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Extracellular ATP affects chlorophyll fluorescence of kidney bean (*Phaseolus vulgaris*) leaves through Ca^{2+} and H_2O_2-dependent mechanism

H.-Q. FENG+, Q.-S. JIAO, K. SUN, L.-Y. JIA, and W.-Y. TIAN

College of Life Science, Northwest Normal University, Lanzhou, 730070, China

Abstract

Extracellular ATP (eATP) has been considered as an important extracellular compound to mediate several physiological processes in plant cells. We investigated the effects of eATP on chlorophyll (Chl) fluorescence characteristics of kidney bean (*Phaseolus vulgaris*) leaves. Treatment with exogenous ATP at 1 mM showed no significant effect on the maximal photochemical efficiency of PSII. However, the treatment significantly enhanced the values of the PSII operating efficiency (Φ_{PSII}), rate of photosynthetic electron transport through PSII (ETR), and photochemical quenching (q_P), while the values of the nonphotochemical quenching (q_N) and quantum yield of regulated energy dissipation of PSII (Y_{NPQ}) significantly decreased. Our observations indicated that eATP stimulated the PSII photochemistry in kidney bean leaves. Similarly, the treatment with exogenous Ca^{2+} or H_2O_2 at 1 mM caused also the significant increase in Φ_{PSII}, q_P, and ETR and the significant decrease in q_N and Y_{NPQ}. $LaCl_3$ (an inhibitor of Ca^{2+} channels) and dimethylthiourea (a scavenger of H_2O_2) abolished the effects of exogenous ATP. The results suggest that the role of eATP in enhancing the PSII photochemistry could be related to a Ca^{2+} or H_2O_2 signaling pathway.

Additional key words: photosynthesis; reactive oxygen species; signaling molecules.

Introduction

ATP is mainly produced by intracellular organelles, such as the mitochondria and chloroplasts, and it serves as energy currency to support the energy-requiring biochemical reactions for cell growth and development. In the last decades, it is found that animal, plant, and microbial cells can secrete ATP from the cytosol into the extracellular matrix (Parish and Weibel 1980, Boyum and Guidotti 1997, Thomas *et al*. 2000).

In plant cells, the release of ATP into the extracellular matrix occurs by multiple mechanisms, including ATP-binding cassette (ABC) transporters and vesicular exocytosis (Thomas *et al*. 2000, Kim *et al*. 2006). Some studies have demonstrated that changes in the concentration of extracellular ATP (eATP) can affect growth, development, biotic and abiotic stress responses, viability, thigmotropism, and gravitropism of plant cells (Tanaka *et al*. 2010a). In addition, it has been reported that eATP can stimulate the accumulation of many important intracellular signaling molecules, such as Ca^{2+}, nitric oxide, and reactive oxygen species (ROS) (Demidchik *et al*. 2003, 2009, Jeter *et al*. 2004, Song *et al*. 2006, Foresi *et al*. 2007). It is believed that plant eATP affects the physiological processes and intracellular signaling molecules through its interaction with membrane-associated receptor proteins, because eATP cannot freely diffuse across the plasma membrane due to its high charge (Tanaka *et al*. 2010a). A recent study by Choi *et al*. (2014) revealed that the *Arabidopsis* DORN1 protein (Does not Respond to Nucleotides 1, a lectin receptor kinase I.9) binds eATP with high affinity and is required for some eATP-induced physiological responses.

Although current studies have revealed the effects of eATP on some physiological processes of plants, the lack of knowledge about the responses of photosynthesis to eATP is remarkable. The primary step of photosynthesis is to absorb light and transfer excitation energy to the reaction centres of PSII to drive the primary photochemical reactions. In reality, however, absorbed light energy

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+Corresponding author; tel: 86-130-087-62166, e-mail: hanqing_feng@hotmail.com
Abbreviations: DMTU – dimethylthiourea; eATP – extracellular ATP; ETR – the rate of photosynthetic electron transport through PSII; F_v/F_m – the maximal photochemical efficiency of PSII; q_N – the nonphotochemical quenching; q_P – the photochemical quenching; ROS – reactive oxygen species; Y_{NO} – the quantum yield of nonregulated energy dissipation of PSII; Y_{NPQ} – the quantum yield of regulated energy dissipation of PSII; Φ_{PSII} – the PSII operating efficiency.
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generally exceeds that required by plants. The excess of light energy, if it is not dissipated, can lead to overreduction of PSII, which can consequently cause oxidative damage to the photosynthetic apparatus. To avoid this damaging effect, the excess light energy can be dissipated as harmless heat by nonphotochemical quenching of excitation energy in PSII (Dat *et al*. 2000, Baker *et al*. 2007). Recently, Chivasa *et al*. (2010) revealed that application of AMP-PCP (a specific competitive inhibitor of eATP) caused a marked suppression in the expression of the proteins belonging to the subunits of PSII, suggesting that eATP could be a potential regulator of the PSII photochemistry. However, direct evidence is still lacking.

In the present work, we investigated the effect of exogenous ATP on the PSII photochemistry by measuring the yield of Chl fluorescence. Possible involvement of Ca^{2+} and H_2O_2, which act as the downstream signaling molecules of eATP, was also studied. We believe that understanding the role of eATP would be helpful to expand further the current knowledge concerning of plant eATP and its role in the mechanisms for the regulation of photosynthesis.

Materials and methods

Plants material and culture condition: Seeds of kidney bean (*Phaseolus vulgaris* L. Nongpu.12), provided by Guangzhou Academy of Agricultural Science, China, were sterilized with 2% NaClO for 20 min and then washed with distilled water 5–6 times to remove the remaining NaClO. The seeds were germinated at 26°C on damp gauze. The germinated seeds were planted in plastic pots (one seed per pot) containing loam soil:vermiculite:perlite mixture (2:1:1, v/v/v) and grown in a growth chamber under the following environmental conditions: 25/20°C day/night temperature, 150 ± 10 μmol(photon) m^{-2} s^{-1} PAR, photoperiod of 12 h, and relative humidity of 50%. Seedlings were watered daily to maintain adequate soil moisture. Two-week old-seedlings with fully expanded, primary leaves were used for experiments.

Treatments of bean leaves: Leaf surface of the primary leaves of the seedlings was sprayed with several different chemical solutions:

Treatment	Chemical solution
Control	Deionized water
1	1 mM ATP
2	1 mM $LaCl_3$ (an inhibitor of Ca^{2+} channels)
3	1 mM ATP + 1 mM $LaCl_3$
4	1 mM $Ca(NO_3)_2$
5	1 mM DMTU (dimethylthiourea, H_2O_2 scavenger)
6	1 mM ATP + 1 mM DMTU
7	1 mM H_2O_2

All solutions were prepared by dissolving the compounds in deionized water. Spray application was performed with a backpack sprayer until the leaf was wet and solution ran off (about 1 ml per plant). After the treatments, the leaves were incubated for 10 min at 25°C under a light intensity of 150 μmol(photon) m^{-2} s^{-1} and 50% relative humidity. Control seedlings were treated with deionized water under the same conditions.

Measurement of Chl fluorescence: After incubation with different chemical solutions or deionized water for 10 min, the Chl fluorescence parameters of the treated primary leaves were measured by using a portable fluorometer (*IMAGING-PAM*, *Walz*, Germany), as described previously by Donnini *et al*. (2013) and Demmig-Adams (1996). The maximal photochemical efficiency of PSII (F_v/F_m) was defined as $(F_m - F_0)/F_m$, where F_m is the maximum fluorescence emission from the dark-adapted state measured with a pulse of saturating light, and F_0 is the minimal fluorescence from the dark-adapted state. The PSII operating efficiency (Φ_{PSII}) was defined as $(F_m' - F_s)/F_m'$, where F_m' is the maximum fluorescence emission from the light-adapted state measured with a pulse of saturating light, and F_s is the steady-state level of fluorescence emission at the given irradiance. The nonphotochemical quenching (q_N) was defined as $(F_m - F_m')/(F_m - F_0')$, where F_0' is the minimal fluorescence of the light-adapted state measured with a far red pulse. The photochemical quenching (q_P), was defined as $(F_m' - F_s)/(F_m' - F_0')$. As Φ_{PSII} represents the number of electrons transferred per photon absorbed by PSII, the rate of photosynthetic electron transport through PSII (ETR) can be calculated as ETR [mol(e^-) m^{-2} s^{-1}] = Φ_{PSII} × PAR × 0.5 × 0.84. The quantum yield of nonregulated energy dissipation of PSII (Y_{NO}), was defined as F_s/F_m, and the quantum yield of regulated energy dissipation of PSII (Y_{NPQ}), was defined as $1 - \Phi_{PSII} - Y_{NO}$. Experiments were repeated independently at least three times and the statistical significance of the measurements for the treatments was determined by using *t*-test.

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

The effects of eATP on the Chl fluorescence: The eATP at 1 mM showed no significant effect on the F_v/F_m (Table 1). However, application of eATP significantly increased the value of the Φ_{PSII}. Because the PSII operating

efficiency is directly related to the rate of the linear electron flux (Baker *et al*. 2007), it is not surprising that the increase in Φ_{PSII} induced by eATP was followed by a significant increase in the ETR. eATP caused a significant decrease in the q_N, indicating that an increase in eATP can decrease the dissipation of absorbed light energy as heat. The significant decrease in q_N by eATP was followed by a significant decrease in the Y_{NPQ}, whereas the value of the Y_{NO} did not significantly change after ATP treatment. The q_P is an estimate of the portion of oxidized PSII centres (Maxwell and Johnson 2000). Treatment with 1 mM ATP significantly increased the value of q_P (Table 1).

The effects of eATP on Chl fluorescence could be associated with Ca^{2+}: Previous studies proposed that the perception of eATP by plant cells can lead to an influx of Ca^{2+} from the extracellular space through plasma membrane Ca^{2+}-permeable channels. The influx of Ca^{2+} acts as an early signaling step for the eATP-mediated physiological events (Dichmann *et al*. 2000, Demidchik *et al*. 2009, Tanaka *et al*. 2010b). First, we investigated whether Ca^{2+} affects the Chl fluorescence of the leaves. The results showed that, similarly to the treatment with eATP, the treatment with exogenous Ca^{2+} at 1.0 mM also caused a significant increase in Φ_{PSII}, ETR, and q_P (Figs. 1*A*,*B*, 2) and a significant decrease in q_N and Y_{NPQ} (Fig. 1*C*,*D*). We used $LaCl_3$ (an inhibitor of Ca^{2+} channels) to further investigate whether the function of eATP could be dependent on Ca^{2+}. Sole application of 1.0 mM $LaCl_3$ had no significant effects on the Chl fluorescence parameters (data not shown). The result showed that the values of Φ_{PSII}, ETR, and q_P in the leaves subjected to the combined treatment with ATP plus $LaCl_3$ were significantly lower than those in the leaves treated with ATP alone (Figs. 1*A*,*B*, 2). The values of q_N and Y_{NPQ} in the leaves subjected to this combined treatment were significantly higher than those in the ATP-treated leaves (Fig. 1*C*,*D*). The Chl fluorescence parameters in the leaves subjected to the combined treatment with ATP plus $LaCl_3$ were similar to those in the controls (Figs. 1*A–D*, 2). The results indicated that the addition of the inhibitor of Ca^{2+} channels could abolish the effects of eATP.

Table. 1. The effects of exogenous 1 mM ATP on the chlorophyll fluorescence parameters. Each value represents the mean ± SD of at least three independent experiments. F_v/F_m – the maximal photochemical efficiency of photosystem II; Φ_{PSII} – the PSII operating efficiency; q_P – the photochemical quenching; ETR – the rate of photosynthetic electron transport through PSII; q_N – the nonphotochemical quenching; Y_{NPQ} – the quantum yield of regulated energy dissipation of PSII; Y_{NO} – the quantum yield of nonregulated energy dissipation of PSII. * – statistically significant differences between the ATP-treated leaves and the control leaves (CK).

Parameter	CK	ATP
F_v/F_m	0.796 ± 0.004	0.797 ± 0.005
Φ_{PSII}	0.423 ± 0.008	0.476 ± 0.005*
q_P	0.601 ± 0.010	0.654 ± 0.002*
ETR	30.867 ± 0.551	34.767 ± 0.321*
q_N	0.476 ± 0.019	0.384 ± 0.021*
Y_{NPQ}	0.224 ± 0.0113	0.165 ± 0.009*
Y_{NO}	0.353 ± 0.011	0.359 ± 0.007

The effects of eATP on the Chl fluorescence is associated with H_2O_2: It has been reported that the perception of eATP by plant cells can also enhance the production of H_2O_2 (Kim *et al*. 2006, Song *et al*. 2006; Demidchik *et al*. 2009, Lim *et al*. 2014). Thus, one may assume that the effects of eATP on Chl fluorescence could be also associated with H_2O_2. Our present work showed that the

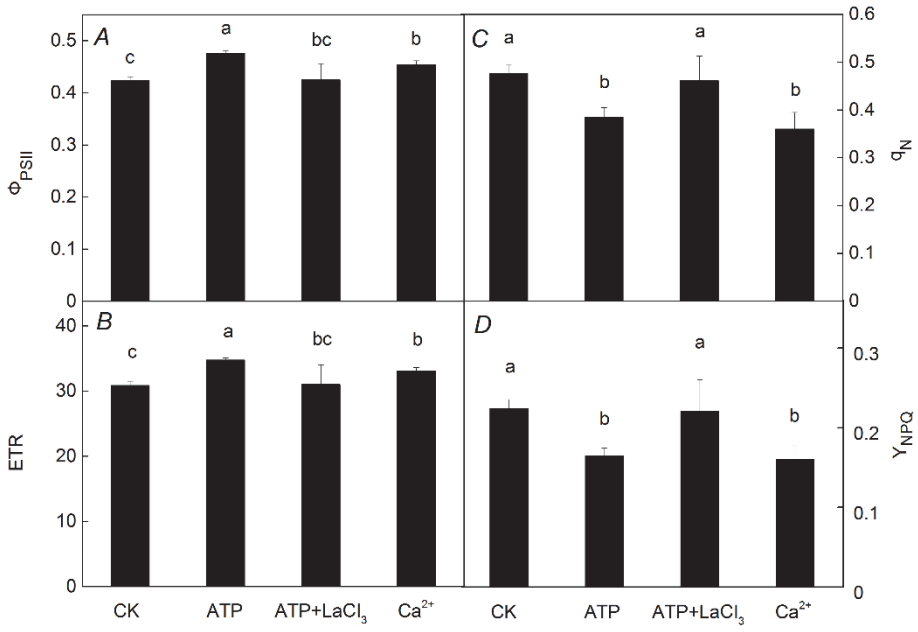

Fig. 1. The effects of ATP, ATP+ $LaCl_3$, and Ca^{2+} on the operating efficiency of PSII (Φ_{PSII}) (*A*), the rate of photosynthetic electron transport through PSII (ETR) (*B*), the nonphotochemical quenching (q_N) (*C*), and the quantum yield of regulated energy dissipation of PSII (Y_{NPQ}) (*D*) of bean leaves. Each value represents the mean ± SD of at least three independent experiments. *Different letters* denote significant differences (P<0.05).

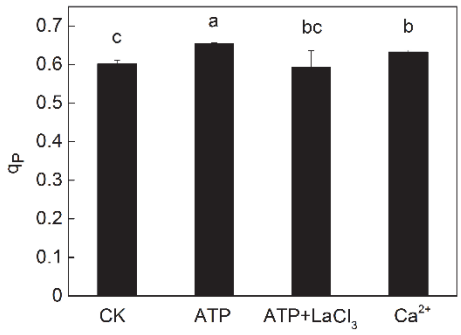

Fig. 2. The effects of ATP, ATP + $LaCl_3$, and Ca^{2+} on the photochemical quenching (q_P) of bean leaves. Each value represents the mean ± SD of at least three independent experiments. *Different letters* denote significant differences (P<0.05).

treatment with exogenous H_2O_2 at 1.0 mM also led to a significant increase in Φ_{PSII}, ETR, and q_P (Figs. 3*A*,*B*, 4) and to a significant decrease in q_N and Y_{NPQ} (Fig. 3*C*,*D*). DMTU (a scavenger of H_2O_2) at 1.0 mM had no significant effect on any of the Chl fluorescence parameters (data not shown). By comparison, the combined treatment with exogenous ATP and DMTU decreased the values of Φ_{PSII}, ETR, and q_P, but increased the values of q_N and Y_{NPQ}, compared to the treatment with ATP alone. The Chl fluorescence parameters after the combined treatment with ATP and DMTU were similar to those in the control leaves, indicating that the application of DMTU almost completely abolished the effects of eATP on Φ_{PSII}, ETR, q_P, q_N, and Y_{NPQ} (Figs. 3*A*–*D*, 4).

Discussion

Some studies in plant cells reported that eATP is secreted into the surrounding medium in the nanomole range (Tanaka *et al*. 2010a). In the extracellular fluid present at *Arabidopsis* wound sites, Song *et al*. (2006) observed that eATP concentration can reach 0.04 mM. However, it has been shown that the concentration of eATP may transiently approach a millimolar concentration in the extracellular matrix adjacent to the ATP release site (Joseph *et al*. 2003). As the eATP measured in the surrounding medium or extracellular fluid is the result of diffusion from tissues, the eATP concentration are assumed to be higher at surface of the cell (Tanaka *et al*. 2010a). Yegutkin *et al*. (2006) found by using a novel intrinsic ATP sensor that eATP concentration at the membrane surface of lymphocytes cells were 1000-fold higher than that in the surrounding medium.

In the present work, eATP at 1 mM, the concentration found to efficiently trigger the physiological response of plant leaves, such as the decreased resistance to viral infection (Chivasa *et al*. 2009), was used to investigate its possible effects on the PSII photochemistry. The results showed that although 1 mM ATP had no effect on the F_v/F_m ratio, this treatment significantly increased the values of Φ_{PSII} and ETR, indicating that an increase in eATP can enhance the portion of photons used in photochemistry and photosynthetic electron transport.

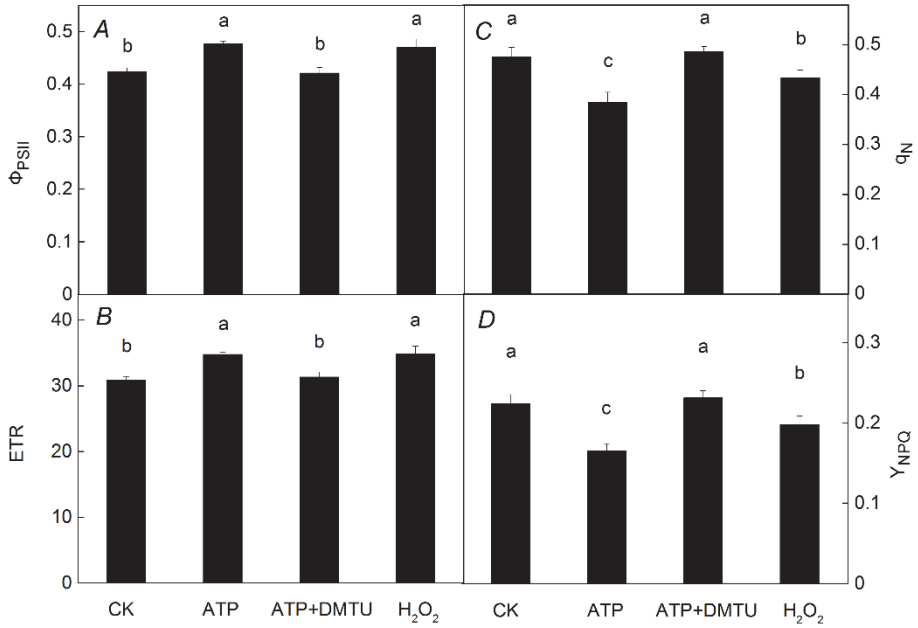

Fig.3. The effects of ATP, ATP + DMTU, and H_2O_2 on the operating efficiency of PSII (Φ_{PSII}) (*A*), the rate of photosynthetic electron transport through PSII (ETR) (*B*), the nonphotochemical quenching (q_N) (*C*), and the quantum yield of regulated energy dissipation of PSII (Y_{NPQ}) (*D*) of bean leaves. Each value represents the mean ± SD of at least three independent experiments. *Different letters* denote significant differences (P<0.05).

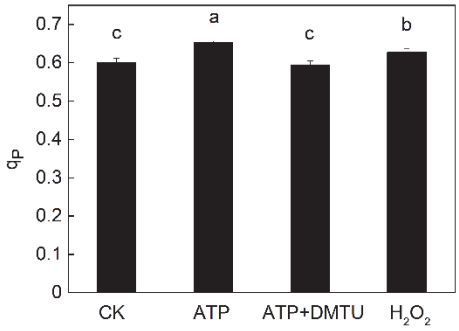

Fig. 4. The effects of ATP, ATP + DMTU, and H_2O_2 on the photochemical quenching (q_P) of bean leaves. Each value represents the mean ± SD of at least three independent experiments. *Different letters* denote significant differences (P<0.05).

The enhancement in Φ_{PSII} and ETR by eATP was followed with a significant decrease in q_N, apparently reflecting that, upon eATP stimulation, more photons were used in photochemistry rather than dissipated as heat. It seems that such effects of eATP could be attributed to an increase in the portion of open PSII reaction centres (with Q_A oxidized), because treatment with 1 mM ATP significantly increased the value of q_P. It should also be noted that eATP caused a decrease in Y_{NPQ}, whereas Y_{NO} did not significantly change. Thus, we suggested that the decrease in the dissipation of absorbed light energy by eATP was primarily due to regulated nonphotochemical energy dissipation mechanism.

It should be noted that plant cells possess apoplastic nucleotidases and apyrases to hydrolyse eATP (Riewe *et al*. 2008). Sun *et al*. (2012) revealed that after treatment of *Populus euphratica* cells with exogenous 1 mM ATP for 20 min, the concentration of eATP in the medium decreased by about 30%. Thus, it could be expected that a similar decrease also occurred after the incubation of bean leaves with 1 mM ATP for 10 min.

Due to its high charge, eATP cannot passively diffuse across the plasma membrane (Tanaka *et al*. 2010a). However, in animal cells, it has been demonstrated that eATP, through activating the receptor proteins located in the plasma membrane, initiates an increase in the concentration of $[Ca^{2+}]_{cyt}$ (cytosolic free calcium), which acts as an early signaling step for the eATP-mediated physiological events (Dichmann *et al*. 2000). In plant cells, it is also observed that treatment with eATP can result in the specific accumulation of $[Ca^{2+}]_{cyt}$ (Demidchik *et al*. 2009, Tanaka *et al*. 2010b). A main mechanism for the eATP-induced increase of $[Ca^{2+}]_{cyt}$ is that perception of eATP by plant cells stimulates an influx of Ca^{2+} from the extracellular space through plasma membrane Ca^{2+}-permeable channels (Demidchik *et al*. 2009).

Under normal conditions, the concentration of Ca^{2+} in the extracellular space ranges from 0.1 to 1 mM (Hepler 2005). Sun *et al*. (2012) reported that 1 mM ATP induced a rapid and continuous net Ca^{2+} influx in *P. euphratica* cells. Similar to the effects of eATP, exogenous Ca^{2+} at 1 mM [a concentration reported to efficiently induce an elevation of $[Ca^{2+}]_{cyt}$ in leaf tissue (Staxen *et al*. 1999)] also led to the significant increase in Φ_{PSII}, ETR, and q_P and to the significant decrease in q_N and Y_{NPQ}. $LaCl_3$ (an inhibitor of Ca^{2+} channels) abolished the effects of exogenous ATP on Chl fluorescence parameters. Thus, it is suggested that eATP could affect the PSII photochemistry through a Ca^{2+}-dependent mechanism. For example, recent studies reported that the thylakoid-localized calcium-sensing receptor is crucial for the formation of the photosynthetic electron transport system (Petroutsos *et al*. 2011, Wang *et al*. 2014). Thus, it is possible that an increase in eATP could enhance the concentration of Ca^{2+}, which increases the PSII photochemistry through stimulating certain Ca^{2+}-dependent physiological events (such as the thylakoid-localized calcium-sensing receptor). However, actual mechanism for Ca^{2+} involvement in the eATP-induced changes of the PSII photochemistry is still unknown and must be determined by further work.

The perception of eATP by plant cells can also lead to an increased production of ROS (Kim *et al*. 2006, Song *et al*. 2006, Demidchik *et al*. 2009, Lim *et al*. 2014). Demidchik *et al*. (2009) proposed that eATP primarily induces the production of extracellular superoxide anion through activating plasma membrane NADPH oxidases. Then, part of the superoxide anion is converted to H_2O_2, which can enter the cytosol and subsequently lead to the accumulation of intracellular H_2O_2. In plant cell cultures, a steady-state, micromolar H_2O_2 concentration exists in the culture medium (Kärkönen and Koutaniemi 2010). It is found that at 1 mM or higher concentrations of exogenous H_2O_2 is required to induce Ca^{2+} influx and to increase intracellular H_2O_2 in the plant (Demidchik *et al*. 2007, Costa *et al*. 2010). The present work showed that the effects of exogenous 1 mM H_2O_2 on Φ_{PSII}, ETR, q_P, q_N, and Y_{NPQ} were similar to those of ATP or Ca^{2+}. Similar to the behavior of $LaCl_3$, DMTU (a scavenger of H_2O_2) almost completely abolished the effects of eATP on the Chl fluorescence parameters. Thus, it seems that the effects of eATP on the PSII photochemistry could be also associated with a H_2O_2 signaling pathway.

Previous study has revealed that the increase in ROS contents by eATP can enhance the concentration of Ca^{2+} by activating an ROS-sensitive Ca^{2+}-permeable channel at the plasma membrane (Demidchik *et al*. 2009), an observation consistent with other findings that H_2O_2 has an ability to enhance the concentration of Ca^{2+} in plant cells (Rentel 2004, Takeda 2008). Thus, it is likely that, upon eATP stimulation, H_2O_2 could enhance the PSII photochemistry through triggering an elevation of Ca^{2+}. We can not exclude the possibility that H_2O_2 could affect the PSII photochemistry through other mechanisms independent of Ca^{2+}. Nevertheless, the results presented here demonstrated that eATP can affect Chl fluorescence characteristics of kidney bean leaves through Ca^{2+} and H_2O_2-dependent mechanism.

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