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Depletion of extracellular ATP affects the photosystem II photochemistry and the role of salicylic acid in this process

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Abstract

In the present work, treatment with AMP-PCP (β,γ-methyleneadenosine-5'-triphosphate – a competitive inhibitor of the extracellular ATP pool) or apyrase (an ATP-degrading enzyme) decreased the PSII operating efficiency (Φ_{PSII}), photochemical quenching (q_p), and maximum quantum efficiency of PSII photochemistry (F_v/F_m) of *Arabidopsis* leaves illuminated with high light intensity. Mutation of extracellular ATP receptor, DORN1, also caused decreases of Φ_{PSII}, q_p, and F_v/F_m when the leaves were exposed to high light. Either the treatment with AMP-PCP and apyrase or the mutation in DORN1 increased the salicylic acid content in the illuminated leaves. Salicylic acid deficiency in *NahG* transgenic mutant made the PSII photochemistry more sensitive to AMP-PCP or apyrase. These results indicate that extracellular ATP depletion can affect the PSII photochemistry, whereas salicylic acid can relieve this effect.

Additional key words: chlorophyll fluorescence; extracellular ATP; photosystem II; salicylic acid.

Introduction

Adenosine-5'-triphosphate (ATP) has been regarded as an intracellular energy currency molecule for many years. In the last decades, however, it has been demonstrated that intracellular ATP can be secreted by animal and plant cells from the cytosol into the extracellular matrix by vesicular exocytosis, ATP-binding cassette (ABC) transporters, or gap-junction hemichannels (Parish and Weibel 1980, Thomas *et al*. 2000, Dutta *et al*. 2002, Lazarowski *et al*. 2003, Kim *et al*. 2006). Application of exogenous ATP can influence many physiological processes of both animal and plant cells (such as cell growth, development, viability, and immune responses to biotic stress) (Lustig *et al.* 1993, Khakh and Burnstock 2009, Tanaka *et al.* 2010a) and stimulate the accumulation of many important intracellular signaling molecules, including intracellular Ca^{2+}, nitric oxide (NO), and reactive oxygen species (ROS) (Dichmann *et al.* 2000, Silva *et al.* 2006, Demidchik *et al.*, 2009). Thus, it is suggested that extracellular ATP (eATP) is an important signaling component for animal and plant cells.

Because ATP has high charge and thus cannot freely diffuse across the plasma membrane (Tanaka *et al.* 2010a,b), it is believed that eATP triggers these physiological events through binding and interacting with a membrane-associated receptor protein(s). In animal cells, it is known that eATP is perceived by plasma membrane P2-type purinoceptors (Abbracchio *et al.* 2006, Khakh and North 2006). However, genomic sequence-based surveys for canonical P2-type receptors in plants failed to detect any likely candidates for an eATP receptor. Recent work by Choi *et al.* (2014) revealed that plant DORN1 protein (Does not Respond to Nucleotides 1, a lectin receptor kinase I.9) binds eATP with high affinity and is required for many eATP-induced physiological responses.

Photosynthesis plays a central role in metabolic processes of plants. The primary step of photosynthesis is to absorb light and transfer excitation energy to the reaction centers of PSII to drive the primary photochemical reactions. Chivasa *et al.* (2010) revealed that treatment of tobacco leaves with competitive inhibitor of eATP markedly suppressed the expression of the proteins belonging to the subunits of PSII. However, whether depletion of eATP can actually affect PSII photochemistry at physiological levels is still unknown. On the other hand, some signaling molecules, which have ability to mediate PSII photochemistry, seem to be also in association with eATP. For example, previous work showed that salicylic acid (SA) accumulates in plant leaves during acclimation

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Abbreviations: ABC – ATP-binding cassette; AMP-PCP – β,γ-methyleneadenosine-5'-triphosphate; ATP – adenosine-5'-triphosphate; Chl – chlorophyll; DORN – does not respond to nucleotides; eATP – extracellular ATP; F_v/F_m – maximum quantum efficiency of PSII photochemistry; *NahG* – salicylate hydroxylase gene; Psbp – prostatic steroid binding protein; q_p – photochemical quenching; ROS – reactive oxygen species; SA – salicylic acid; WT – wild-type; Φ_{PSII} – the PSII operating efficiency.

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to high light (Karpinski *et al.* 2003). Some studies reported that exogenous SA treatment can retard the degradation of D1 protein and PSII functional injury during the responses of plants to high light (Zhao *et al.* 2011, Wang *et al.* 2014). Furthermore, by using the mutants with altered SA contents, Mateo *et al.* (2006) revealed that SA plays an important role for optimal acclimation of PSII to light. Surprisingly, Chivasa *et al.* (2009) reported that the changes of eATP concentration can affect the biosynthesis of SA. Thus, if eATP can affect the PSII photochemistry, it is expected that SA could play a role in this process. However, only few studies have investigated this issue.

In the present work, we used β,γ-methyleneadenosine-5'-triphosphate (AMP-PCP), apyrase, and *dorn* 1-3 mutant to investigate the effects of eATP on PSII photochemistry and the role of SA in this process.

Materials and methods

Plant material and culture conditions: The seeds of *Arabidopsis thaliana* wild-type (WT, Columbia Col-0), *NahG* transgenic mutant (expressing the bacterial *nahG* gene, which encodes the enzyme salicylate hydroxylase that inactivates salicylic acid by converting it to catechol), and *dorn* 1-3 mutant (in which T-DNA disrupted the *dorn1* gene at nucleotide 92 of the open reading frame) were sown on pre-wetted pots (one seed per pot) that contained loam soil:vermiculite:perlite (2:1:1; v/v) for 4 d at 4°C. After then, the plants were cultivated at 22/18°C on a 16-h light/8-h dark regime at PAR of 150 ± 10 μmol(photon) $m^{-2} s^{-1}$. Relative air humidity was maintained at about 70%, and seedlings were watered daily to maintain adequate soil moisture. For experiments, 7-week-old plants (having young flowers) were used.

Treatments: Under dark conditions, the leaves of the plants were sprayed with 200 μM AMP-PCP (a nonhydrolysable analogue of ATP that can exclude eATP from binding sites of eATP receptors, Chivasa *et al.* 2005, 2009, 2010) that was dissolved in water, or the leaves were sprayed with 20 units (one unit of apyrase hydrolyzes 1 micromole of ATP per min at pH 6.5) mL^{-1} apyrase (an ATP-degrading enzyme; its exogenous application can effectively reduce the eATP content, Chivasa *et al.* 2005, 2009, 2010) dissolved in MES buffer (pH 6.5). The leaves were sprayed with water or MES buffer alone as the controls for AMP-PCP or apyrase treatment, respectively. Spray applications were performed until leaf tissues were wet and the solution ran off. The sprayed solution that ran off from the leaf surface was rapidly removed and the leaves were maintained wet for 2 h by adding these solutions when necessary. After then, the residual solution on the leaves was removed and the plants were transferred from the dark to one of three light intensities [150, 400, or 800 μmol(photon) $m^{-2} s^{-1}$] for 3 h or they were kept in the dark for the same time.

Chlorophyll (Chl) fluorescence parameters were measured by using Chl fluorometer (*PAM-2000, Walz,* Effeltrich, Germany) as described by Maxwell and Johnson (2000).

For the measurement of the PSII operating efficiency (Φ_{PSII}) and the photochemical quenching (q_p), the leaves of the plants were continuously illuminated with the actinic light at the given light intensity [0, 150, 400, or 800 μmol(photon) $m^{-2} s^{-1}$] for 3 h after the treatment with chemical solutions. The steady-state level of fluorescence emission (F_s) was measured at each light level and a saturating pulse was imposed to determine the maximal fluorescence level in the light-adapted leaves (F_m'). The actinic light was removed and the minimal fluorescence level in the light-adapted state (F_0') was determined by illuminating the leaves with far-red pulse. Φ_{PSII} was defined as $(F_m' - F_s)/F_m'$ and q_p was defined as $(F_m' - F_s)/(F_m' - F_0')$.

For the measurement of maximum quantum efficiency of PSII photochemistry (F_v/F_m), the plants were adapted to darkness for 30 min after the treatment with chemical solutions and exposure to different light intensities for 3 h (as described above). The minimum fluorescence yield in the dark-adapted state (F_0) was measured by switching on the measuring light. Once F_0 in the dark-adapted state become constant, a pulse of saturating flash was applied to determine the maximum fluorescence emission from the dark-adapted state (F_m). F_v/F_m was defined as $(F_m - F_0)/F_m$.

Salicylic acid (SA) analysis: Leaves were harvested and, after removal of the midribs, weighed, frozen in liquid nitrogen, and stored at –80°C. The tissue of 1 g was homogenized with 2.5 mL of 90% methanol and the sample was centrifuged at 12,000 × *g* for 15 min. The pettlet was resuspended again with 90% methanol and centrifuged at 12,000 × *g* for 15 min. The combined supernatant from these two centrifugations was dried under N_2. The residue was resuspended with 2.5 mL of 5% TCA (trichloroacetic acid) and centrifuged at 12,000 × *g* for 10 min. The supernatant was collected and partitioned with 5 mL of mixture solution containing ethyl acetate, cyclopentane, and isopropanol with the volume ratio at 50:49.5:0.5%. The organic phase was collected and dried under N_2 and then resuspended with a mobile phase of 55% methanol. The quantification of SA by high-performance liquid chromatography (HPLC) with fluorescence detection was performed according to previously published methods (Surplus *et al.* 1998). SA was separated on a *Columbus* 5 μm C_{18} column with 250 × 4.6 mm (*Phenomenex, Torrance,* CA, USA) with a mobile phase flow rate of 1.2 mL min^{-1}. The quantitation of SA was obtained using a HPLC fluorescence spectrometer (*PerkinElmer 200, PerkinElmer Inc.*, Wellesley, MA, USA) with excitation and emission wavelengths set at 313 and 405 nm, respectively.

Statistical analysis: The results are expressed as the mean ± standard deviation (SD). The data were statistically evaluated with *t*-test methods. The difference was considered to be statistically significant when $P<0.05$.

Results

Effects of AMP-PCP and apyrase on PSII photo-

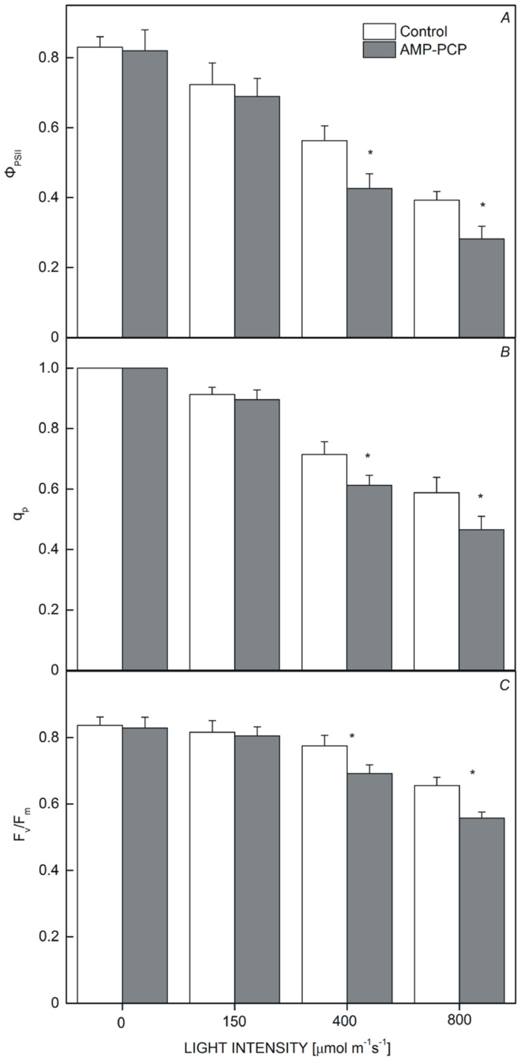

Fig. 1. The effects of AMP-PCP on Φ_{PSII}, qp, and F_v/F_m of WT leaves. The values represent means of at least four individual experiments.* – statistically significant differences (at $P<0.05$) between the controls and the leaves treated with AMP-PCP at the same light intensity. AMP-PCP – β,γ-methyleneadenosine-5'-triphosphate; F_v/F_m – maximum quantum efficiency of PSII photochemistry; q_p – photochemical quenching; Φ_{PSII} – the PSII operating efficiency.

chemistry under different light intensities: We observed that the treatment with AMP-PCP had no significant effects on the values of Φ_{PSII}, q_p, and F_v/F_m of the WT leaves under dark or at light intensity of 150 μmol(photon) m^{-2} s^{-1}, compared to the controls (treatment with water). However, at the light intensity of 400 or 800 μmol(photon) m^{-2} s^{-1}, AMP-PCP significantly decreased the values of Φ_{PSII}, q_p, and F_v/F_m, compared to the controls (Fig. 1).

Similar to the treatment with AMP-PCP, the leaf treatment of WT plants with apyrase did not significantly changed the values of Φ_{PSII}, q_p, and F_v/F_m under dark or at light intensity of 150 μmol(photon) m^{-2} s^{-1}, whereas the treatment with apyrase significantly decreased the values of Φ_{PSII}, q_p, and F_v/F_m at the light intensity of 400 or 800 μmol(photon) m^{-2} s^{-1}, compared to the treatment with MES (Fig. 2).

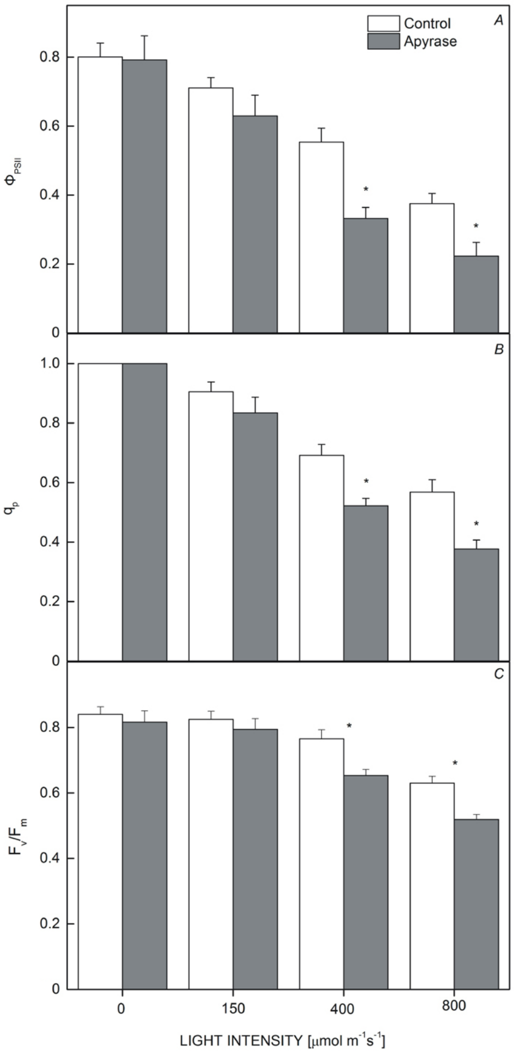

Fig. 2. The effects of apyrase on Φ_{PSII}, qp, and F_v/F_m of WT leaves. The values represent means of at least four individual experiments.* – statistically significant differences (at $P<0.05$) from the controls and the leaves treated with apyrase at the same light intensity. F_v/F_m – maximum quantum efficiency of PSII photochemistry; WT – wild type; q_p – photochemical quenching; Φ_{PSII} – the PSII operating efficiency.

Difference in PSII photochemistry between WT and *dorn*1-3 mutant: Recently, DORN1, a lectin receptor kinase, has been found to recognize eATP in *Arabidopsis* and is required for the eATP-induced responses (Choi *et al.* 2014). In the present work, the *dorn* 1-3 mutant plants, in which T-DNA disrupted the *dorn1* gene at nucleotide 92 of the open reading frame (Choi *et al.* 2014), were used to further investigate whether depletion of eATP can actually affect the PSII photochemistry at physiological levels. The results showed that there was no significant difference in the values of Φ_{PSII}, q_p, and F_v/F_m between the WT leaves and *dorn*1-3 mutant leaves at the dark or at the light intensity of 150 μmol(photon) m^{-2} s^{-1} (Fig. 3). However, at light intensity of 400 or 800 μmol(photon) m^{-2} s^{-1}, the values of

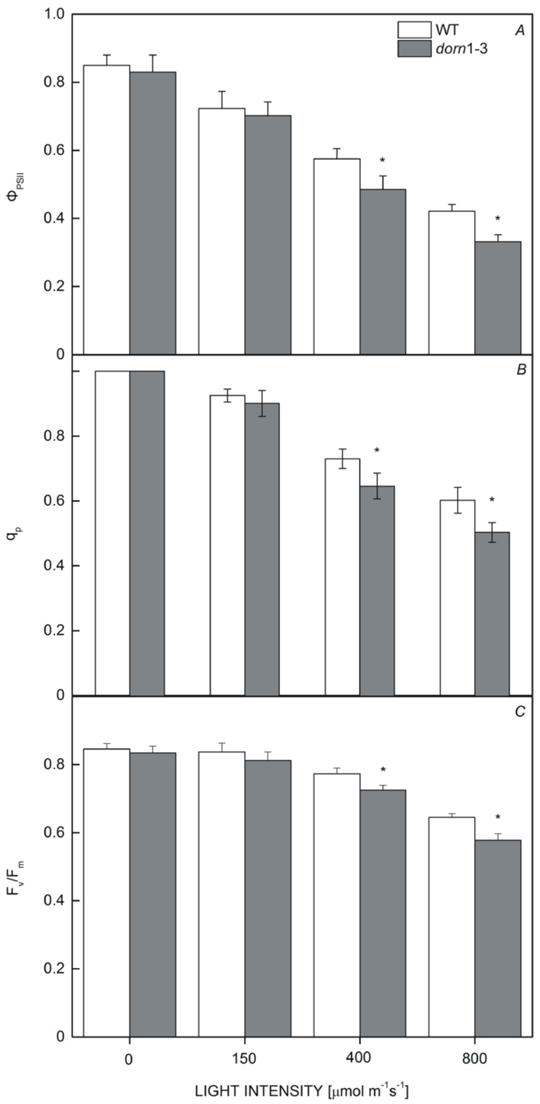

Fig. 3. The difference in Φ_{PSII}, q_p, and F_v/F_m between the WT leaves and *dorn*1-3 mutant leaves under different light intensities. The values represent means of at least four individual experiments. * – statistically significant differences (at $P<0.05$) from the WT leaves and *dorn*1-3 mutant leaves at the same light intensity. dorn – does not respond to nucleotides; F_v/F_m – maximum quantum efficiency of PSII photochemistry; q_p – photochemical quenching; WT – wild type; Φ_{PSII} – the PSII operating efficiency.

Φ_{PSII}, q_p, and F_v/F_m in the leaves of *dorn*1-3 mutant plants were significantly lower than those in the WT plant leaves (Fig. 3).

eATP affects SA biosynthesis: Compared to the dark, light illumination at 150, 400, or 800 μmol(photon) m^{-2} s^{-1} increased the leaf SA contents (Fig. 4). Under the dark conditions, the treatment with AMP-PCP or apyrase did not significantly affect the leaf SA content of WT plants. However, at light intensity of 150, 400, or 800 μmol(photon) m^{-2} s^{-1}, the treatment with AMP-PCP or apyrase significantly enhanced the leaf SA content in the WT plants, compared to the treatment with water or MSE, respectively (Fig. 4).

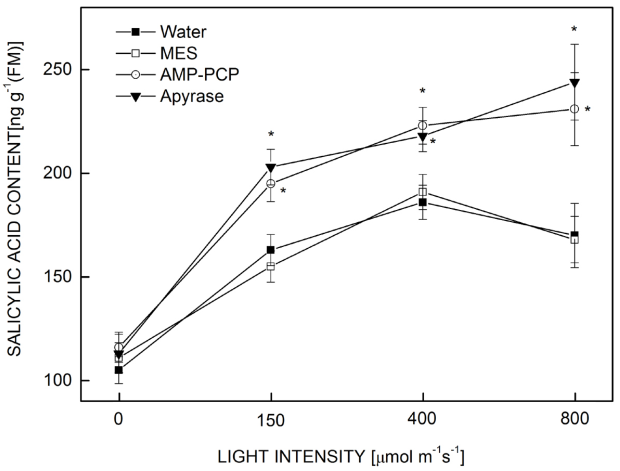

Fig. 4. The effects of AMP-PCP and apyrase on SA content of WT leaves under different light intensities. The values represent means of at least four individual experiments.* – statistically significant differences (at $P<0.05$) from the chemically treated leaves and their controls at the same light intensity. SA – salicylic acid; WT – wild type.

The *dorn*1-3 mutant plants were also used to further investigate the possible effects of eATP depletion on SA biosynthesis. The results showed that there was no significant difference in the leaf SA content between WT and *dorn*1-3 mutant at the dark. However, at light intensity of 150, 400, or 800 μmol(photon) m^{-2} s^{-1}, the leaf SA content in the *dorn*1-3 mutant was significantly higher than that in the WT plants (Fig. 5).

SA deficiency affects the sensibility of PSII photochemistry to AMP-PCP and apyrase: We used the SA-deficient *NahG* transgenic mutant to further study the possible role of SA in the AMP-PCP and apyrase-induced changes of PSII photochemistry under different light intensities. The SA content in the leaves of *NahG* transgenic mutant was significantly lower than that in the leaves of the WT plants at any light intensity tested (Fig. 1S, *supplement*).

At dark or at light intensity of 150 μmol(photon) m^{-2} s^{-1}, there was no significant difference in the values of the Φ_{PSII}, q_p, and F_v/F_m between WT and *NahG* transgenic mutant. However, at light intensity of 400 or 800 μmol(photon) m^{-2} s^{-1}, the values of the Φ_{PSII}, q_p, and F_v/F_m in the WT plants were significantly higher than those of the *NahG* transgenic mutant (Fig. 6). These observations indicate that SA plays a role in optimizing the PSII photochemistry when light intensity is enhanced.

The effect of AMP-PCP on the operation of PSII photochemistry in the WT plants was compared to that in the *NahG* transgenic mutant. The effects of AMP-PCP on the Φ_{PSII} (or q_p and F_v/F_m) were presented by calculating the difference value of Φ_{PSII} (or q_p and F_v/F_m) between the AMP-PCP- and H_2O-treated leaves, or by calculating the ratio of the Φ_{PSII} (or q_p and F_v/F_m) in the AMP-PCP-treated leaves to that in the H_2O-treated leaves (Table 1S, *supplement*). The results showed that, under dark, AMP-PCP had no

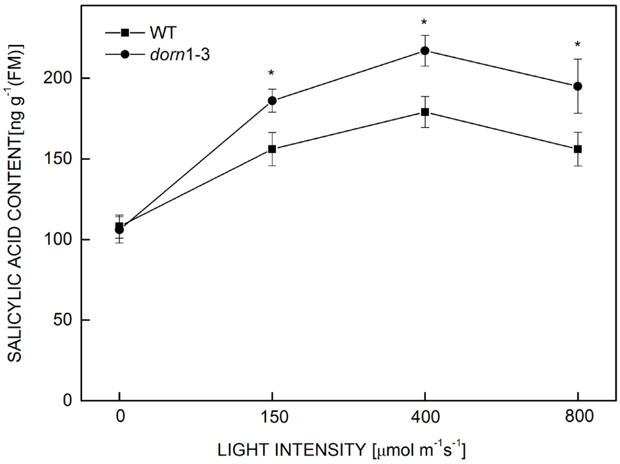

Fig. 5. The difference in SA content between WT and *dorn*1-3 mutant leaves under different light intensities. The values represent means of at least four individual experiments.* – statistically significant differences (at $P<0.05$) between the WT and *dorn*1-3 mutant at the same light intensity. dorn – does not respond to nucleotides; SA – salicylic acid; WT – wild type.

significant effects on the values of Φ_{PSII}, q_p, and F_v/F_m in either the WT leaves or the leaves of the *NahG* transgenic mutant (Table 1S). At light intensity of 150 μmol(photon) m^{-2} s^{-1}, although AMP-PCP still had no significant effects on the Φ_{PSII}, q_p, and F_v/F_m in the WT leaves, AMP-PCP caused significant declines of the Φ_{PSII} and q_p in the *NahG* transgenic mutant leaves (Table 1S). At light intensity of 400 or 800 μmol(photon) m^{-2} s^{-1}, although AMP-PCP caused significant decreases of Φ_{PSII}, q_p, and F_v/F_m in the WT leaves, the AMP-PCP-induced decreases of Φ_{PSII}, q_p, and F_v/F_m in the *NahG* transgenic mutant leaves were more dramatic than those in the WT leaves (Table 1S).

Similar results were obtained when AMP-PCP was replaced by apyrase (Fig. 7; Table 2S, *supplement*).

Discussion

AMP-PCP is a non-hydrolysable analogue of ATP and thus can exclude eATP from binding sites of eATP receptors, while apyrase is an ATP-degrading enzyme and thus its application can effectively reduce the eATP content (Chivasa *et al.* 2005, 2009, 2010). DORN1 is responsible for recognizing eATP in *Arabidopsis* cells and thus is required for the eATP-induced responses (Choi *et al.* 2014). The present work showed that treatment of *Arabidopsis* leaves with AMP-PCP and apyrase or the mutation in *dorn1* had no significant effects on the values of Φ_{PSII}, q_p, and F_v/F_m of *Arabidopsis* leaves that were maintained under dark or illuminated with low light intensity [150 μmol(photon) m^{-2} s^{-1}] (Figs. 1–3). However, under higher light intensity [400 or 800 μmol(photon) m^{-2} s^{-1}], these chemical treatments or the mutation in *dorn1* significantly decreased the values of Φ_{PSII}, q_p, and F_v/F_m (Figs. 1–3). These observations indicate that, under the higher light intensity, a depletion of eATP can reduce the proportion of photons used in photochemistry, render PSII reaction centers more "closed" (or reduced), cause damage to PSII reaction centers, and increase the susceptibility to photoinhibition. Moreover, during the photoinhibitory light treatment [400 or 800 μmol(photon) m^{-2} s^{-1}], the rate of PSII photoinhibition (as measured by the decrease in F_v/F_m) was also lower in the *dorn*1-3 mutants than that in the wild type in the presence of lincomycin (the chloroplast protein-synthesis inhibitor) (Fig. 3S, *supplement*). This indicate that eATP depletion could also impair the repair of the damaged PSII. In conclusion, depletion of eATP can decrease the PSII photochemistry under the higher light intensity.

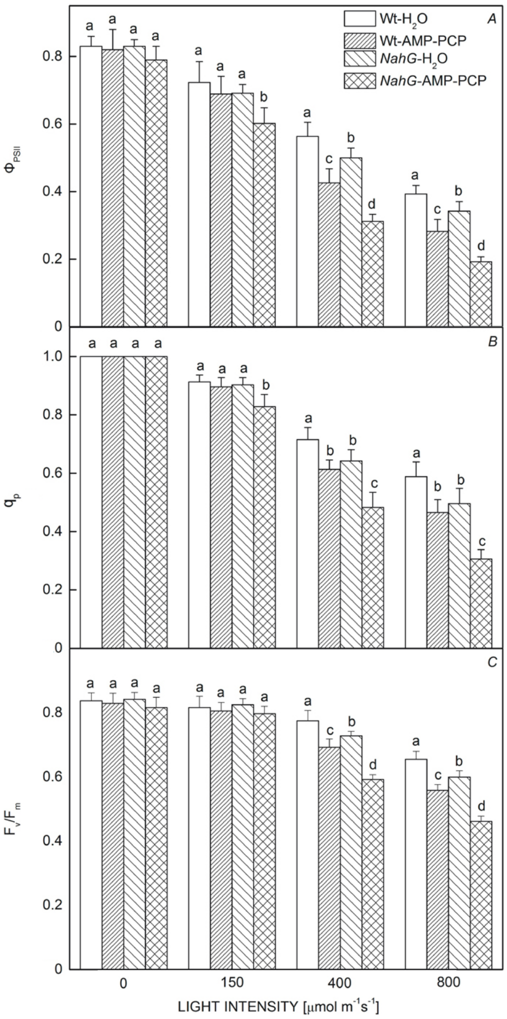

Fig. 6. The Φ_{PSII}, qp, and F_v/F_m in the H_2O or AMP-PCP-treated WT and *NahG* leaves under different light intensities. The values represent means of at least four individual experiments. The means denoted by *the same letter* did not significantly differ at $P<0.05$ at the same light intensity. F_v/F_m – maximum quantum efficiency of PSII photochemistry; *NahG* – salicylate hydroxylase gene transgenic mutant; q_p – photochemical quenching; WT – wild type; Φ_{PSII} – the PSII operating efficiency.

By using two-dimensional echocardiographic methods coupled with tandem mass spectrometry analysis, Chivasa *et al.* (2010) found that the depletion of eATP by AMP-PCP markedly suppressed the expression of Psbp and

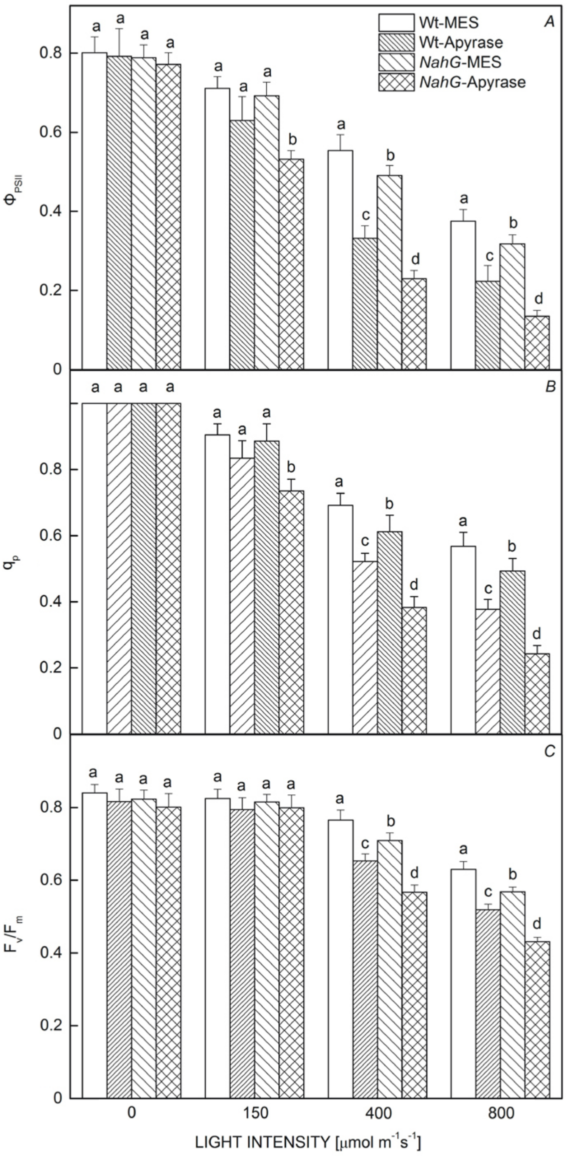

Fig. 7. The Φ_{PSII}, q_p, and F_v/F_m in the MES or apyrase-treated WT and *NahG* leaves under different light intensities. The values represent means of at least four individual experiments. The means denoted by *the same letter* did not significantly differ at $P<0.05$ at the same light intensity. *NahG* – salicylate hydroxylase gene transgenic mutant; F_v/F_m – maximum quantum efficiency of PSII photochemistry; q_p – photochemical quenching; WT – wild type; Φ_{PSII} – the PSII operating efficiency.

other proteins which belong to the subunits of PSII. Some works have reported that the proteins are essential for the regulation and stabilization of PSII. For example, the plant, which lacked Psbp, showed a marked decrease in the quantum yield of PSII and was hypersensitive to light (Ifuku *et al.* 2005). Thus, the observed decrease of the PSII photochemistry due to eATP depletion could be attributed to the changes of expression of the proteins related to the acclimation of PSII to light.

SA is an important signaling molecule to activate plant defense responses against biotic and abiotic stresses (Gaffney *et al.* 1993, Yuan and Lin 2008). When the PSII photochemistry was decreased by the increase in light intensity, the leaf SA content increased (Fig. 4). Previous works showed that *Arabidopsis* plants acclimated to short-time high light (sHL) contained higher concentrations of SA, and this increased SA was thought to improve the operating efficiency of PSII during acclimatory responses to sHL (Karpinski *et al.* 1999, Mateo *et al.* 2006). Chivasa *et al.* (2009) found that changes of eATP content can affect the content of endogenous SA. Thus, in the present work, we also studied the role of SA in the reduction of the PSII photochemistry by the depletion of eATP. When the PSII photochemistry was suppressed by the depletion of eATP *via* chemical treatment or mutation in *dorn1*, the leaf SA content was enhanced (Fig. 4). From these observations, however, it was not possible to distinguish whether the enhanced SA content under the conditions of the eATP depletion is required to cope with the decrease of the PSII photochemistry or *vice versa*.

To distinguish between these two possibilities, the effect of eATP depletion on the PSII photochemistry in the WT plants was compared to that in the *NahG* transgenic mutant. In the WT leaves illuminated with the light intensity at 150 μmol(photon) m^{-2} s^{-1}, the treatment with either AMP-PCP or apyrase did not significantly changed the values of the Φ_{PSII} and q_p (Figs. 6,7; Tables 1S,2S). At the same light intensity, however, AMP-PCP or apyrase significantly decreased the values of the Φ_{PSII} and q_p in the leaves of *NahG* transgenic mutant (Figs. 7,8; Tables 1S, 2S). At higher light intensity [400 or 800 μmol(photon) m^{-2} s^{-1}], although the treatment with AMP-PCP or apyrase caused significant decreases of the Φ_{PSII}, q_p, and F_v/F_m in both the WT plants and the *NahG* transgenic mutant, the decreases of Φ_{PSII}, q_p, and F_v/F_m were more dramatic in the *NahG* transgenic mutant than that in the WT plants (Tables 1S, 2S). These observations suggest that the SA deficiency renders the PSII photochemistry more sensitive to the depletion of eATP. Thus, the enhanced content of SA could play a role in relieving the decrease of the PSII photochemistry by the depletion of eATP.

Although little is known on the role of SA in optimizing acclimation of PSII to light, Mateo *et al.* (2006) reported the SA contents at high light were strictly correlated to H_2O_2 and glutathione contents. Some works have revealed that H_2O_2 can promote protonation of PQ and thus efficiently provoke the plant acclimatory responses to high light (Karpinski *et al.* 1999, 2000). An accumulation of glutathione was thought to limit the increased generation of active oxygen species upon high-light conditions and thus enhance the high light acclimation by alleviating the oxidative stress (Mateo *et al.* 2006). Thus, the role of SA for optimal acclimation of PSII to light could be attributed to H_2O_2 and glutathione. Regardless of how complex is the mechanism for the function of SA in mediating light acclimation of plants, the present findings suggest that eATP depletion can affect the PSII photochemistry and SA could function in relieving this effect. This seems helpful in further evaluating the importance of extracellular ATP and SA in affecting the photochemistry responses to high light.

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