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BRIEF COMMUNICATION

# Extracellular ATP alleviates the salicylic acid-induced inhibition of cell viability and respiration through a $Ca^{2+}$-dependent mechanism

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## Abstract

Extracellular ATP (eATP) has been considered as signalling compound to mediate several physiological processes. Here we show that eATP played a role in alleviating the salicylic acid (SA)-induced inhibition of cell viability and respiration in tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension cells. Exogenous SA at higher concentrations (0.05, 0.1, 0.3, 0.5, or 0.7 mM) caused a significant reduction in respiratory $O_2$ uptake or cell viability. The addition of exogenous ATP alleviated the SA-induced reductions in cell viability and respiration, but the effect was dependent on the concentrations of either exogenous SA or ATP. Further study shows that the alleviative effects of exogenous ATP were abolished by the addition of $GdCl_3$ (an inhibitor of $Ca^{2+}$ channels) or EGTA (a $Ca^{2+}$ chelator). These results suggest that this role of extracellular ATP could be related to a $Ca^{2+}$ signalling pathway.

*Additional key words*: *Nicotiana tabacum*, tobacco suspension cells.

ATP is usually localised in intracellular spaces, however, 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 either *via* ATP-binding cassette transporters or vesicular exocytosis (Thomas *et al*. 2000, Kim *et al*. 2006). Furthermore, this extracellular ATP (eATP) was found to be important for several physiological processes in plants, including cell growth, development, thigmotropism, and gravitropism (Tang *et al*. 2003, Wolf *et al*. 2007, Weerasinghe *et al*. 2009, Tonón *et al*. 2010). Some studies have reported that eATP can stimulate the accumulation of many important intracellular signalling molecules, such as cytosolic free calcium ions, nitric oxide, and reactive oxygen species (Foresi *et al*. 2007, Demidchik *et al*. 2009).

Salicylic acid (SA) is a phenolic compound that is an important effector molecule in plants (Klessig and Malamy 1994). The application of SA has long been known to induce plant defence responses to bacterial or viral pathogens by activating the expression of pathogenesis-related (*PR*) genes (Delaney *et al*. 1994, Murphy *et al*. 1999). SA was also found to increase the plant resistance to abiotic stresses, such as chilling, high salinity, and heavy metal excess (Wang *et al.* 2009, Moussa and El-Gamal 2010, Tari *et al*. 2010). Otherwise, some studies showed that the application of exogenous SA, especially at higher concentrations, inhibits respiratory $O_2$ uptake and decreased cell viability (Xie and Chen 1999, Robson and Vanlerberghe 2002, Tari *et al*. 2011). It is thought that such behaviour of SA could play a role in mounting the hypersensitive cell death known as defence response to restrict pathogen growth (Alvarez 2000, Xie and Chen 1999).

Recently, Chivasa *et al*. (2009) reported that the exogenous application of ATP suppresses the SA-induced expression of *PR* genes suggesting that eATP acts as negative regulator of the SA-induced defence response to pathogens. However, whether eATP could also have an antagonistic effect on the SA-induced

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*Abbreviations*: eATP - extracellular ATP; SA - salicylic acid.
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inhibition of cell viability or respiration has not been extensively studied. In the present study, we demonstrate that eATP played a role in alleviating inhibition of cell viability and respiration induced by higher concentrations of SA, and such a role of eATP could be dependent on a $Ca^{2+}$-mediated signalling pathway.

Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell suspension cultures were grown at 25 °C on a rotary shaker in the dark in an MS medium (Murashige and Skoog 1962) supplemented with 3 % (m/v) sucrose and 0.4 mg $dm^{-3}$ 2,4-dichlorophenoxyacetic acid. The cell suspension cultures were subcultured by a 10-fold dilution in a fresh MS medium every 7 d. Three days after subculture, the cells were used for all experiments.

In the first experiment, the cell suspensions were subjected to different concentrations of SA (0, 0.02, 0.05, 0.1, 0.3, 0.5, or 0.7 mM). In the second experiment, the cell suspensions were subjected to different concentrations of ATP (0.001, 0.01, or 0.05 mM). In the third experiment, the cell suspensions were subjected to 0, 0.02, 0.05, 0.1, 0.3, 0.5 or 0.7 mM SA, each of which contained 0.001, 0.01, or 0.05 mM ATP (Table 1). In the fourth experiment, the cell suspensions were subjected to 0.3 mM $GdCl_3$, 5.0 mM EGTA, 0.1 mM SA containing both 0.3 mM $GdCl_3$ and 0.05 mM ATP, or 0.1 mM SA containing both 5.0 mM EGTA and 0.05 mM ATP, respectively. The treated cell suspensions were then incubated at 25 °C in the dark for 10 h. The cell suspensions that were subjected to deionized water and incubated under the same conditions were used as control.

Cell viability was determined using the Evans blue staining assay (Yang *et al*. 2004). The cell suspensions after the treatments were stained with a 0.25 % (m/v) Evans blue solution for 8 min and then washed with a phosphate buffered saline (PBS ) solution. The dye bound to dead cells was extracted and solubilized with a solution containing 1 % (m/v) sodium dodecyl sulphate (SDS) and 50 % (v/v) methanol at 50 °C for 0.5 h. The concentration of extracted dye corresponded to the absorbance measured at 600 nm.

The steady rate of respiratory $O_2$ uptake was measured and calculated as described by Bingham and Farrar (1989). In brief, the cells in the culture medium were transferred to an air-tight cuvette. The respiratory $O_2$ uptake of the cells was monitored in the absence of any respiratory inhibitors by using a Clark-type oxygen electrode (*SP-2 type*, constructed by the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China).

The results are expressed as means ± standard deviations (SD). The data were analysed using the Kruskall–Wallis one-way analysis of variance test. $P < 0.05$ was considered statistically significant.

The application of 0.02 or 0.05 mM SA had no significant effect on the cell viability, whereas SA at higher concentrations (0.1, 0.3, 0.5, or 0.7 mM) caused a significant reduction in cell viability (Table 1). Similarly, the respiratory $O_2$ uptake of the cell suspensions decreased with the increase in the concentration of exogenous SA. It should be noted that the respiratory $O_2$ uptake of cell suspensions was more sensitive to exogenous SA than the cell viability, because 0.05 mM SA caused a significant decrease in the respiratory $O_2$ uptake but did not significantly change the cell viability (Table 1).

Table 1. The effects of exogenous SA, ATP, or the combinations of SA plus ATP on the cell viability and respiratory $O_2$ uptake. Means ± SD of at least four individual experiments. Different letters denote significant differences between the treatments with different concentrations of SA in the absence of exogenous ATP ($P < 0.05$); * - statistically significant differences between the SA-treated cells and the SA + ATP-treated cells under the same concentration of SA ($P < 0.05$).

| Parameter | SA [mM] | 0 mM ATP | 0.001 mM ATP | 0.01 mM ATP | 0.05 mM ATP |
|---|---|---|---|---|---|
| Cell viability [%] | 0 | 100.0 ± 2.3$^a$ | 99.3 ± 1.5 | 99.3 ± 1.7 | 99.2 ± 1.3 |
| | 0.02 | 100.1 ± 2.7$^a$ | 99.2 ± 1.4 | 100.1 ± 1.0 | 100.5 ± 3.1 |
| | 0.05 | 97.2 ± 3.0$^a$ | 97.9 ± 0.6 | 98.6 ± 2.0 | 100.0 ± 1.4 |
| | 0.10 | 83.3 ± 4.1$^b$ | 92.2 ± 4.0* | 92.9 ± 1.3* | 96.3 ± 2.3* |
| | 0.30 | 71.3 ± 2.6$^c$ | 70.8 ± 3.9 | 81.9 ± 2.3* | 95.0 ± 3.4* |
| | 0.50 | 61.9 ± 3.2$^d$ | 61.8 ± 6.3 | 70.2 ± 4.7* | 85.5 ± 7.2* |
| | 0.70 | 53.8 ± 3.5$^e$ | 54.5 ± 4.3 | 53.7 ± 2.4 | 60.6 ± 4.3* |
| Respiratory $O_2$ uptake | 0 | 14.1 ± 1.3$^a$ | 14.2 ± 1.9 | 14.9 ± 1.8 | 14.8 ± 2.0 |
| [μmol($O_2$) $kg^{-1}$(f.m.) $s^{1}$] | 0.02 | 14.2 ± 1.8$^a$ | 13.4 ± 1.6 | 13.6 ± 1.8 | 15.1 ± 2.6 |
| | 0.05 | 11.3 ± 0.6$^b$ | 10.1 ± 1.3 | 11.8 ± 1.7 | 13.4 ± 1.2* |
| | 0.10 | 9.3 ± 0.7$^c$ | 9.3 ± 1.3 | 10.5 ± 2.1 | 12.5 ± 1.0* |
| | 0.30 | 7.0 ± 1.1$^d$ | 7.0 ± 1.4 | 7.2 ± 1.4 | 10.6 ± 1.0* |
| | 0.50 | 4.1 ± 0.8$^e$ | 3.4 ± 0.9 | 4.2 ± 0.5 | 8.1 ± 0.8* |
| | 0.70 | 2.7 ± 0.5$^f$ | 2.8 ± 0.5 | 2.5 ± 0.9 | 3.9 ± 0.8* |

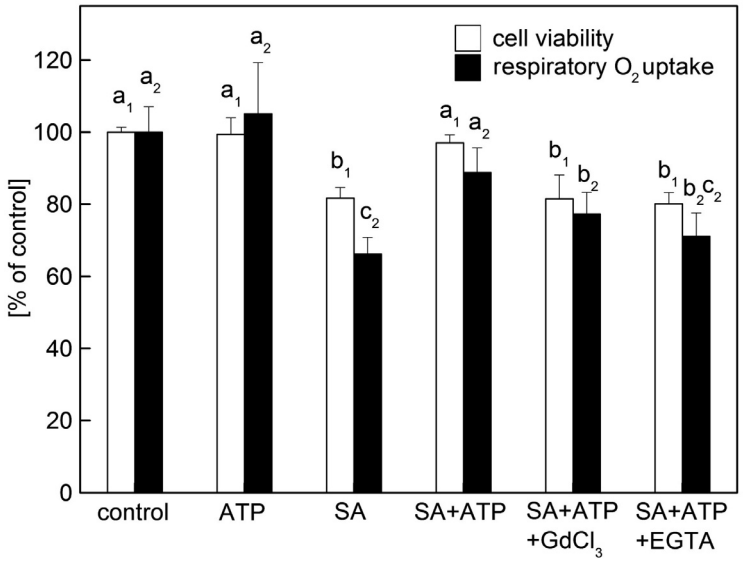


Fig. 1. The effects of different treatments on the cell viability and respiratory $O_2$ uptake. Cell suspensions were exposed to 0.05 mM ATP, 0.1 mM SA, 0.1 mM SA containing 0.05 mM ATP (SA+ATP), 0.1 mM SA containing both 0.05 mM ATP and 0.3 mM $GdCl_3$ (SA+ATP+$GdCl_3$), or 0.1 mM SA containing both 0.05 mM ATP and 5.0 mM EGTA (SA+ATP+EGTA). The values of the cells without any chemical treatment are denoted as control (100 %). The values of cell viability and respiratory $O_2$ uptake represent means of at least four individual experiments. Significant differences ($P < 0.05$) of the same parameter among the different treatments are marked with different letters followed by the same number.

We subsequently studied whether eATP could affect the SA-induced inhibition of cell viability and respiratory $O_2$ uptake. The treatment with exogenous ATP (0.001, 0.01, or 0.05 mM) alone had no significant effects on the cell viability and respiratory $O_2$ uptake (Table 1). Then, the viability and respiration in the cells exposed to SA alone were compared with those in the cells exposed to the combined treatment with SA plus ATP. The results show that exogenous ATP at 0.001, 0.01, or 0.05 mM significantly alleviated the decrease in cell viability induced by 0.1 mM SA. Exogenous ATP at 0.01 or 0.05 mM significantly alleviated the decrease in cell viability induced by 0.3 or 0.5 mM SA. However, the decrease of cell viability induced by 0.7 mM SA was alleviated only by 0.05 mM exogenous ATP (Table 1). The addition of low concentrations of exogenous ATP (0.001 or 0.01 mM) had no significant effects on the respiratory $O_2$ uptake of the SA-treated cells, whereas ATP at 0.05 mM significantly alleviated the reduction in respiratory $O_2$ uptake of the cells treated with 0.05, 0.1, 0.3, 0.5, or 0.7 mM SA (Table 1).

Due to the high charge of eATP, it cannot passively diffuse across the plasma membrane (Tanaka *et al*. 2010). However, it has been demonstrated that eATP, through the activation of its receptor proteins located in the plasma membrane, initiates an increase in the $Ca^{2+}$ content, which acts as early signalling step for eATP-mediated physiological events (Dichmann *et al*. 2000, Demidchik *et al*. 2009, Choi *et al*. 2014). In the present study, $GdCl_3$ (an inhibitor of $Ca^{2+}$ channels) and EGTA (a $Ca^{2+}$ chelator) were used to examine whether the role of eATP in alleviating the SA-induced inhibition of respiration and cell viability could be dependent on $Ca^{2+}$. The concentrations of $GdCl_3$ and EGTA used here were 0.3 mM and 5.0 mM, respectively, which have been reported to significantly restrict the eATP-induced increase of $Ca^{2+}$ (Sun *et al*. 2012). The application of $GdCl_3$ or EGTA themselves had no significant effects on any of the experimental parameters (data not shown). Then $GdCl_3$ or EGTA was added into the cell suspensions that were treated with 0.1 mM SA plus 0.05 mM ATP (Fig. 1), because SA at this concentration had caused a significant inhibition in both cell viability and respiration, and exogenous ATP at this concentration alleviated this inhibition. The result shows that the addition of $GdCl_3$ or EGTA almost completely abolished the alleviative effect of eATP on the SA-induced decreases in cell viability (Fig. 1). Combining this observation with previous finding that an exogenous application of ATP increases $Ca^{2+}$ content (Demidchik *et al*. 2009), it is suggested that an increase in $Ca^{2+}$ content by eATP could trigger certain physiological events to interfere with the negative effect of SA on cell viability.

$GdCl_3$ or EGTA partly abolished the alleviative effect of eATP on the SA-induced decreases in respiration. It was reported that $Ca^{2+}$ can stimulate the mitochondrial transmembrane potential and respiration by increasing the availability of mitochondrial substrates (McCormack *et al*. 1990, Rutter *et al*. 1996, Jouaville *et al*. 1999, Logan and Knight 2003). Sun *et al*. (2012) also observed that eATP induces an increase in the mitochondrial transmembrane potential by triggering $Ca^{2+}$ influx. Thus,

the alleviative effect of eATP on the SA-induced inhibition of respiration could also be partly attributed to a positive regulation of the mitochondrial functions by $Ca^{2+}$. In detail, it should be noted that eATP only at a higher concentration (0.05 mM) alleviated the SA-induced decrease of respiration. In comparison, besides at this higher concentration, eATP at lower concentrations (0.001 or 0.01 mM) also alleviated the SA-induced decrease of cell viability, as observed in the cells treated with 0.1, 0.3 or 0.5 mM SA (Table 1). And, the alleviative effect of eATP on the SA-induced decreases in respiration was not completely abolished by $GdCl_3$ or EGTA. Thus, it is possible that SA at a higher concentration could inhibit respiration *via* other mechanisms which are not affected by the eATP-induced increase of $Ca^{2+}$ content.

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