をそれぞれ 3 種用いて比較したところ，熱耐性の高いと言われている C4 種は PSII 活性を高く維持し,脂質の過酸化度も低かった。また，C4 型種は葉が厚く，活性酸素種を生成させにくい葉の構造をもっていることもその高い熱ストレス耐性に関係していると思われる。

これらの結果をまとめると，イネ科の芝草草種の熱ストレス耐性には活性酸素種の生成抑制やその消去，葉の構造な様々な特性が関与しているが，すべての実験結果は，熱ストレスによる障害には活性酸素種の生成に伴う酸化ストレスが関係していることが明らかとなった

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