Compensatory acclimated mechanisms of photoprotection in a *Xa* mutant of *Lycopersicon esculentum* Mill.

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Abstract

To probe the role of xanthophylls in non-photochemical quenching (NPQ) and the compensatory acclimated photoprotection mechanisms, a tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) *Xa* mutant with deficit in lutein (L) and neoxanthin (N) contents was used. The *Xa* mutant showed lowered NPQ, an increased degree of de-epoxidation state [(A+Z)/(V+A+Z)], and decreases of photosystem 2 (PS2) antenna size. Although the *Xa* mutant had a CO_2 assimilation rate similar to that of Ailsa Craig, it exhibited a much larger stomatal conductance (g_s) than Ailsa Craig. Decreased electron flux in PS2 (J_{PS2}) for the *Xa* mutant was associated with electron flux for photorespiratory carbon oxidation (J_o) and alternative electron flux in PS2 (J_a) while electron flux for photosynthetic carbon reduction (J_c) was not different from Ailsa Craig. Moreover, the *Xa* mutant also exhibited higher activities of antioxidant enzymes, higher contents of ascorbate and glutathione, and lower contents of reactive oxygen species. Hence some compensatory acclimated mechanisms of photoprotection operated properly in the lack of NPQ and xanthophylls.

Additional key words: chlorophyll fluorescence; CO_2 concentration; net photosynthetic rate; non-photochemical quenching; photoprotection; photosynthetic electron flux; quenching; reactive oxygen species; stomatal conductance; tomato; xanthophyll cycle.

Introduction

Although radiant energy is required for photosynthesis, it is harmful if absorption of this energy exceeds the capacity for utilization, causing photo-oxidative damage to the photosynthetic apparatus. Excitation energy that is not used for photochemistry and not dissipated as fluorescence or heat will be transferred improperly to oxygen or neighbouring molecules, creating reactive oxygen species (ROS) or toxic radicals (Asada 1999, Niyogi 1999, Mullineaux and Karpinski 2002), which may lead to oxidative damage of the reaction centre of photosystem 2 (PS2) and photoinhibition (Krause 1988, Andersson *et al.* 1992, Aro *et al.* 1993). Accordingly, plants have evolved multiple photoprotective mechanisms to prevent and alleviate the harmful effects of the excess of energy and ROS on the photosynthetic apparatus. Until now, the role of processes such as thermal dissipation of excess absorbed energy, the D1 repair cycle, photorespiration, the water-water cycle, and cyclic electron flow around PS2 has been well established in the photoprotection (Schnettger *et al.* 1994, Osmond *et al.* 1997, Asada 1999, Niyogi 1999, Miyake and Yokota 2001, Müller *et al.* 2001, Külheim *et al.* 2002, Zhou *et al.* 2004, 2006).

The thermal dissipation of excess absorbed photon energy, measured as the non-photochemical quenching of chlorophyll (Chl) fluorescence (NPQ), is an important photoprotective mechanism in the prevention of photo-

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Abbreviations: A – antheraxanthin; APX – ascorbate peroxidase; C_a – ambient CO_2 concentration; C_i – intracellular CO_2 concentration; Chl – chlorophyll; DHAR – dehydroascorbate reductase; F_m – maximal fluorescence yields; F_m', F_s – maximal and steady-state fluorescence yields in a light-adapted state; g_s – stomatal conductance; GR – glutathione reductase; H_2O_2 – hydrogen peroxide; J_a – alternative electron flux in PS2; J_c – electron flux for photosynthetic carbon reduction; J_o – electron flux for photorespiratory carbon oxidation; J_{PS2} – electron flux in PS2; L – lutein; LHC – light-harvesting complex; MDA – malondialdehyde; MDAR – monodehydroascorbate reductase; N – neoxanthin; NPQ – non-photochemical quenching; P_N – the rate of CO_2 assimilation; PPFD – photosynthetic photon flux density; $O_2^{\cdot-}$ – superoxide anion; ROS – reactive oxygen species; SOD – superoxide dismutase; V – violaxanthin; Z – zeaxanthin; Φ_{PS2} – quantum efficiency of PS2 photochemistry.
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oxidative damage to the photosynthetic apparatus. NPQ is a cooperative phenomenon which is not yet fully understood at the mechanism level. NPQ is a feedback regulatory mechanism triggered by a build-up of the proton gradient (ΔpH) across the thylakoid membrane (Demmig-Adams *et al.* 1990, Niyogi *et al.* 1998). In addition, the protonation of PsbS following a fall in the lumen pH is probably involved in the conformational change that is required for NPQ (Li *et al.* 2004). Another feature of NPQ is its dependence on the very precise macromolecular organization of the PS2 antenna (Horton 1999, Horton and Ruban 2005). Xanthophylls in light-harvesting complexes (LHCs) have a critical role in NPQ. The extent of NPQ is strongly regulated by the contents of zeaxanthin (Z) and antheraxanthin (A) that are formed from the de-epoxidation of violaxanthin (V) to Z *via* the xanthophyll cycle (Demmig-Adams *et al.* 1990, Niyogi *et al.* 1998). In this cycle, the lumen enzyme violaxanthin de-epoxidase (VDE) is activated when the pH in the chloroplast lumen falls below a certain threshold and then converts V to Z *via* A (Demmig-Adams and Adams 1996, Müller *et al.* 2001, Holt *et al.* 2004). However, not all thermal dissipation depends on operation of the xanthophyll cycle; some studies show that lutein (L) (Niyogi *et al.* 1997, Pogson *et al.* 1998, Pogson and Rissler 2000, Cheng 2003) and neoxanthin (N) (Frank *et al.* 1999) have an unsuspected role in NPQ.

Xanthophylls have also important functions as antioxidants and components of LHCs. Xanthophylls which are bound to membrane can quench triplet Chl and singlet oxygen, thus inhibiting lipid peroxidation and stabilizing membranes (Frank and Cogdell 1996, Niyogi *et al.* 1999). Furthermore, contents of antioxidant molecules such as xanthophylls, tocopherols, ascorbate, and glutathione as well as enzymes such as ascorbate peroxidase (APX) and superoxide dismutase (SOD) increased during photo-acclimation, a mechanism for plants to acclimate themselves to excessive irradiance (Niyogi *et al.* 1999, Golan *et al.* 2006).

Using xanthophyll biosynthetic mutants is a powerful tool to get insight into the mechanistic contribution of xanthophylls to photoprotection (*Arabidopsis thaliana*; Golan *et al.* 2006). Although several xanthophyll biosynthetic mutants are available in tomato, few studies have used these mutants to elucidate the relation between xanthophyll biosynthesis and photoprotection. In our study, tomato *Xa* mutant with altered carotenoid metabolism was used to probe the contribution of specific xanthophylls to NPQ, and to provide insight into the compensatory acclimated photoprotection mechanisms when the xanthophylls were deficient. Accordingly, CO_2 assimilation, electron flux in photosynthetic apparatus, and NPQ were quantified by simultaneously analyzing gas exchange and Chl fluorescence parameters. The contents of pigments, the activities of important antioxidant enzymes, the contents of some key antioxidants, and the extent of lipid peroxidation were also determined.

Materials and methods

Plants: Tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) and its *Xa* mutant were obtained from Tomato Genetics Resource Center (California University, Davis, USA). *Xa* mutant has an altered expression of lycopene β-cyclase (Thorup *et al.* 2000). Homozygous *Xa* plants were not viable and dwarfed with yellow leaves. The experiment was made during the summer months in a greenhouse at Zhejiang University, China. Seeds were sown in growth medium containing a mixture of peat and perlite (7 : 3, v : v) in trays. When the first true leaf fully expanded, seedlings were transplanted to 8 000 cm^3 pots containing the same growth medium. Each pot had two seedlings. The plants were irrigated daily with half-strength Enshi nutrient solution (Yu and Matsui 1997). The environmental conditions were as follows: a 12 h photoperiod, temperatures of 25/18 °C (day/night), photosynthetic photon flux density (PPFD) of 800 μmol(photon) m^{-2} s^{-1}, and the relative humidity of 80 %. Throughout the experiment, fully expanded leaves at similar development stages were selected for gas exchange and Chl fluorescence measurements, and biochemical assays. Meanwhile, leaves were sampled, frozen quickly in liquid nitrogen, and stored at –86 °C prior to biochemical assays. Each treatment had 18 plants with three replicates.

Gas exchange and Chl fluorescence were measured at different ambient CO_2 concentrations (C_a) of 100 to 400 μmol(CO_2) mol^{-1} with a CO_2 supply controller. Gas exchange and Chl fluorescence in response to different C_a were determined simultaneously on the 8th leaf with portable infrared gas exchange analyzer equipped with the leaf chamber Chl fluorometer attachment (*LI-6400*; *LI-COR*, Lincoln, NE, USA). Artificial irradiation was supplied to the leaf from a red-blue LED source attached to the sensor head. Leaf CO_2 assimilation rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), steady state fluorescence yield (F_s), light adapted maximum fluorescence (F_m'), and the quantum efficiency of photochemical energy dissipation (Φ_{PS2}) were measured at 1 000 μmol(photon) m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and 25 °C. After the measurements of the irradiance-adapted parameters, the plants were placed in a dark room and were adapted blackly for more than 30 min. Then, a saturating pulse [8 000 μmol(photon) m^{-2} s^{-1}] was applied over 0.8 s and the maximal fluorescence yields (F_m) was recorded. NPQ was calculated as $(F_m - F_m')/F_m$ according to Demmig-Adams and Adams (1996).

Rate of alternative electron flow: The electron transport

through PS2 (J_{PS2}) was calculated as $J_{PS2} = \Phi_{PS2}$ α PPFD according to Harley *et al.* (1992), where leaf absorption (α) was measured as described by Miyake and Yokota (2000). The rates of electron flux for photosynthetic carbon reduction cycle (J_c) and photorespiratory carbon oxidation cycle (J_o) were estimated as described by Miyake and Yokota (2000). An alternative flux (J_a), caused by electrons that were not used by the carboxylation and/or oxygenation cycles in the total electron flux driven by PS2, was estimated from $J_{PS2} - (J_c+J_o)$ (Miyake and Yokota 2000).

Biochemical assays: Carotenoids were extracted with 80 % acetone, and were analyzed by HPLC according to Thayer and Björkman (1990). Ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (E = 2.8 mM cm^{-1}). Superoxide dismutase (SOD, EC 1.15.1.1) was estimated by the photochemical method as described by Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of the rate of *p*-nitro blue tetrazolium chloride reduction at 560 nm. Glutathione reductase (GR, EC 1.6.4.2) was assayed according to Madamanchi and Alscher (1991) by following the decrease in absorbance at 340 nm caused by NADPH oxidation (E = 6.2 mM cm^{-1}). Monodehydroascorbate reductase (MDAR, EC 1.6.5.4) was measured by monitoring the decrease at 340 nm due to the NADH oxidation (E = 6 mM cm^{-1}) (Arrigoni *et al.* 1981). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was determined according to Dalton *et al.* (1986).

Ascorbate and glutathione contents were determined according to Sgherri *et al.* (2000) and Tommasi *et al.* (2001), respectively. The content of hydrogen peroxide (H_2O_2) was measured by monitoring the A_{410} of titanium-peroxide complex following the method of Patterson *et al.* (1984). Superoxide anion ($O_2^{\cdot-}$) was assayed according to the modified method of Elstner and Heupel (1976). Malondialdehyde (MDA) was determined following Cakmak and Marschner (1992).

Results

Deficit in N and L synthesis was observed in the *Xa* mutant. N and L contents in the *Xa* mutant were significantly lower by 26 and 48 % than those in Ailsa Craig, respectively (Table 1). In comparison, the content of A in the *Xa* mutant was 3-fold higher than that in Ailsa Craig (Table 1). Although the contents of V, Z, and xanthophyll cycle pool size (V+A+Z) in the *Xa* mutant were not obviously different from those in Ailsa Craig, the *Xa* mutant showed a significantly higher de-epoxidation state [(A+Z)/(V+A+Z)], which increased from 0.18 to 0.37 (Table 1).

Table 1. Xanthophyll (for abbreviations see p. 28) analysis of Ailsa Craig and *Xa* mutant tomato leaves. Means±SD of three independent measurements. Values followed by different letters indicate significant difference at 5 % level.

Pigment	Ailsa Craig [g kg^{-1}(FM)]	*Xa* mutant
N	46.8±6.7a	12.1±2.9b
V	40.3±8.3a	36.5±10.1a
A	4.8±1.9b	14.9±3.8a
Z	4.1±2.1a	6.0±3.6a
L	148.2±16.9a	71.1±11.5b

There were no significant differences in P_N between Ailsa Craig and *Xa* mutant (Fig. 1*A*), however, the *Xa* mutant showed a 50 % higher g_s (Fig. 1*B*). Meanwhile, the *Xa* mutant exhibited a slightly higher C_i than Ailsa Craig (Fig. 1*C*). As C_a decreased, P_N and C_i in Ailsa Craig and the *Xa* mutant both decreased linearly. However, g_s remained relatively constant across the C_a examined.

The total electron flux in PS2 (J_{PS2}) was divided into electron flux for photosynthetic carbon reduction (J_c), electron flux for photorespiratory carbon oxidation (J_o), and alternative electron flux (J_a). As C_a decreased from 400 μmol(CO_2) mol^{-1} to 100 μmol(CO_2) mol^{-1}, J_{PS2} and J_c in Ailsa Craig and the *Xa* mutant both decreased gradually while a contrasting trend was found in J_o and J_a. The *Xa* mutant always had a lower J_{PS2} than Ailsa Craig across the C_a examined (Fig. 2*A*). In contrast, there were no differences in J_c between Ailsa Craig and *Xa* mutant (Fig. 2*B*). As compared to Ailsa Craig, the *Xa* mutant showed much lower J_o at 400 μmol(CO_2) mol^{-1} C_a, but the differences in J_o gradually disappeared with decreasing C_a (Fig. 2*C*). Moreover, significantly lower J_a was observed in the *Xa* mutant compared to Ailsa Craig, especially under low C_a (Fig. 2*D*).

We further investigated the changes of NPQ in two genotypes after exposure to different C_a. The *Xa* mutant always had a significantly lower NPQ than Ailsa Craig within the C_a examined. Meanwhile, NPQ in both Ailsa Craig and *Xa* mutant increased gradually with decreasing C_a (Fig. 3).

To determine if the antioxidant system could compensate for the decline in NPQ and xanthophyll content in the *Xa* mutant, we compared the activities of the ROS-scavenging enzymes or the levels of antioxidant molecules in two genotypes (Tables 2 and 3). The activities of SOD, APX, GR, MDAR, and DHAR in the *Xa* mutant were significantly higher than those in Ailsa Craig (Table 2). Moreover, the *Xa* mutant also had higher ascorbate and glutathione contents than Ailsa Craig (Table 3). Resultantly, both H_2O_2 and O_2 contents

appeared to be lower in the *Xa* mutant than in Ailsa Craig, but no significant differences in MDA contents were observed between Ailsa Craig and *Xa* mutant (Table 3).

Discussion

Xanthophylls are important components in plant photoprotection processes (Niyogi *et al.* 1997). The pivotal role for Z (and A) or the de-epoxidation of the xanthophyll cycle in NPQ is well-established and any alterations in xanthophyll cycle pool size (V+A+Z), especially the contents of Z and A, would have an impact on NPQ induction (Demmig-Adams *et al.* 1990, Niyogi *et al.* 1998). In the present study, we used the *Xa* mutant deficient in L and N to study the relation of specific xanthophylls and NPQ in plants. We found that the *Xa* mutant displayed significant accumulation in A relative to Ailsa Craig (Table 1), which might be due to increased flux into the β-carotene branch of the xanthophyll biosynthesis pathway arisen from impaired L synthesis. Similar results were found in *Arabidopsis* (Pogson *et al.* 1996, Golan *et al.* 2006) and *Chlamydomonas* (Niyogi *et al.* 1997). The accumulation of substantial A as an intermediate in the xanthophyll biosynthesis pathway is probably a characteristic phenotype of L-deficient mutants. In comparison, V, Z, and (V+A+Z) contents in the *Xa* mutant were not obviously different from those in

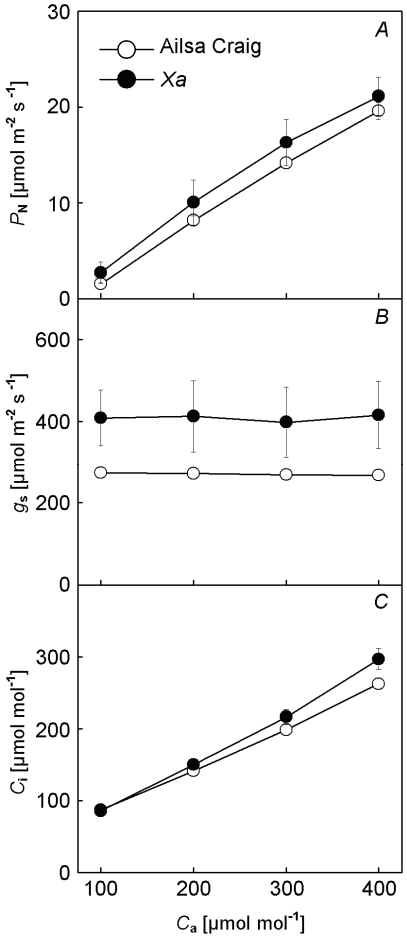

Fig. 1. The CO_2 assimilation rate, P_N (*A*), stomatal conductance, g_s (*B*), and intracellular CO_2 concentration, C_i (*C*) in Ailsa Craig (○) and *Xa* mutant (●) tomato leaves under different ambient CO_2 concentrations (C_a). Measurements were made at 25 °C and 1 000 μmol(photon) m^{-2} s^{-1}. Means of three replicates with standard deviations (SD) shown by vertical bars. SD is not shown when the value is smaller than the point.

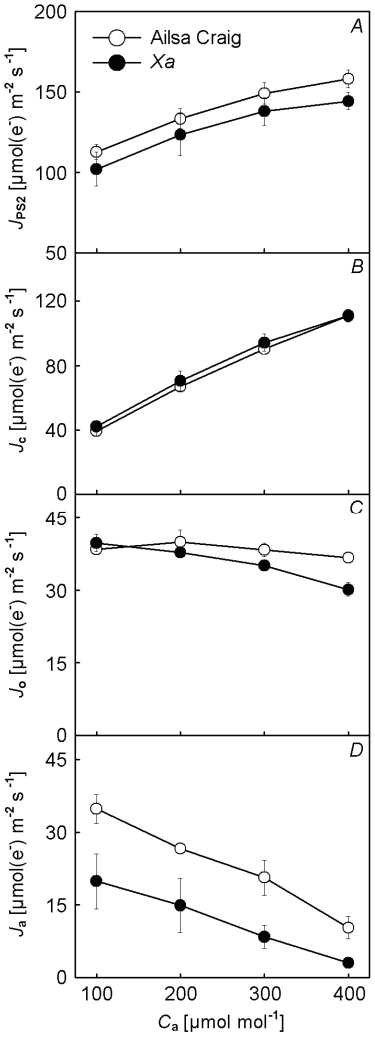

Fig. 2. The total electron flux in PS2, J_{PS2} (*A*), electron flux for photosynthetic carbon reduction, J_c (*B*), electron flux for photorespiratory carbon oxidation, J_o (*C*), and alternative electron flux in PS2, J_a (*D*) in Ailsa Craig (○) and *Xa* mutant (●) tomato leaves under different ambient CO_2 concentrations (C_a). Measurements were made at 25 °C and 1 000 μmol(photon) m^{-2} s^{-1}. Means of three replicates with standard deviations (SD) shown by vertical bars. SD is not shown when the value is smaller than the point.

Ailsa Craig (Table 1). Accordingly, higher de-epoxidation state [(A+Z)/(V+A+Z)] was observed in the *Xa* mutant, which was obtained in the L-deficient *lut1* and *lut2* mutants of *Arabidopsis* under moderate irradiance (Pogson *et al.* 1996). Despite increased A content and de-epoxidation state [(A+Z)/(V+A+Z)], the *Xa* mutant showed significantly compromised NPQ (Fig. 3). Hence, reduced NPQ could not be attributed to an alteration in xanthophyll cycle activity or the absolute xanthophyll cycle pigment contents since no decreases were found within them, which is in agreement with a study by Lokstein *et al.* (2002). L and N also contribute to NPQ in addition to xanthophyll cycle activity or the absolute contents of xanthophyll cycle pigments (Niyogi *et al.* 1997, Pogson *et al.* 1998, 2000, Frank *et al.* 1999, Cheng 2003). However, Lokstein *et al.* (2002) reported that a direct involvement of L in mechanism of NPQ is unlikely. Further study is needed before we could establish the relation of L and NPQ.

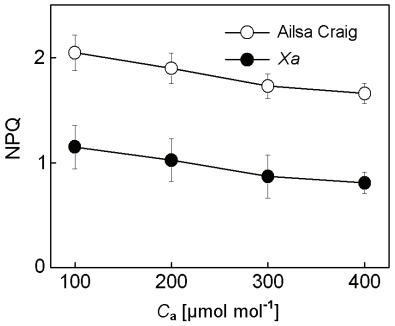

Fig. 3. The non-photochemical quenching (NPQ) in Ailsa Craig (○) and *Xa* mutant (●) tomato leaves under different ambient CO_2 concentrations (C_a). Measurements were made at 25 °C and 1 000 μmol(photon) m^{-2} s^{-1}. Means of three replicates with standard deviations shown by vertical bars.

Table 2. The activities of antioxidant enzymes (SOD, APX, DHAR, MDAR, and GR) in Ailsa Craig and *Xa* mutant tomato leaves. Means±SD of three independent measurements. Values followed by distinct letter indicate significant difference at 5 % level.

Plant	SOD [unit mg^{-1}(protein)]	APX [mmol kg^{-1}(protein) s^{-1}]	DHAR	MDAR	GR
Ailsa Craig	11.8±0.58b	8.72±0.10b	2.28±0.18b	0.48±0.03b	0.93±0.05b
Xa	14.1±0.19a	10.47±0.56a	2.82±0.07a	0.65±0.02a	1.20±0.02a

L and N play an important role in structural function in the assembly of LHCs (Pogson *et al.* 1996, 1998, Lokstein *et al.* 2002). Absence of L and N causes decreases of the Chl accumulation and the functional Chl antenna size (Pogson *et al.* 1998, Juergen *et al.* 2001, Golan *et al.* 2006). In our study, deficit in L and N resulted in the decreases of Chl content and the increases of Chl *a*/*b* ratio (data not shown) which reflect a smaller size of photon harvesting antenna of PS2 (Golan *et al.* 2006). Accordingly, the reduction of PS2 antenna size in the *Xa* mutant is probably responsible for the compromised NPQ, which is consistent with previous findings in a Chl *b*-less barley mutant (Hartel *et al.* 1996) and xanthophyll biosynthetic mutants of *A. thaliana* (Lokstein *et al.* 2002).

A decrease in NPQ would bring about an increased activity of other photoprotection mechanisms. We found that the *Xa* mutation was associated with a smaller PS2 antenna complex and consequently a lower capacity for photon harvesting, and unchanged P_N (Fig. 1*A*). A lower capacity for photon harvesting would decrease the amount of photons absorbed, whereas a relatively higher P_N would lower the amount of absorbed radiant energy that exceeds the capacity for energy utilization in photosynthesis, thus reducing the amount of irradiance stress experienced by plants. In addition, the higher g_s (Fig. 1*B*) and slightly higher C_i (Fig. 1*C*) in the *Xa* mutant relative to Ailsa Craig may be an acclimation of photosynthetic apparatus to keep high photosynthesis and protect the photosynthetic apparatus from the photo-oxidative damage.

Table 3. The contents of ascorbate, glutathione, and H_2O_2, $O_2^{\cdot-}$ production rate, and lipid peroxidation (expressed as MDA contents) in Ailsa Craig and *Xa* mutant tomato leaves. Means±SD of three independent measurements. Values followed by distinct letter indicate significant difference at 5 % level.

Parameter	Ailsa Craig	*Xa*
Ascorbate [mmol kg^{-1}(FM)]	3.62±0.12b	4.20±0.08a
Glutathione [μmol kg^{-1}(FM)]	164.6±4.2b	237.9±43.9a
H_2O_2 [mmol kg^{-1}(FM)]	110.6±4.3a	90.3±2.1b
$O_2^{\cdot-}$ [μmol kg^{-1}(protein) s^{-1}]	19.5±0.5a	17.5±0.7b
MDA [mmol kg^{-1}(FM)]	0.11±0.01a	0.11±0.01a

Quantification of the fate of photon energy absorbed by leaves is important in understanding the mechanism of photoprotection in chloroplast. Our analysis showed that the *Xa* mutant had lower J_{PS2} relative to Ailsa Craig (Fig. 2*A*), which might be due to a smaller PS2 antenna size and a lower capacity for photon harvesting in the *Xa* mutant (data not shown). However, J_c in the *Xa* mutant did not differ from Ailsa Craig (Fig. 2*B*), which further confirmed that the *Xa* mutant protected the photosynthetic apparatus from the photo-oxidative damage by maintaining high photosynthetic carbon reduction as indicated by unaffected P_N (Fig. 1*A*). Accompanied with lower J_{PS2} (Fig. 2*A*) and unaffected J_c (Fig. 2*B*), the *Xa* mutant showed lower J_o (Fig. 2*C*) and J_a (Fig. 2*D*) as

compared to Ailsa Craig. Several studies have shown that electron transport to O_2 is the main component for J_a and is an effective way to dissipate excess energy (Lovelock and Winter 1996, Park *et al.* 1996, Asada 1999, Zhou *et al.* 2004). The decreases of J_o and J_a in the *Xa* mutant indicated that photorespiration and water-water cycle are not enhanced to compensate the decreases of flux to NPQ. Nevertheless, electron transport to O_2 simultaneously carries hazards inherent in the formation of reactive oxygen species (ROS). Accordingly, lower J_o and J_a in the *Xa* mutant also suggested that production of ROS would be less compared to Ailsa Craig, thus reducing the amount of ROS damage to the photosynthetic apparatus. This is probably a compensatory acclimated mechanism of photoprotection for the *Xa* mutant.

To identify if antioxidant system could also copensate for decreases in NPQ and xanthophylls, we compared the antioxidant systems in two genotypes. Results showed that the higher activities of antioxidant enzymes (SOD, APX, GR, MDAR, and DHAR) (Table 2) and also higher contents of ascorbate and glutathione observed in the *Xa* mutant provided additional protection against ROS-initiated lipid peroxidation, as evidenced by the lower contents of H_2O_2 and $O_2^{\cdot-}$ and similarly MDA relative to Ailsa Craig (Table 3). Ascorbate and glutathione were found in the high irradiance-grown *npq* (Golan *et al.* 2006) and ascorbate-deficient *vtc2* mutants (Müller-Moulé *et al.* 2004) of *A. thaliana*, respectively, in response to high irradiance. Our results support the hypothesis that the increases of antioxidant capacity in the *Xa* mutant may be another compensatory acclimated mechanism of photoprotection for NPQ and xanthophyll deficit.

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