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Great promoting effect of high irradiance from germination on flowering in *Arabidopsis thaliana* – a process of photo-acclimation

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Abstract

Arabidopsis thaliana L., *ch1-1* (chlorophyll *b*-less mutant), *gi-1* (*GI* deficient mutant), *cry2-1* (blue-light-photoreceptor *CRY2* deficient mutant), and Columbia (Col; wild ecotype) were grown under broad range of irradiances (*I*) from the beginning of germination and the effect of *I* on the survival, development, and flowering was studied. Under low and moderate *I* (<300 μmol m^{-2} s^{-1}), flowering time and plant size at flowering showed great variations among *ch1-1*, *gi-1*, *cry2-1*, and Col, whereas under higher *I* (>500 μmol m^{-2} s^{-1}), these characteristics were almost the same. Hence under high *I*, development and flowering of *ch1-1*, *gi-1*, *cry2-1*, and Col converged to almost the same state. Flowering time was negatively correlated with *I*, and under high *I* acclimation in *A. thaliana* was associated with a decrease in chlorophyll (Chl) content and increases in xanthophyll cycle pool and membrane-bound APX activity (EC 1.11.1.11) suggesting that an increase in oxidative stress induces earlier flowering. The plants of *gi-1* and *cry2-1* survived but Col and *ch1-1* died under 1 000 μmol m^{-2} s^{-1}, showing that mutants deficient in *GI* or *CRY2* are more photo-stress-tolerant than Col and the Chl *b*-less mutant. Hence high *I* promotes in plants of *Arabidopsis* raised from germination till flowering the development and flowering time involving modulation of the photosynthetic apparatus, and this promoting effect is independent of the functions of flower-inducing *GI* or *CRY2* gene. This can be regarded as photo-acclimation of *A. thaliana* for survival and reproduction under high *I*.

Additional key words: chlorophyll; flowering time; leaf number; rosette; xanthophyll de-epoxidation.

Introduction

Plant growth and development are regulated by both environmental and endogenous developmental programs (Lacey 1986, Koornneef *et al.* 1998). During their life cycle, plants are exposed to varying *I*. Acclimation to changing *I* is necessary, especially for the survival of sedentary organisms like plants. Plants need light not only for photosynthesis but also for precise regulation of their development (Kagawa *et al.* 2001). Stress such as high *I* or chilling generally impairs carbon fixation and the excess of photon energy not used for carbon fixation leads to the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, hydroxyl radicals, and singlet oxygen (Elstner and Osswald 1994, Niyogi 1999).

An increase in *I* for a short period can be beneficial for photosynthetic yield in low-*I*-adapted plants (Long *et al*. 1994). However, prolonged exposure to high *I* can result in an increase in ROS generation (Karpinski *et al.* 1997). ROS have many adverse effects on the plants but they are also required for some developmental processes (Ogawa and Iwabuchi 2001, Lokhande *et al.* 2003). Many antioxidant enzymes and antioxidants play an important role in removing ROS. The capacity of the antioxidant defence system increases under adverse conditions (Jung *et al.* 2000), but when ROS accumulation exceeds the capacity of antioxidant systems that remove ROS, irreversible

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Abbreviations: AsA – ascorbate; APX – ascorbate peroxidase; AZ/VAZ – de-epoxidation rate; Chl – chlorophyll; FLT – flowering time; FM – fresh mass; H_2O_2 – hydrogen peroxide; *I* – irradiance; LN – number of leaves per plant; PS – photosystem; RD – rosette diameter; ROS – reactive oxygen species; tAPX – membrane-bound APX; VAZ – xanthophyll cycle pool.
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photodamage may occur to plant cells (Karpinski *et al.* 1999). Additionally, under excess *I*, carotenoids of xanthophyll cycle [violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z)] play an important role in safe dissipation of excess energy as heat. Moreover, a decrease in lumen pH under high *I* causes de-epoxidation of V to Z *via* A and excess energy is dissipated as heat (Demmig-Adams *et al*. 1995, Gandul-Rojas *et al.* 2004).

Genetic analyses of *Arabidopsis* have identified a number of genes involved in flower induction (reviewed in Reeves and Coupland 2000). The circadian clock acts as a sensor in day-length measurement and is also involved in the regulation of floral transition (Thomas and Vince-Prue 1997, Mizoguchi *et al.* 2005). In *Arabidopsis*, *LATE ELONGATED HYPOCOTYLE* (*LHY*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and *EARLY FLOWERING3* (*ELF3*) genes play a central role in generating circadian rhythms. These circadian clock genes regulate *GIGANTEA* (*GI*), *CONSTANS* (*CO*), and *FLOWERING LOCUS T* (*FT*) genes and thus promote flowering (Jeong and Clark 2005). *CRYPTOCHROME2* (*CRY2*) and *PHYTOCHROME A* (*PHYA*) stabilize the CO protein and also promote flowering (Searle and Coupland 2004). *GI* and *CRY2* regulate *CO* at the post-transcriptional level or act independently for flower induction (Suarez-Lopez *et al.* 2001). Thus, *CO* plays a key role in activating transcription of several flower-inducing genes including *FT* (Samach *et al.* 2000).

Many reports indicate the promoting effect on flowering of high *I*, in which *A. thaliana* was grown under 500 μmol m^{-2} s^{-1} in long days (Ogawa *et al.* 2004) or under 600 μmol m^{-2} s^{-1} in short days (Bailey *et al.* 2001). However, the effects of low to extremely high *I* (~1 000 μmol m^{-2} s^{-1}) on the survival, development, and flowering of *A. thaliana* from the beginning of germination have not yet been investigated. To understand such effects, we carried out experiments with *A. thaliana* Columbia (Col) wild ecotype and three mutants, *ch1-1*, *gi-1*, and *cry2-1* (Table 1), investigated their morphological and physiological parameters, and analysed inter-relationships between them.

Materials and methods

We selected *ch1-1*, *gi-1*, *cry2-1*, and Columbia (Col) (Table 1) of *Arabidopsis thaliana* L. to see the effects of low to extremely high *I* on their survival, development, and flowering. All the mutants used are in the wild ecotype Col background. Seeds of *ch1-1*, *gi-1*, *cry2-1*, and Col were grown in plastic round pots (height 7.5 cm, diameter 4.5 cm) containing vermiculite. The pots were kept for cold dark pre-treatment at 4 ºC for 4 d in growth chambers (*Nippon Medical and Chemical Instruments Co.*). After pre-treatment, pots were transferred to different *I* under long-day conditions (50, 150, 300, 500, 700, and 1 000 μmol m^{-2} s^{-1}; 16/8 h light/dark cycle) at 22 ºC. Low (50 μmol m^{-2} s^{-1}) and moderate (150 and 300 μmol m^{-2} s^{-1}) *I* was provided by *NEC FL20SEX-N-HG* lamp (*Nec*, Japan). High *I* (500, 700, and 1 000 μmol m^{-2} s^{-1}) was provided by *Mitsubishi Osram MLBOC400C-U* lamp (*Mitsubishi*, Yokohama, Japan). On the sowing day, each tray was sufficiently supplied with 2 500-fold diluted *HYPONeX* nutrient solution (*HYPONeX*, Osaka, Japan). Nutrient solution (500 cm^3) was supplied to each tray once a week until the end of the experiment. Trays were watered well according to the necessity of watering under different *I*. Plants were harvested around flowering time (FLT). The day of harvest was different for each *I* treatment according to the FLT. At harvest, FLT, growth parameters such as rosette diameter (RD), the number of leaves per plant (LN), and fresh mass (FM) were determined. The day when flower buds were visually seen was recorded as FLT at each *I*. The growth experiment at each *I* was conducted 5 times for replication. One or two samples were taken from each experiment and as the result, the number of pooled samples in each *I* was $n = 5$ or 10.

Preparation of membrane-bound ascorbate peroxidase (APX) fraction and assay of APX activity: Frozen leaves were ground to fine powder by using a mortar and a pestle in liquid nitrogen. Plant tissue powder (50 mg) was homogenized with 500 mm^3 of 50 mM potassium phosphate buffer (pH 7.8) containing 50 mM ascorbate (AsA) and 1 mM EDTA. The homogenate was mixed well and centrifuged at 250 rps for 10 min. The pellet was collected and solubilised in 500 mm^3 of 50 mM potassium phosphate buffer (pH 7.8), 0.5 % (v/v) *Triton X-100*, and 50 mM AsA. After centrifugation at 250 rps for 10 min, the supernatant was collected and defined as the membrane fraction (enzyme extract). The activity of membrane-bound APX (tAPX) should be the same as that of thylakoid-bound APX (Amako *et al.* 1994, Matsuki *et al*. 2003).

The tAPX activity (EC 1.11.1.11) was determined according to Nakano and Asada (1987) using an UV-visible spectrophotometer (*Beckman DU 7400*). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide (H_2O_2), and 50 mM AsA and 10 mm^3 enzyme extract in a total volume of 1 cm^3. The reaction was started by adding 0.1 mM H_2O_2 and performed at a temperature of 25 °C. The oxidation rate of AsA was determined by the decrease in absorbance at 290 nm, which is the typical wavelength for AsA. The tAPX activity was expressed using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for AsA at 290 nm.

Extraction and quantification of pigments (Chl *a*, Chl *b*, V, A, and Z) was done according to a modified method of Gilmore and Yamamoto (1991). Pigments were extracted by grinding the frozen samples in a mortar

Table 1. The summary of wild type Columbia (Col) and three mutants used in this study.

Name	Characteristics	References
Col	wild type, original habitat: N38.5°, E92.5°, little response to vernalization, early flowering	Bowman 1993
ch1-1	Chl-*b*-less mutant, very small light harvesting antenna	Tanaka and Tanaka 2000
gi-1	deficient in *GI* (a flower-inducing gene), insensitive to photoperiod and vernalization, late flowering	Blázquez *et al.* 2003, Izawa *et al.* 2003, Jeong and Clark 2005
cry2-1	deficient in *CRY2* (blue-light-photoreceptor), day-neutral, *i.e*. insensitive to photoperiod, late flowering	Suarez-Lopez *et al.* 2001, Yanovsky and Kay 2002, Jeong and Clark 2005

with 100 % acetone. The extract was centrifuged at 240 rps for 3 min, and the supernatant was used for separating chlorophyll (Chl) and xanthophylls with HPLC (*Shimadzu*, Kyoto, Japan) on a *Shim-pack* CLC column (150 mm long, 6 mm i.d.; *Shimadzu*, Kyoto, Japan).

Statistical analysis: To check how the parameters, FLT, LN, RD, FM, Chl, Chl *a/b*, tAPX activity, VAZ, and AZ/VAZ (dependent variable, *y*) are affected by different *I* (independent variable, *x*), linear regression analysis was performed: $y = a_0 + a_1x$, where a_0 and a_1 are regression coefficients. Residual analyses were performed to study normality of the variables and homoscedasticity of residuals. A log-transformation of the variables was made when necessary.

In order to investigate the effects of each *I* on the parameters and to check differences among *ch1-1, gi-1*, *cry2-1*, and Col in five *I* treatments, a Bonferroni multiple comparison was performed in ANOVA. In ANOVA, the variables were almost normally distributed and no transformation of the variables was made. All the statistical analysis was performed with *SPSS for Windows* (*SPSS*, Chicago, IL, USA).

Results

***I* dependence on establishment of Col, *ch1-1*, *gi-1*, and *cry2-1*: linear regression analysis**: The late-flowering mutants *gi-1* and *cry2-1* grew under all tested irradiances, whereas wild ecotype Col and Chl *b*-less mutant *ch1-1* died under 1 000 µmol m^{-2} s^{-1} at the stage of two cotyledons just after germination (Fig. 1). Flowering time and the rate of plant growth in terms of LN, RD, and FM decreased with an increase in *I* in Col and all the three mutants (p<0.01 for Col and each mutant) (Figs. 1 and 2).

Total Chl ($a+b$) content decreased with an increase in *I* in Col and all three mutants (p<0.01 for Col and each mutant), although in Col and *cry2-1* it increased suddenly only under 300 µmol m^{-2} s^{-1} and then decreased (Fig. 3*A*). Chl *a/b* ratio did not show differences in trend with *I*; it increased from 50 to 300 µmol m^{-2} s^{-1} and then decreased under higher *I* in Col, *gi-1*, and *cry2-1* (Fig. 3*B*).

The soluble APX activity did not show any specific trend with an increase in *I* (data not shown). In *cry2-1*, the tAPX activity and VAZ increased from 50 to 500 µmol m^{-2} s^{-1} and showed small decrease under 700 and 1 000 µmol m^{-2} s^{-1}. In total, tAPX activity, VAZ, and AZ/VAZ generally increased with an increase in *I* in Col and all the three mutants (p<0.01 for Col and each mutant) (Fig. 4).

Comparison between late-flowering mutants, Chl *b*-less mutant, and Col under low to extremely high *I*: Part of the full statistical results of the Bonferroni multiple comparisons among *ch1-1, gi-1, cry2-1*, and Col under five *I* ranges (Figs.1, 3, and 4) showed that under low and moderate *I* (<300 µmol m^{-2} s^{-1}), FLT, LN, and RD of *ch1-1*, *gi-1*, or *cry2-1* were different from those of Col (p<0.01 for each I); however, under high *I* (>500 µmol m^{-2} s^{-1}), those of *gi-1* and/or *cry2-1* were the same as those of Col (Fig. 1). FM was different between Col and *gi-1* or *cry2-1* only under 50 and 150 µmol m^{-2} s^{-1} (p<0.01 for each I) but under higher *I* there was no difference in FM among these genotypes (Fig. 1*D*).

Under low and moderate *I*, total Chl was different between *ch1-1, gi-1, cry2-1*, and Col (p<0.01 for each *I*), whereas under higher *I* there was no difference in total Chl (Fig. 3*A*). Chl *a/b* ratio of Col was always higher than that of *gi-1* or *cry2-1* under low, moderate, and extremely high *I* (p<0.01 for each *I*) (Fig. 3*B*).

Under low *I*, tAPX activity was not different between Col and *ch1-1*, *gi-1*, or *cry2-1*, whereas under moderate and high *I*, it was different (p<0.01 for each *I*) (Fig. 4*A*). Under low and moderate *I*, VAZ showed similar values for the tested genotypes, but under higher *I* differences in VAZ were found (p<0.01 for each *I*) (Fig. 4*B*). Under low *I*, AZ/VAZ of Col was different from that of *ch1-1* and *cry2-1*, but under moderate and high *I*, it was different from that of *gi-1* and *cry2-1* (p<0.01 for each *I*) (Fig. 4*C*).

Discussion

Plant development and flowering under low to extremely high *I*: FLT, LN, RD, and FM at flowering significantly decreased with an increase in *I* suggesting that the vegetative developmental period gets shorter (Fig. 1). Under low and moderate *I* (<300 µmol m^{-2} s^{-1}), FLT was 40–70 d, LN 20–40, RD 4–7 cm, and FM 150–570 mg, showing great variations among *ch1-1*, *gi-1*, *cry2-1*, and Col, whereas under higher *I* (>500 µmol m^{-2} s^{-1}), FLT was 15–20 d, LN 7–10, RD 0.4–0.7 cm, FM 2–8 mg, showing much smaller variations among them. Thus the development and flowering of late flowering mutants, *gi-1* and *cry2-1*, and Chl *b*-less mutant *ch1-1* converge to the same state as early-flowering Col under extremely high *I* (~1 000 µmol m^{-2} s^{-1}).

Under high *I*, the rosette of *A. thaliana* became compact with a shorter petiole probably to escape from high *I*, whereas under low *I*, plants grew bigger in RD with a longer petiole to maximise *I* interception (Fig. 1*C*) (Bailey *et al.* 2001). In *A. thaliana*, during the induction of flowers, shoot apical meristem stops producing leaves and instead starts producing the floral meristem that gives rise to flowers (Ma 1998). Therefore, the number of leaves (LN) is commonly used as a measure of FLT (Blázquez *et al.* 2003, Ogawa *et al.* 2004). In our study, LN showed also a significant negative correlation with *I* and positive correlation with FLT in all the three mutants and Col (Fig. 1*A*,*B*). This result also indicates that the increase in *I* forced *A. thaliana* to stop leaf production and start early flowering; therefore, FM at flowering decreased significantly with an increase in *I* (Fig. 1*D*).

Lacey (1986) reported that relative growth rate and plant size determine flowering time. In contrast to this, in our experiment, under higher *I* (>500 µmol m^{-2} s^{-1}) not only Col and *ch1-1* but also late flowering mutants were forced to flower early even though they were small in size and age (Figs. 1 and 2). This result suggests that *I* is important for flower induction which is independent of plant size (or RGR).

Effect of low to extremely high *I* on photo-acclimation of *A. thaliana*: The photo-acclimation in Col and all the three mutants with an increase in *I* is associated with a decrease in Chl content and increases in tAPX activity, VAZ, and AZ/VAZ, suggesting that an increase in oxidative stress causes a decrease in Chl content in *A. thaliana* with an increase in *I* (Figs. 3*A* and 4). This might be one of the photo-acclimation processes to avoid further photo-damage caused under high *I* by scavenging ROS or by dissipating excess energy as heat. In accordance with our results, under stressful conditions increases in antioxidant enzymes, VAZ, or AZ/VAZ are always negatively correlated with Chl content in wheat, moong, and four *A. thaliana* ecotypes (Lokhande *et al.* 2003, Moharekar *et al.* 2003).

The negative correlations between FLT and tAPX activity, VAZ, or AZ/VAZ in Col and all the three mutants (Figs. 1*A* and 4) suggest that an increased oxidative stress with increasing *I* induced earlier

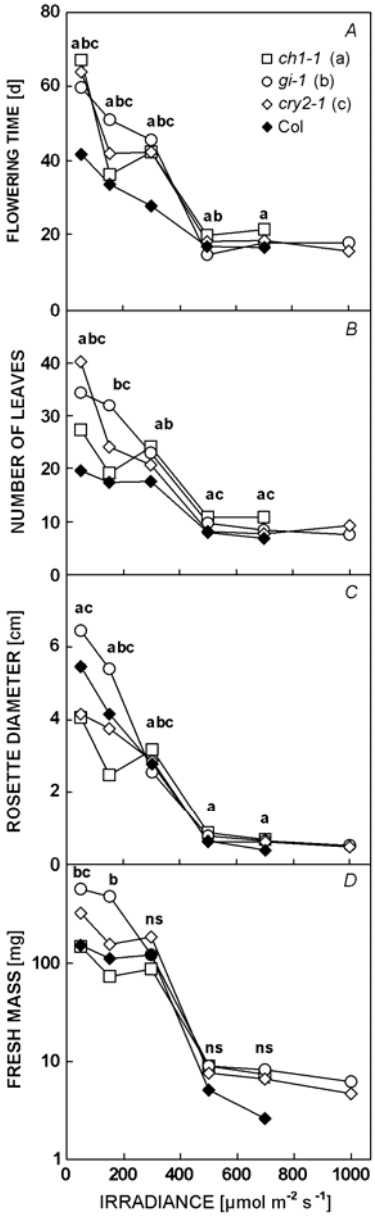

Fig. 1. Effects of irradiance (*I*) on (*A*) flowering time (FLT) (n = 10 in each *I*), (*B*) the number of leaves (LN) (n = 5 in each *I*), (*C*) rosette diameter (RD) (n = 5 in each *I*), and (*D*) fresh mass (FM) (n = 5 in each *I*) at flowering. Symbols of *ch1-1* and Col are missing under 1 000 µmol m^{-2} s^{-1} because they died under such extremely high *I*. Mean ± SE for each mutant and ecotype; total n for regression analysis of FLT was 60 and that for RD, LN, and FM was 30. In all figures, a, b, and c indicate significant differences between Col and each of *ch1-1*, *gi-1*, and *cry2-1*, respectively, at each *I* as part of the full results of the Bonferroni multiple comparison (p<0.05). ns, not significantly different.

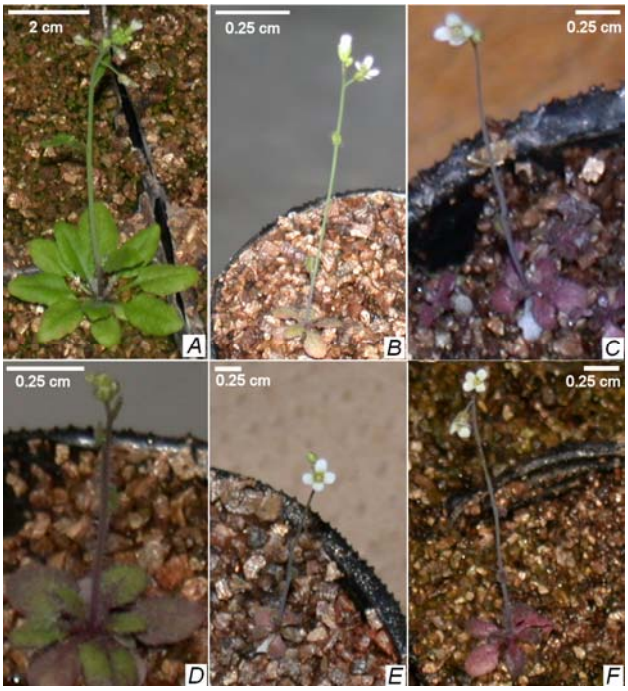

Fig. 2. Effects of irradiance on plant size at flowering of (*A*) Col under 50 µmol m^{-2} s^{-1} [42 d after germination, rosette diameter at flowering (RD) 5.4 cm; the number of leaves per plant at flowering (LN) 20, fresh mass per plant at flowering (FM) 151.2 mg)]; (*B*) Col under 500 µmol m^{-2} s^{-1} (17 d after germination; RD 0.65 cm; LN 8; FM 5.09 mg); (*C*) Col under 700 µmol m^{-2} s^{-1} (16 d after germination; RD 0.39 cm; LN 7; FM 2.5 mg); (*D*) *ch1-1* mutant under 700 µmol m^{-2} s^{-1} (21 d after germination; RD 0.7 cm; LN 11; FM 7.36 mg); (*E*) *cry2-1* mutant under 1 000 µmol m^{-2} s^{-1} (15 d after germination; RD 0.5 cm; LN 9; FM 4.62 mg); and (*F*) *gi-1* mutant under 1 000 µmol m^{-2} s^{-1} (18 d after germination; RD 0.54 cm; LN 7; FM 6 mg).

flowering. Previously, we reported a negative correlation between oxidative stress and FLT in four *A. thaliana* ecotypes (Lokhande *et al.* 2003). The plants of *gi-1* and *cry2-1* survived but Col and *ch1-1* died under 1 000 µmol m^{-2} s^{-1}, indicating that the mutants deficient in *GI* or *CRY2* are more photo-stress-tolerant than Col and Chl *b*-less mutant. In our previous study, we reported that late-flowering north ecotypes of *A. thaliana* were more photo-stress-tolerant than early-flowering south ones (Lokhande *et al.* 2003). Additionally, a late-flowering north ecotype Old-1 (original habitat: N53°, E8°; cf. early-flowering Col, N38.5°, E92.5°) survived even under 1 000 µmol m^{-2} s^{-1} from the beginning of germination (FLT, 11 d; LN, 7; RD, 0.47 cm) (Moharekar *et al.*, unpublished).

The Chl *a/b* ratio increased in the plants grown under 50 to 300 µmol m^{-2} s^{-1} (Fig. 3*B*), indicating that light-harvesting antenna size gets smaller in response to increased *I*, a well-known process (Tanaka and Tanaka 2000). However, Chl *a/b* ratio decreased in the plants grown under higher *I* (>500 µmol m^{-2} s^{-1}). This is probably because plants were grown under extremely high *I* from the beginning of germination and such conditions might have affected light-harvesting antenna size or it might be a kind of acclimation process to extremely high *I*.

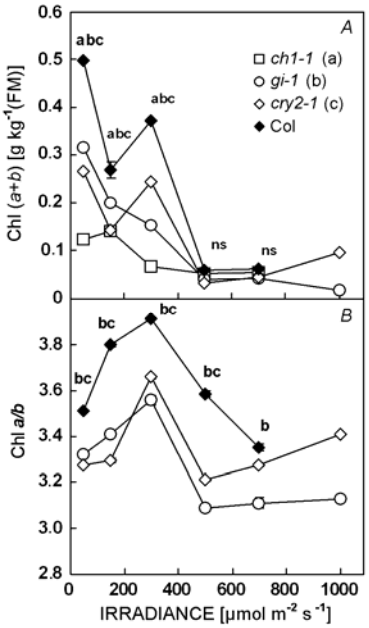

Fig. 3. Effects of irradiance (*I*) on (*A*) total chlorophyll (Chl) and (*B*) Chl *a/b* ratio at flowering. Symbols of *ch1-1* and Col are missing under 1 000 µmol m^{-2} s^{-1} because they died under such extremely high *I*. Means ± SE for each mutant and ecotype (n = 5 in each *I*); total n for regression analysis was 30. In all figures, a, b, and c indicate significant differences between Col and each of *ch1-1*, *gi-1*, and *cry2-1*, respectively, at each *I* as part of the full results of the Bonferroni multiple comparison (p<0.05). ns, not significantly different.

Photosystem-dependent and GI- or CRY2-independent flower induction: a process of photo-acclimation in *A. thaliana*: The FLT in *ch1-1* was delayed under low and moderate *I* (<300 µmol m^{-2} s^{-1}) and was promoted under higher *I* (>500 µmol m^{-2} s^{-1}), suggesting that *I* also affects flowering through PS. This indicates that sufficient photon energy was harvested at PS2 even in *ch1-1* under higher *I* (>500 µmol m^{-2} s^{-1}), and thus the level of ROS was saturated enough to promote early flowering through modulation of the photosynthetic apparatus (Fig. 4*C*). We found that under low and moderate *I* flowering of *gi-1* and *cry2-1* was significantly delayed than Col but under higher *I* (>500 µmol m^{-2} s^{-1}) their flowering occurred at the same time as in early-flowering Col (Fig. 1*A*). This suggests that high *I* induces early flowering independently of *GI* or *CRY2* gene (Table 1). Probably, high *I* might activate *CO* or *FT* directly, not through *GI* or *CRY2*, for early flowering or it directly switches the plant for flowering, when plants are grown under such high *I* from the very beginning of germination. To our knowledge, this is the first report that shows the great promoting effect of extremely high *I*

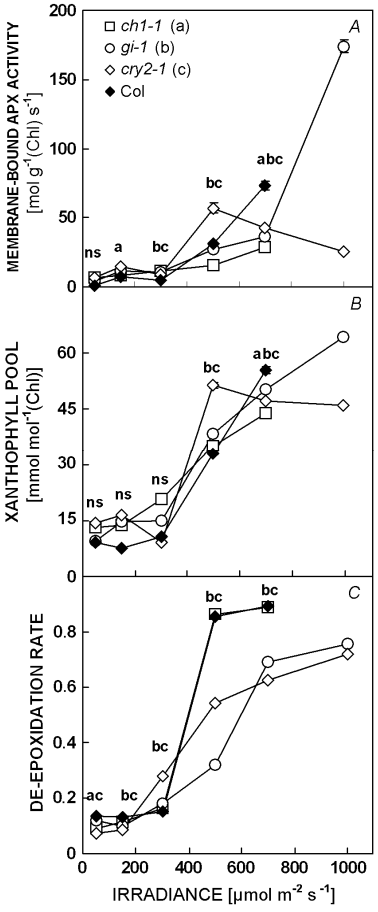

Fig. 4. Effects of irradiance (*I*) on (*A*) membrane-bound APX (tAPX) activity, (*B*) xanthophyll cycle pool (VAZ), and (*C*) de-epoxidation rate (AZ/VAZ) at flowering. Symbols of *ch1-1* and Col are missing under 1 000 µmol m^{-2} s^{-1} because they died under such extremely high *I*. Means ± SE for each mutant and ecotype (n = 5 in each *I*); total n for regression analysis was 30. In all figures, a, b, and c indicate significant differences between Col and each of *ch1-1*, *gi-1*, and *cry2-1*, respectively, at each *I* as part of the full results of the Bonferroni multiple comparison (p<0.05). ns, not significantly different.

(~1 000 µmol m^{-2} s^{-1}) from the beginning of germination on plant development and early flowering at an extremely small size and age in *ch1-1*, *gi-1*, *cry2-1*, and Col in long-days. This process is independent of the functions of *GI* or *CRY2* gene but involves modulation of the photosynthetic apparatus. This can be regarded as photo-acclimation of *A. thaliana* for survival and reproduction under high *I*. The detailed mechanism, however, remains to be elucidated.

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