

Extracellular ATP Functions as an Endogenous External Metabolite Regulating Plant Cell Viability

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ATP is a vital molecule used by living organisms as a universal source of energy required to drive the cogwheels of intracellular biochemical reactions necessary for growth and development. Animal cells release ATP to the extracellular milieu, where it functions as the primary signaling cue at the epicenter of a diverse range of physiological processes. Although recent findings revealed that intact plant tissues release ATP as well, there is no clearly defined physiological function of extracellular ATP in plants. Here, we show that extracellular ATP is essential for maintaining plant cell viability. Its removal by the cell-impermeant traps glucose–hexokinase and apyrase triggered death in both cell cultures and whole plants. Competitive exclusion of extracellular ATP from its binding sites by treatment with β,γ -methyleneadenosine 5'-triphosphate, a nonhydrolyzable analog of ATP, also resulted in death. The death response was observed in *Arabidopsis thaliana*, maize (*Zea mays*), bean (*Phaseolus vulgaris*), and tobacco (*Nicotiana tabacum*). Significantly, we discovered that fumonisin B1 (FB1) treatment of *Arabidopsis* triggered the depletion of extracellular ATP that preceded cell death and that exogenous ATP rescues *Arabidopsis* from FB1-induced death. These observations suggest that extracellular ATP suppresses a default death pathway in plants and that some forms of pathogen-induced cell death are mediated by the depletion of extracellular ATP.

INTRODUCTION

ATP is a ubiquitous, energy-rich compound in all cells of living organisms. It is found both within organelles, such as mitochondria and chloroplasts, and in the cytoplasm of higher organisms. The energy derived from ATP is used to drive a myriad of vital biochemical reactions that are fundamental to the survival of cells and whole organisms. The presence of intracellular ATP has been recognized since its discovery in living cells in 1929 (Fiske and Subarrow, 1929). However, cells can secrete ATP to the extracellular matrix in the absence of cytolysis, an observation first reported in animal cells in 1959 (Holton, 1959) and only recently in a plant species, *Arabidopsis thaliana* (Thomas et al., 2000).

In animals, nonlytic release of ATP by nonsecretory cells occurs under quiescent conditions (Beigi and Dubyak, 2000; Schwiebert et al., 2002), after mechanical stimulation (Grierson and Meldolesi, 1995; Homolya et al., 2000; Yegutkin et al., 2000), or after treatment with agonists such as bradykinin, acetylcholine, and serotonin (Yang et al., 1994). Touch stimulation and

osmotic stress elicit increased ATP release from *Arabidopsis* (Jeter et al., 2004), suggesting conservation of ATP release in response to mechanical stimulation between plants and animals. Neuronal cells and secretory cells such as mast cells and pancreatic acinar cells release ATP via regulated exocytosis (Leitner et al., 1975; Unsworth and Johnson, 1990; Sorensen and Novak, 2001). The mechanisms by which plant cells and nonsecretory animal cells release ATP are still poorly understood, but there is evidence for the involvement of ABC transporters and anion channels (Abraham et al., 1993; Roman et al., 1997; Thomas et al., 2000; Dutta et al., 2002, 2004).

In animal cells, extracellular ATP is an absolute requirement for several physiological processes, such as neurotransmission, platelet aggregation, regulation of blood vessel tone, cell growth and expansion, differentiation and function of immune cells, and muscle contraction (Gordon, 1986; Redegeld et al., 1999). In these cells, extracellular ATP funnels into signaling pathways via two mechanisms. First, it participates in extracellular phosphorylation (Redegeld et al., 1999). For example, extracellular phosphorylation is an absolute requirement in T cell-mediated immune responses. Specific abolition of ectophosphorylation, without affecting intracellular phosphorylation, by the ectoprotein kinase inhibitor K-252b is accompanied by a loss of cytolytic activity of cytotoxic T lymphocytes for target cells (Redegeld et al., 1997). Second, extracellular ATP participates in signal transmission via the activation of P2X and P2Y plasma membrane nucleotide binding receptors known as purinoceptors (Burnstock and Kennedy, 1985). P2X receptors are ligand-gated ion channels that gate extracellular cations in response to ATP,

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whereas P2Y receptors are G protein-coupled receptors. Some physiological processes are dependent on the activation of purinoceptors by extracellular ATP to initiate signaling cascades. For example, extracellular ATP binds to P2X₇ receptors to activate astrocyte Gln release, which modulates synaptic activity and participates in brain intercellular signaling (Duan et al., 2003).

Although extracellular ATP released by intact plant tissues (Thomas et al., 2000) has no clearly defined function, two lines of evidence suggest that it may have physiological significance. The first is based on electrophysiological studies using patch-clamp techniques. Extracellular ATP depolarizes *Arabidopsis* root hair membranes (Lew and Dearnaley, 2000) and triggers an increase of cytosolic Ca²⁺ (Demidchik et al., 2003), implicating a plant homolog of animal purinoceptors; however, plant purinoceptors have yet to be identified. This Ca²⁺ influx mediates the activation of *Arabidopsis* gene expression induced by exogenous ATP (Jeter et al., 2004). The second line of evidence is based on proteomic studies of *Arabidopsis* extracellular matrix proteins. Identification of secreted, extracellular phosphoproteins in the *Arabidopsis* extracellular matrix (Chivasa et al., 2002; Ndimba et al., 2003) suggests the participation of ATP in extracellular signaling via phosphorylation.

Thus, we initiated studies to examine the role of extracellular ATP in plants via the specific removal of ATP from the extracellular matrix of cell suspension cultures and intact plants. In this article, we report the discoveries that (1) a physiological role of extracellular ATP in plants is to govern cell viability by the suppression of a default death pathway, and (2) a mechanism by which fumonisins B1 (FB1) induces death in *Arabidopsis* is the depletion of extracellular ATP.

RESULTS

Cell-Impermeant ATP Traps Trigger Death in *Arabidopsis* Cell Cultures

We confirmed that plant cells indeed secrete ATP to the extracellular matrix by showing that *Arabidopsis* cell cultures fed with [³²P]H₃PO₄ synthesized and secreted radiolabeled ATP to the extracellular matrix within 1 h (Figure 1A). Six hours later, the level of radiolabeled ATP in the extracellular medium remained the same (Figure 1A, lanes 3 and 6), indicating that the cells tightly regulate extracellular ATP levels. Using Evans blue staining, we found no changes in the integrity of the plasma membrane of the suspension cells within the first hour during which the cells took up radioactive phosphate and released radioactive ATP (Figure 1C). This finding shows that the radiolabeled ATP in the growth medium was actively secreted by the cells and did not result from passive release attributable to cell death. Moreover, the fact that labeled ATP remained the same between 1 and 7 h from the addition of labeled phosphate rules out release via cell death but suggests a highly controlled dynamic balance between secretion and hydrolysis by extracellular enzymes.

To establish its physiological function, we selectively depleted extracellular ATP from cell suspension cultures and plants using cell-impermeant ATP traps. The ATP traps used were apyrase (a

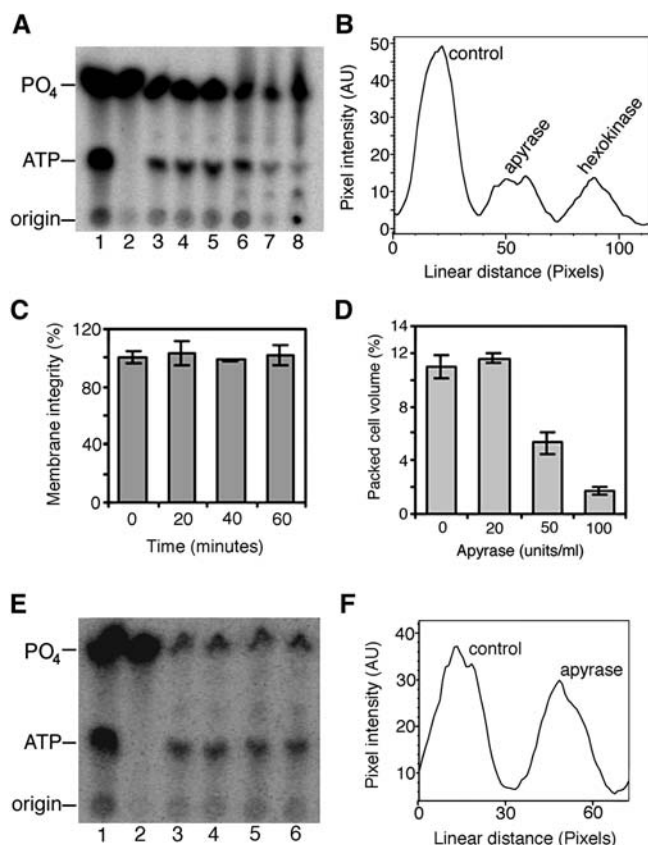


Figure 1. Extracellular ATP and Cell Viability of *Arabidopsis* Cell Cultures.

(A) ATP and phosphate (PO₄) standards (lane 1) and [³²P]H₃PO₄ fed to three independent cell cultures and medium aliquots taken at time 0 (lane 2) and 1 h later (lanes 3 to 5) showing the de novo synthesis and secretion of ATP. At 1 h, solutions of ATP traps were added, and 6 h later, medium samples were taken from the control culture (lane 6) and from cultures treated with 100 units/mL apyrase (lane 7) and glucose-hexokinase (lane 8).

(B) Line graph of a pixel intensity profile showing relative levels of ATP in the 6-h samples (lanes 6 to 8) in **(A)**.

(C) Plasma membrane integrity, measured by Evans blue staining, of cell cultures in **(A)** over the first 1 h.

(D) Dose response of cell viability to treatment with apyrase.

(E) ATP and phosphate standards (lane 1), [³²P]H₃PO₄ (lane 2), and medium aliquots 1 h after adding radioactive phosphate (lanes 3 and 4) and just before adding 20 units/mL apyrase. Medium from control cells (lane 5) and cells treated with 20 units/mL apyrase (lane 6) 6 h after enzyme treatment.

(F) Line graph of a pixel intensity profile across the ATP bands in lanes 5 and 6 of **(E)**.

Data and error bars represent means ± SD (*n* = 3). AU, arbitrary units.

hydrolase that breaks down ATP, via ADP, to AMP) and the glucose-hexokinase system. Hexokinase phosphorylates glucose in a reaction consuming a molecule of ATP. However, the plant extracellular matrix is acidic and replete with proteases, factors that could potentially inhibit the enzymatic activity of the ATP traps. Thus, we verified that both apyrase and glucose-hexokinase traps effectively reduced the extracellular ATP level

in these cell cultures (Figures 1A and 1B). In initial experiments, we noted that cell death ensued within 24 to 48 h of incubating *Arabidopsis* cell cultures with apyrase, as confirmed by propidium iodide and fluorescein diacetate staining (data not shown). Quantitative estimation of cell death/viability was obtained by measuring the ability of the cultures to grow subsequent to the treatment. Cell cultures treated for 72 h were diluted 30-fold with fresh growth medium to diminish apyrase activity, and the cell density (packed cell volume) was determined after another 96 h of growth.

Using this method, we observed a significant dose-dependent reduction of *Arabidopsis* cell culture viability in response to apyrase (Figure 1D). Effects of ATP removal have previously been confounded with the effects of buffer salts in lyophilized commercial preparations of ATP-degrading enzymes (Redegeld et al., 1991). However, we found that dialyzed and nondialyzed apyrase had indistinguishable effects on cell viability (Figure 2A), indicating that cell death was not attributable to buffer salts. Treatment of the cell cultures with BSA equivalent to the amount of apyrase used did not cause any cell death (Figure 2B), demonstrating that the death response did not result from the presence of a foreign protein in the cultures. In addition, when apyrase was denatured by boiling for 5 min before treating the cultures, there was no cell death (Figure 2A), revealing that the death response requires the active enzyme. Moreover, neither AMP nor ADP, the products of ATP hydrolysis by apyrase, caused the death of the cultures (Figure 2B). This indicates that death was in response to the depletion of ATP and not to the accumulation of ADP or AMP. The correlation of extracellular ATP depletion with cell death was further demonstrated by the observation that 20 units/mL apyrase, not enough to cause death (Figure 1D), barely reduced the level of extracellular ATP in treated cells within 6 h (Figures 1E and 1F), whereas 100 units/mL apyrase effectively reduced ATP (Figures 1A and 1B) and induced cell death (Figure 1D). Because apyrase is cell-impermeant, these results demonstrate that deprivation of extracellular ATP triggers a cell death response in *Arabidopsis*.

Glucose-hexokinase also functions as an ATP trap. As with apyrase, treatments caused a significant dose-dependent death response in *Arabidopsis* cell cultures (Figure 2C). Whereas dialyzed hexokinase evoked the cell death response, neither denatured hexokinase (Figure 2A) nor ADP and glucose-6-phosphate (Figure 2B) elicited the response. Thus, neither buffer salts nor the products of the phosphorylation of glucose by hexokinase engendered cell death. Although glucose can freely diffuse into cells, hexokinase is membrane-impermeant and remains in the external growth medium, resulting in a specific consumption of extracellular ATP. Together, these results reinforce the conclusion that removal of extracellular ATP compromises the viability of *Arabidopsis* cell cultures.

Treatment with a Nonhydrolyzable ATP Analog Induces Cell Death

Because ATP cannot freely diffuse across the plasma membrane (Glynn, 1968; Boldin and Burnstock, 2001), exogenous application of its nonhydrolyzable structural analogs can be used to specifically interfere with reactions requiring extracellular ATP

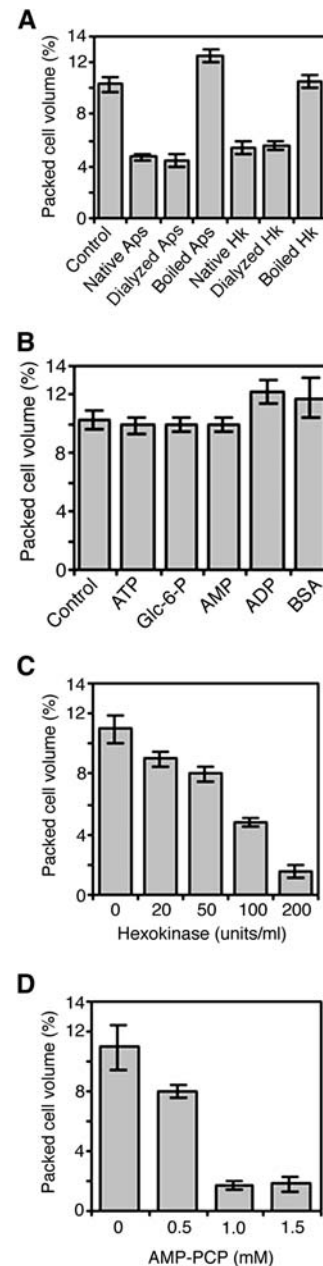


Figure 2. Effects of Various Treatments on the Viability of *Arabidopsis* Cell Cultures.

(A) Effect of dialyzing or denaturing apyrase (Aps; 100 units/mL) and hexokinase (Hk; 200 units/mL) on cell viability.

(B) Effect of various solutions on cell viability. Glc-6-P, glucose-6-phosphate.

(C) Dose response of cell viability to glucose-hexokinase treatment.

(D) Dose response of cell viability to AMP-PCP treatment.

Data and error bars represent means \pm SD ($n = 3$).

hydrolysis (Redegeld et al., 1999). We used $\beta\gamma$ -methyleneadenosine 5'-triphosphate (AMP-PCP), a nonhydrolyzable analog of ATP. Inundation of *Arabidopsis* cell cultures with exogenous AMP-PCP was attended by a concentration-dependent decrease in cell viability (Figure 2D). This finding confirms that extracellular ATP plays a role in cell viability because competitive exclusion from its binding sites by AMP-PCP, as well as its removal by hydrolyzing enzymes, result in cell death.

Extracellular ATP Is Required for the Viability of Intact *Arabidopsis* Plants

Extracellular ATP is also required for viability in planta. Treatment of localized areas of *Arabidopsis* leaves with apyrase, glucose-hexokinase, and AMP-PCP resulted in the collapse of tissue within the treated zone, leading to death between 2 and 4 d after treatment (Figure 3). Localized treatment was used to show the contrast with adjacent living tissue. When entire leaves were treated with the solutions, the whole leaf eventually died (Figure 3). Control leaves treated with BSA, ATP, AMP, ADP, glucose, or glucose-6-phosphate remained healthy (data not shown). To demonstrate that death was not the result of internalization of the



Figure 3. Death of Intact *Arabidopsis* Tissues after Treatment with Extracellular ATP Traps.

Top row, leaves treated on localized areas that developed localized lesions. Bottom row, entire leaves treated and beginning to die. These leaves eventually died except for the controls. Similar results were obtained when the solutions were infiltrated with a needle or needleless syringe. Glc-Hk, glucose-hexokinase. Bar = 6 mm.

ATP traps at the infiltration site caused by mechanical damage, the glucose-hexokinase system applied to the growth medium of hydroponic *Arabidopsis* plants caused death of the roots and some of the shoots (Figures 4 and 5). Similarly, death was triggered in *Arabidopsis* plants grown for 1 week on normal nutrient agar and transferred to nutrient agar supplemented with AMP-PCP (Figure 6). Control plants transferred to agar supplemented with ATP remained viable (Figure 6). Therefore, the cell death response observed in cell suspension cultures caused by extracellular ATP deprivation also occurs in whole plants.

Several Plant Species Require Extracellular ATP for Viability

We investigated the requirement for extracellular ATP in cell viability across plant species. Tobacco (*Nicotiana tabacum*) plants responded to treatment in a similar manner to *Arabidopsis*. Local treatment with apyrase, glucose-hexokinase, and 1 mM AMP-PCP triggered the development of localized lesions (Figure 7). Treating the entire leaf caused the death of the whole leaf. However, treatment with a higher concentration (5 mM) of AMP-PCP had systemic effects: upper leaves that had not received the primary treatment died as well (Figure 7E). An equivalent concentration of ATP did not cause death (Figure 7D). Bean (*Phaseolus vulgaris*) plant leaves treated with apyrase or glucose-hexokinase died as well (Figure 7). Subjecting cell cultures of a monocot, maize (*Zea mays*), to all three treatments elicited the death response (Figure 8), indicating that the requirement of extracellular ATP for viability is not restricted to dicots. We conclude that extracellular ATP has a physiological role in plants that is evolutionarily conserved between distantly related species.

FB1 Triggers Extracellular ATP Depletion That Precedes Cell Death in *Arabidopsis*

Having established that the removal of extracellular ATP from plants or cell suspension cultures triggers death, we wondered whether some forms of plant cell death invoked during normal growth and development or during interaction with other organisms might be mediated by the ablation of extracellular ATP. We decided to investigate the pathogen-induced hypersensitive cell death, which can be simulated by treating plant tissues or cells with pathogen-derived elicitors (Levine et al., 1994). To explore the possibility that diminution of extracellular ATP mediates this response, we used FB1, a programmed cell death-eliciting mycotoxin that switches on active plant defenses in *Arabidopsis* (Stone et al., 2000).

We wanted to monitor the fate of extracellular ATP in *Arabidopsis* tissues during FB1 treatment. Thus, we turned to cell suspension cultures, which offer a simple experimental system in which to sample the extracellular matrix without damaging cells and risking contamination with intracellular metabolites. To achieve this, we used tracer experiments in which cell cultures were spiked with [32 P]ATP and treated with 1 μ M FB1 or 0.014% (v/v) methanol as a control. There was a heightened increase in the hydrolysis of extracellular ATP in FB1-treated cells detectable after 16 h of treatment (Figure 9). This resulted in the rapid

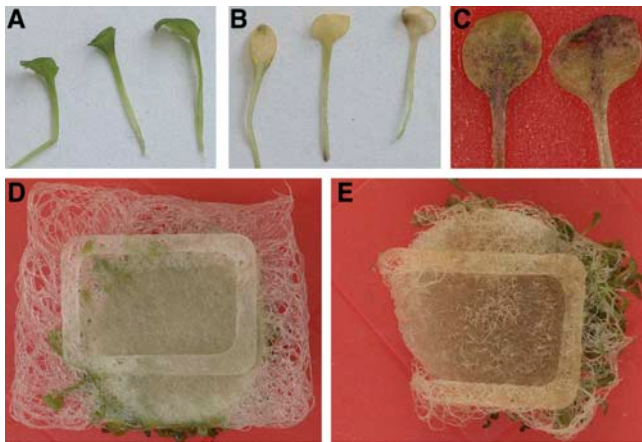


Figure 4. Death of Hydroponic *Arabidopsis* Plants Caused by Glucose-Hexokinase.

- (A) Healthy leaves of plants treated with glucose.
 (B) Dying leaves of plants treated with glucose-hexokinase.
 (C) Close-up photograph of leaves from plants treated with glucose-hexokinase showing both chlorosis and brown lesions of localized tissue death.
 (D) and (E) Roots of glucose-treated plants showing normal growth (D) and retarded roots of glucose-hexokinase-treated plants (E) 7 d after treatment. Fluorescein diacetate staining and microscopy confirmed that the roots in (E) had reduced viability.

decrease of extracellular ATP level in FB1-treated cells to 24 h (Figure 9). The decline in extracellular ATP continued, and by 40 h, labeled ATP was undetectable in FB1-treated cultures, whereas it was still present in control cultures (data not shown). Examination of the integrity of the plasma membrane using Evans blue staining did not show any difference between control cells and FB1-treated cell cultures at 24 h (Figure 9), showing that the extracellular ATP depletion precedes the loss of plasma mem-

brane integrity and cell death, which become obvious 72 h later (Figure 9), when the cells become chlorotic and start losing mitochondrial dehydrogenase activity (data not shown).

Diminution of extracellular ATP before the loss of membrane integrity implies that the machinery for extracellular ATP removal is resident within the extracellular matrix and that this event occurs without leakage of intracellular ATP-degrading enzymes. This observation, placing extracellular ATP depletion upstream of FB1-induced death, was requisite if the hypothesis that FB1-induced death is mediated by the depletion of extracellular ATP was to be upheld.

Exogenous ATP Rescues *Arabidopsis* from FB1-Induced Death

Having established that FB1 treatment activates extracellular ATP depletion in *Arabidopsis* cell cultures, we performed experiments to establish whether this event was a mechanism that mediates FB1-induced cell death. We reasoned that if this cell death response were solely or partially mediated via this removal of extracellular ATP, then it would be interdicted or attenuated by the addition of exogenous ATP concomitant with treatment.

Treatment with 1 μ M FB1 causes a progressive decline in the viability of *Arabidopsis* cell cultures, which becomes obvious at 72 h and is usually complete by 120 h. Death of the entire culture is reflected by the total loss of cellular dehydrogenase activity (Figure 10). In accordance with the prediction that extracellular ATP deficiency participates in FB1-induced death, exogenous ATP added to the cell cultures concurrently with FB1 attenuated cell death (Figure 10A) and delayed it by a minimum of 48 h. The dose response of FB1-induced death to exogenous ATP is shown in Figure 10B. We found that exogenous ATP added to the cells up to 40 h after the addition of FB1 could still rescue the cells from FB1-induced death (Figure 10A). However, when added at 48 h or later, ATP could

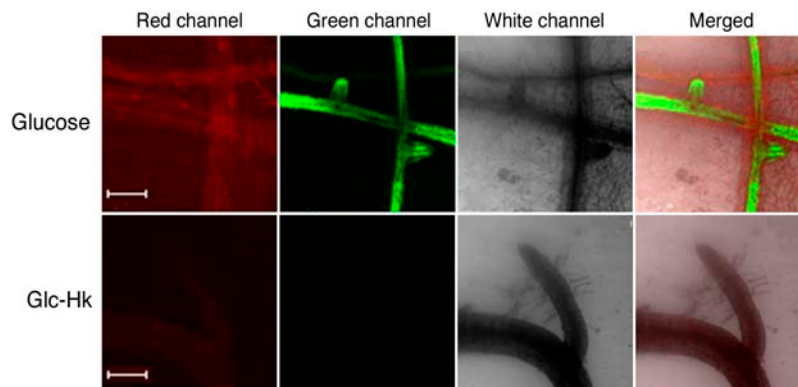


Figure 5. Glucose-Hexokinase Causes the Death of *Arabidopsis* Roots.

Top row, root tissue from plants treated with glucose. Bottom row, root tissue from plants treated with glucose-hexokinase (Glc-Hk). Red channel, confocal images showing autofluorescence of the root tissues. Green channel, confocal images of fluorescein diacetate signals emitted by the same samples. White channel, images of the same samples captured using transmitted light under phase contrast. The extreme right column shows the merged images of the preceding three panels. It is clear that the glucose-hexokinase treatment caused the death of the roots, as there was no fluorescein diacetate signal in these samples. Bars = 200 μ m.

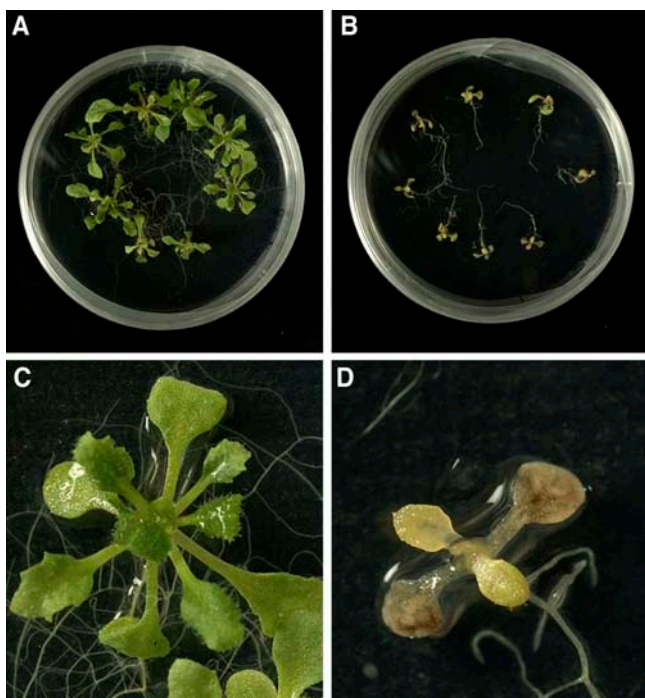


Figure 6. Death Triggered by Noninvasive Application of an ATP Analog.

Arabidopsis plants were germinated on nutrient agar and transferred to plates with agar supplemented with 1 mM ATP (**A**) or 1 mM AMP-PCP (**B**). Photographs were taken after 4 weeks. (**C**) and (**D**) show close-up photographs of representative plants from (**A**) and (**B**), respectively. It is apparent that AMP-PCP caused the death of the treated plants.

not rescue the cells from elicitor-induced death. These results show that even though extracellular ATP depletion commences between 16 and 20 h after FB1 addition, irreversible commitment to death occurs at ~48 h.

Effect of Phosphate-Containing Compounds on FB1-Induced Death

We were interested to determine whether or not the abrogation of FB1-induced death was unique to ATP and not an effect of the degradation products of ATP. It is clear from Figure 11A that exogenous phosphate, AMP, and ADP were unable to rescue the cells from FB1-induced death. This finding indicates that intact ATP, and not ADP, AMP, or phosphate released by nonspecific phosphatases (Bozzo et al., 2002; Olczak et al., 2003), was responsible for the rescue.

Next, we decided to investigate the effects of other nucleoside triphosphates on FB1-induced death. CTP, GTP, and UTP had similar effects on FB1-induced death as ATP had (Figure 11A). There are reports in the literature suggesting that the addition of other nucleoside triphosphates to cell cultures leads to the accumulation of endogenous extracellular ATP via competitive inhibition of ATP hydrolysis by nonspecific ectonucleotide-degrading enzymes (Ostrom et al., 2000; Lazarowski et al., 2003). Thus, we tested the possibility that the other nucleotides

may exert their effects on FB1-induced death indirectly, via the protection of endogenous extracellular ATP from hydrolysis. We treated cell cultures with AMP or UTP for 4 h and measured the level of ATP in the medium using the luciferin–luciferase method. The stock solutions of these compounds were tested for ATP contamination before addition to the cell cultures, and we found that they were not contaminated. We discovered that the ATP in the medium remained the same in AMP-treated cells but accumulated >4.5-fold in UTP-treated cell cultures (Figure 11B). This confirmed the possibility that the nucleotides may have interfered with FB1-induced death by acting as decoy substrates for the ATP-degrading enzymes and allowing ATP accumulation to protect the cells. This possibility is supported by

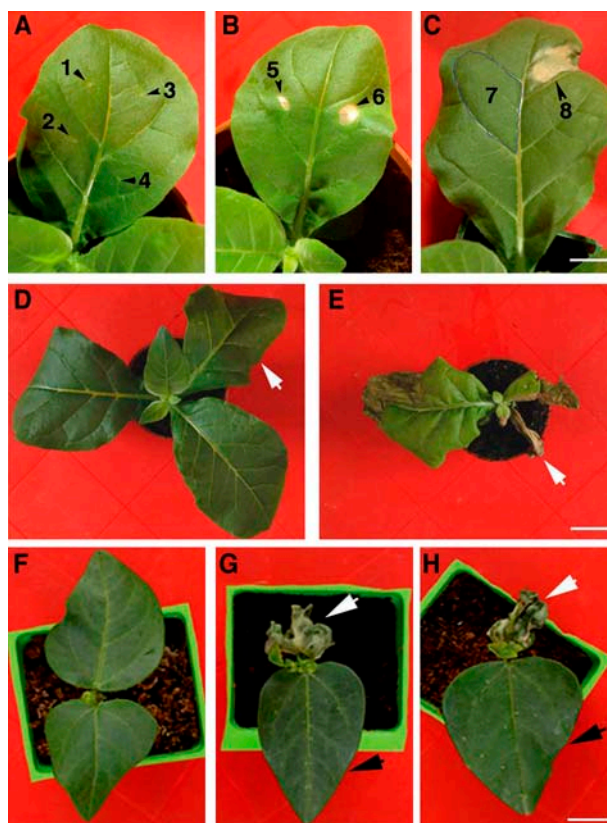


Figure 7. Reaction of Bean and Tobacco to Extracellular ATP Removal.

(**A**) Control treatments with water (1), BSA (2), AMP/ADP (3), and glucose/glucose-6-phosphate/ADP (4) of tobacco.
 (**B**) Local lesions in response to apyrase (5) or glucose-hexokinase (6) treatment of tobacco.
 (**C**) Localized areas of a tobacco leaf treated with ATP (7) or AMP-PCP (8).
 (**D**) Tobacco treated with ATP. The arrow indicates the treated leaf.
 (**E**) Tobacco treated with AMP-PCP on the indicated leaf.
 (**F**) Untreated bean plant.
 (**G**) Water-treated (black arrow) and apyrase-treated (white arrow) bean leaves.
 (**H**) Bean leaves treated with glucose (black arrow) or glucose-hexokinase (white arrow).
 Bars = 2 cm for (**A**) to (**C**), 4 cm for (**D**) and (**E**), and 2 cm for (**F**) to (**H**).

the correlative evidence that AMP, a compound that fails to rescue cells, does not cause the accumulation of endogenous extracellular ATP, whereas UTP, a nucleotide that rescues elicitor-treated cells, does.

ATP Rescues Intact Plant Tissues from FB1-Induced Death

FB1 kills the roots of submerged hydroponic *Arabidopsis* plants, which become stainable with Evans blue 72 h after treatment. In conformity with a requirement for extracellular ATP depletion in FB1-induced death, exogenous ATP added to the plants concurrently with FB1 rescued the roots from death (Figure 12). Dose-response experiments revealed that a minimum of $\sim 400 \mu\text{M}$ ATP is required to rescue the roots of hydroponic *Arabidopsis* plants (Figure 12A). In other experiments, *Arabidopsis* plants were sown on agar plates and transferred 5 d later to plates supplemented with FB1 or FB1 mixed with ATP. At this growth stage, the plants had fully expanded cotyledons. The cotyledons of plants in both treatments died within 1 week, and growth of true leaves proceeded in the plants with FB1 + ATP but not with FB1 only (Figure 12B). This finding shows that FB1 treatment led to the death of these plants, whereas ATP rescued the plants despite the death of the cotyledons.

One interpretation of this is that the plants initially absorbed FB1 into the cotyledons and the lack of uptake of the membrane-impermeant ATP precluded the protection of the cotyledons by extracellular ATP. Because ATP was restricted to the rhizosphere, it rescued the roots from death. After FB1 degradation or depletion from the vicinity of the roots, the roots were able to supply the apical meristem with nutrients and growth resumed. However, ATP did not rescue the plants from FB1-induced growth retardation (Figure 12), demonstrating that ATP does not indiscriminately abolish FB1-induced effects but specifically targets the death response. Exogenous phosphate could not rescue the plants from FB1-induced death (Figure 12), indicating that phosphate cleaved from ATP by nonspecific acid phosphatases, ATPases, or apyrases does not account for the observed

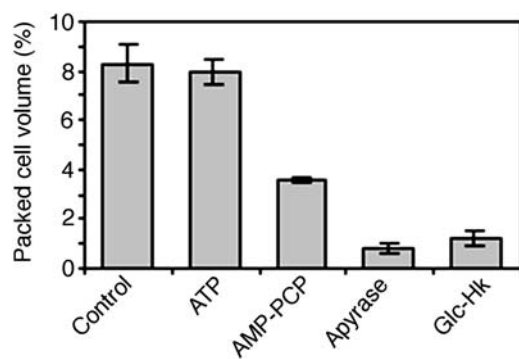


Figure 8. Extracellular ATP Is Required for the Viability of Maize Cell Cultures.

Viability of maize cell suspension cultures after treatment with water (control), 1 mM ATP, 1 mM AMP-PCP, 100 units/mL apyrase, or 50 mM glucose + 200 units/mL hexokinase (Glc-Hk). Data and error bars represent means \pm SD ($n = 3$).

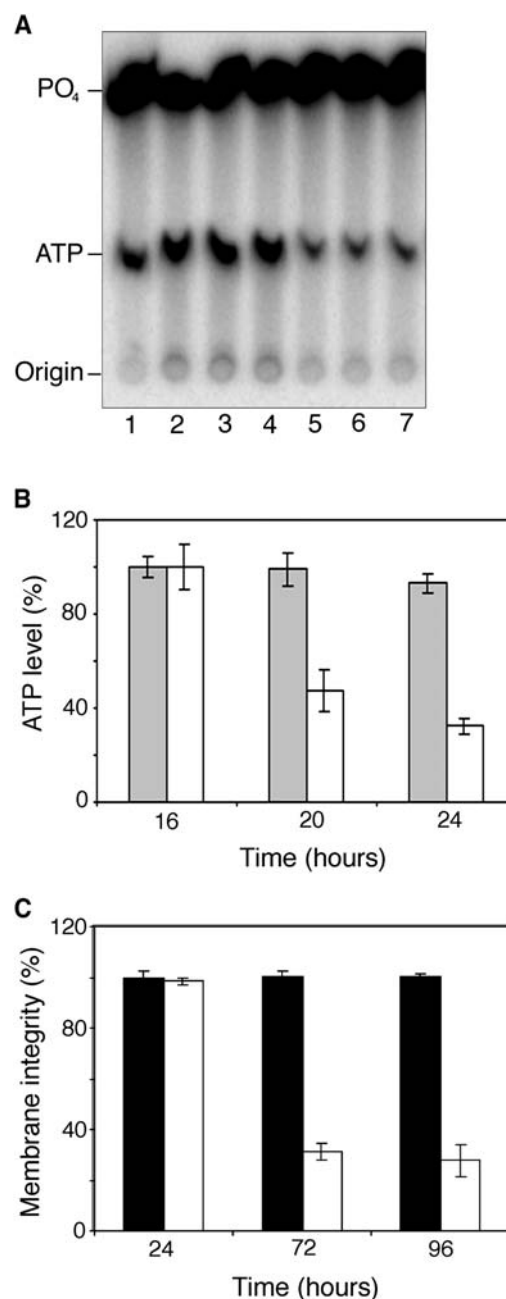


Figure 9. FB1 Treatment Induces the Depletion of Extracellular ATP That Precedes the Loss of Membrane Integrity.

Light-grown *Arabidopsis* cell cultures were treated with methanol (control) or FB1 and spiked with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

(A) Growth medium aliquots from triplicate control (lanes 2 and 3) or FB1-treated (lanes 4 to 6) cultures 24 h after treatment and separated by TLC. Lane 1 contains free phosphate (PO_4) and ATP standards.

(B) Level of radioactive ATP in growth medium (as a percentage of radioactivity initially added to the cell cultures) between 16 and 24 h. Gray bars, control cultures; white bars, FB1-treated cultures.

(C) Changes in the integrity of the plasma membrane over 96 h in control (black bars) and FB1-treated (white bars) cell cultures assayed by the Evans blue method.

Data and error bars represent means \pm SD ($n = 3$).

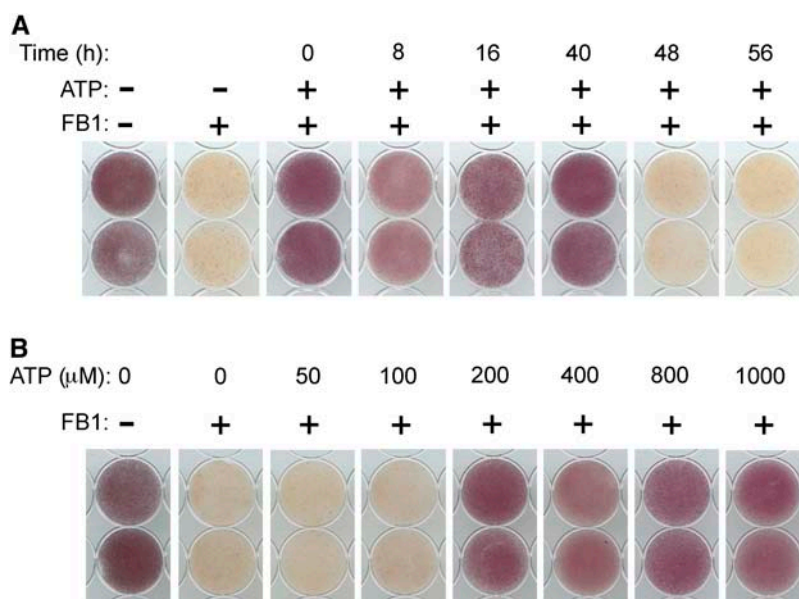


Figure 10. Extracellular ATP Attenuates FB1-Induced Cell Death in *Arabidopsis* Cell Cultures.

(A) Light-grown cell cultures at a density of 5% (w/v) were treated with 1 μ M FB1 at time 0 and spiked with 1 mM ATP at the given times. Cells were harvested at 120 h and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution.

(B) Cell cultures were treated with 1 μ M FB1 at time 0, and increasing concentrations of ATP were added 40 h later. Cells were harvested at 120 h and processed as in **(A)**.

The formation of the purple formazan is an indication of viability; unstained cells are dead; + or - indicates addition or omission of the indicated compound, respectively.

ATP effects. Overall, these results suggest that removal of extracellular ATP is a critical event mediating FB1-induced death in *Arabidopsis*.

Extracellular ATP Depletion Alters the Abundance of Cellular Protein

We wanted to investigate whether there were any changes in the abundance of cellular proteins associated with the removal of extracellular ATP. Total soluble proteins from cells treated with glucose (control) or glucose-hexokinase were analyzed by two-dimensional difference gel electrophoresis and the BVA module of the DeCyder software (GE Healthcare). Protein spots whose abundance reproducibly increased or decreased significantly (*t* test; $P < 0.05$) by a factor of $\geq 20\%$ across five independent biological replicates in response to extracellular ATP removal were identified by mass spectrometry and database searches. A total of 34 protein spots responsive to treatment were identified via mass spectrometry, and 13 of these decreased while the rest increased in abundance (Table 1).

These results reveal that deprivation of extracellular ATP triggers a change in cellular metabolism by engendering significant alterations in the abundance of cellular proteins. The list of responsive proteins includes mitochondrial, cytosolic, chloroplast, and endoplasmic reticulum proteins (Table 1), suggesting a requirement for extracellular ATP in the maintenance of metabolic homeostasis across cellular organelles. Of note was the massive response of several spots of the stress-responsive

glutathione *S*-transferase (GST) proteins. Three spots identified as a single GST (At1g02930) were upregulated, whereas another isoform (At2g30870) found in a single protein spot was downregulated. This finding confirms at the protein level that the presence of extracellular ATP is so vital that its withdrawal elicits a stress response that results in death, as described above.

DISCUSSION

ATP Release

The existence of extracellular ATP in plants has been reported previously (Thomas et al., 2000; Jeter et al., 2004). This study has gone further and demonstrated, using radiolabeling, that ATP is rapidly synthesized and secreted in plant cell cultures and that its level is tightly regulated, presumably by a dynamic balance between active release and hydrolysis or utilization by enzymes in the extracellular matrix. This is supported by the observation that within 1 h of feeding cells with radioactive phosphate, the cells had synthesized and released radioactive ATP and its level remained unchanged 6 h later. In animal cell systems, extracellular ATP homeostasis is governed by a complex interplay of dephosphorylating and transphosphorylating ectoenzyme activities in concert with regulated release from cells. A balance between ectonucleotide pyrophosphatase and nucleoside diphosphokinase has been reported to play a role in regulating extracellular ATP in human and rat cell lines (Lazarowski et al.,

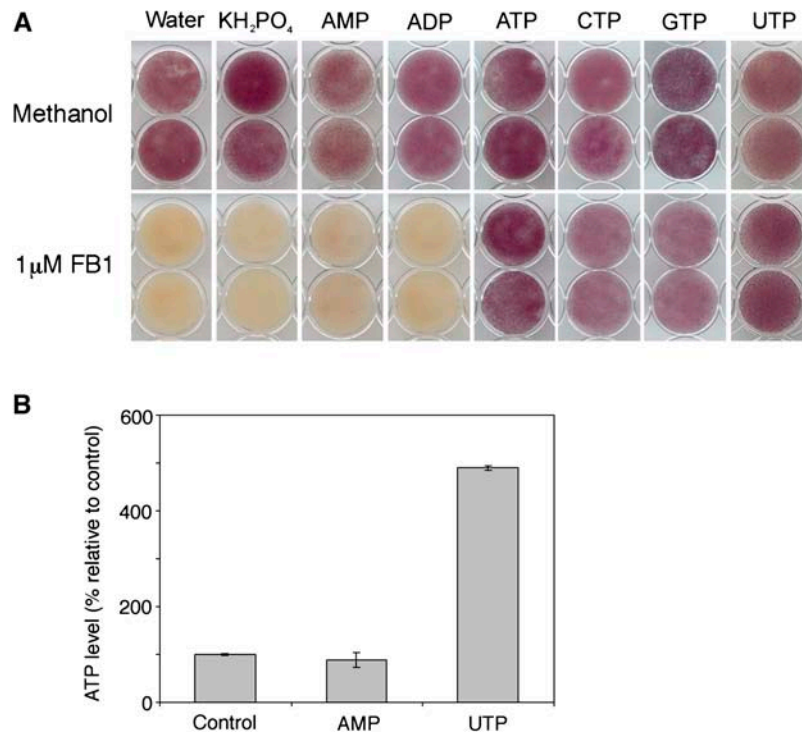


Figure 11. Nucleoside Triphosphates Rescue *Arabidopsis* Cells from FB1-Induced Death.

(A) Light-grown cell cultures adjusted to a density of 5% (w/v) were treated with either 0.014% methanol (control) or 1 μ M FB1 mixed with 1 mM of the indicated compounds. Cells were harvested 120 h later, and viability was determined via the MTT assay.

(B) Cell cultures were treated with water (control), 1 mM AMP, or 1 mM UTP and ATP in the growth medium assayed by the luciferin–luciferase method. Data and error bars represent means \pm SD ($n = 3$).

2000). Extracellular ATP level in human blood is regulated by a balance of extracellular hydrolytic activity of nucleotide pyrophosphatase and 5'-nucleotidase and the opposite extracellular ATP-generating pathway requiring ectoadenylate kinase and ectonucleoside diphosphokinase (Yegutkin et al., 2003). Although ATP hydrolytic enzymes have been identified in the plant extracellular matrix, no extracellular adenylate kinases or nucleoside diphosphokinases have been reported; therefore, a role for extracellular ATP-generating reactions in plant extracellular ATP homeostasis remains to be determined.

Extracellular ATP and Cell Viability

To find the physiological function of ATP in plants, we used a strategy of depleting the level of endogenous extracellular ATP and examining the plant tissues for any attendant responses. Our results have revealed a new role for extracellular ATP in plants, namely, the regulation of plant cell viability. Enzymatic removal of extracellular ATP was consistently followed by death, both in cell cultures and in intact plants of several species. This suggests that extracellular ATP is a crucial factor required to suppress a default cell death pathway in plants. Our results reveal a fundamental difference between the effects of extracellular ATP in plants and animals; although extracellular ATP is a crucial requirement for plant cell viability, it is a cytotoxic factor in

particular animal cells expressing P2X₇ purinoceptors (Ferrari et al., 1997; von Albertini et al., 1998).

The observation that ATP in the pericellular space is vital for sustaining cell viability is consistent with several reports demonstrating that the extracellular matrix is indispensable for cell viability in both plants and animals. For example, perturbation of *Arabidopsis* extracellular arabinogalactans using Yariv reagents induces programmed cell death (Gao and Showalter, 1999), implying that arabinogalactans are part of an extracellular matrix machinery that sustains viability during normal growth. In animals, default cell death is switched on by ablation of adrenocorticotrophic hormone, a hormone that binds to cell surface receptors and normally suppresses the death of adrenocortical cells (Wyllie et al., 1973; Negoescu et al., 1995).

The presence of such a default death pathway regulated by extracellular signal molecules secreted by cells has been demonstrated before in plants. This default death pathway is switched on when plant cells are plated below a critical cell density, but cell-free growth medium from high-density cultures rescues these cells from death (McCabe et al., 1997). The nature of these secreted signals is still unclear, but our study has identified extracellular ATP as a hitherto unknown extracellular metabolite that regulates cell death and is indispensable for viability.

However, in addition to its role in modulating cell death/viability, we predict that extracellular ATP in plants will turn out

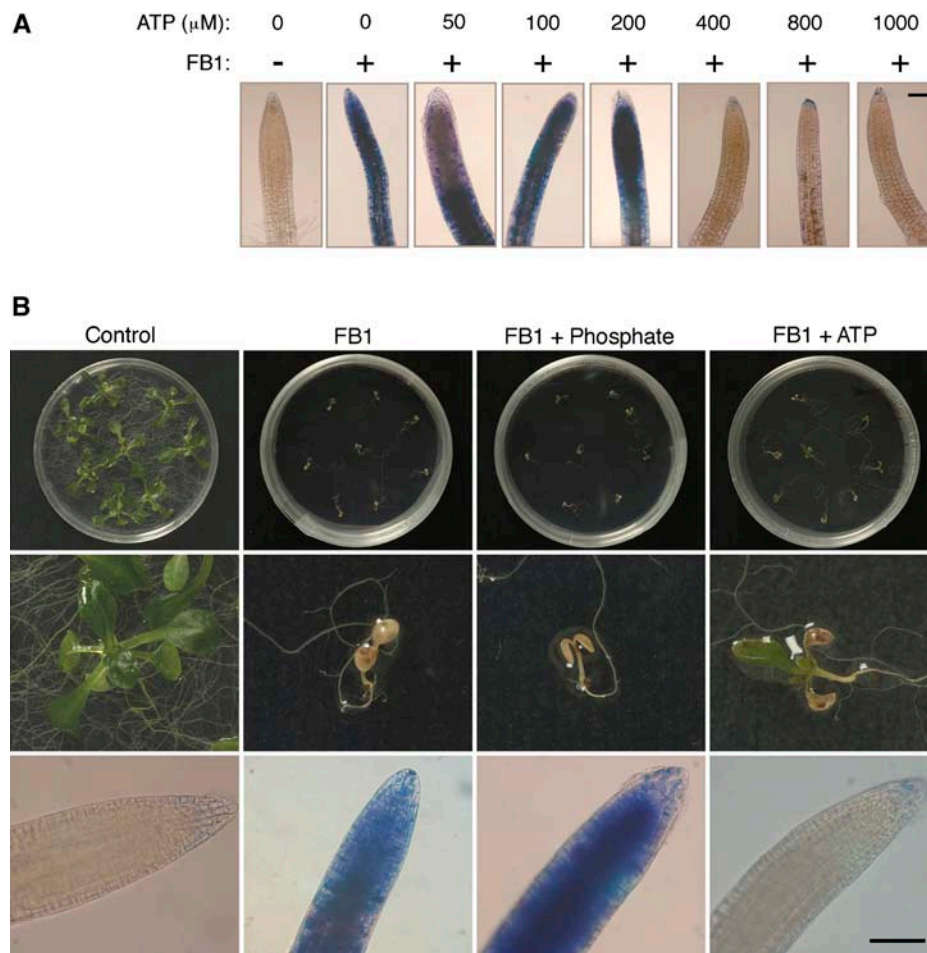


Figure 12. ATP, but Not Phosphate, Rescues *Arabidopsis* Plants from FB1-Induced Death.

(A) Hydroponic plants were treated with methanol (control) or 1 μM FB1 mixed with the indicated concentrations of ATP. Roots were stained 72 h later with Evans blue to determine viability. Bar = 1 μm ; + or – implies addition or omission of the indicated compound, respectively.

(B) *Arabidopsis* plants were germinated on nutrient agar and transferred after 5 d to plates with agar supplemented with 0.014% methanol (control), 1 μM FB1, or 1 μM FB1 mixed with either 1 mM ATP or 1 mM KH_2PO_4 . Top row, retardation of growth in all treatments containing FB1. Middle row, close-up photographs of representative plants from corresponding plates in the top row. Bottom row, root tips from hydroponic plants subjected to the same treatments for 72 h and stained with Evans blue to reveal cell death. It is clear from the middle and bottom rows that only ATP was able to rescue the plants from FB1-induced death. Bar = 1 μm for the bottom row.

to participate in numerous physiological processes depending on cell type and developmental stage, similar to the situation in animal cells (Gordon, 1986; Redegeld et al., 1999). The clues obtained from the observed effects of exogenous ATP on *Arabidopsis* will eventually lead to the discovery of more physiological functions of endogenous extracellular ATP in plants. The effects of exogenous ATP currently known include the inhibition of root gravitropism (Tang et al., 2003) and the activation of mitogen-activated protein kinases and the ethylene biosynthetic pathway (Jeter et al., 2004) in *Arabidopsis*.

How Does Extracellular ATP Sustain Cell Viability?

The mechanism by which extracellular ATP sustains the viability of plant cells is unclear, but we discuss the potential mechanisms

below. Extracellular ATP might directly or indirectly interact with an extracellular protein whose activity is vital for viability and is dependent on ATP. The interaction may serve to trigger a cascade of events that results in the inactivation of a prodeath protein by its binding to a death inhibitor protein. Removal of extracellular ATP from its extracellular interacting protein leads to the disassociation of such a complex, and the death activator triggers death. This model requires cells to perceive the presence of extracellular ATP and activate intracellular biochemical signaling events. There is already evidence that, in *Arabidopsis*, extracellular ATP activates Ca^{2+} influx (Demidchik et al., 2003; Jeter et al., 2004), a universal progenitor of intracellular signaling cascades.

ATP could possibly exert its protective effects by interacting with its extracellular target in one of three ways or a combination

Table 1. Proteins Altered in Abundance by Treatment with Glucose–Hexokinase

Spot No. ^a	Protein Name	Accession ^b	Ratio ^c
Protein folding			
632	Heat shock protein 81-2	At5g56030	−1.50
633	Heat shock protein 81-2	At5g56030	−1.50
636	Heat shock protein 81-2	At5g56030	−1.49
729	Putative heat shock protein 70	At4g24280	1.20
1063	Putative protein disulfide isomerase	At1g21750	1.21
Mitochondrial proteins			
440	Putative aminopeptidase	At1g63770	1.22
1238	Aldehyde dehydrogenase, mitochondrial	At3g48000	1.29
1242	Aldehyde dehydrogenase, mitochondrial	At3g48000	1.25
1959	Malate dehydrogenase (NAD), mitochondrial	At1g53240	1.27
Amino acid metabolism			
482	Nodulin/Gln-ammonia ligase-like	At3g53180	1.42
1135	Putative Ala aminotransferase	At1g17290	1.24
1187	Putative Ala aminotransferase	At1g17290	1.30
1098	Ketol-acid reductoisomerase	At3g58610	1.23
1516	S-Adenosylmethionine synthetase	At3g17390	−1.37
1525	S-Adenosylmethionine synthetase	At3g17390	−1.37
1570	S-Adenosylmethionine synthetase	At3g17390	−1.54
1616	Aminoacylase	At4g38220	1.29
1718	Gln synthetase-related protein	At3g17820	−1.38
Carbohydrate metabolism			
1497	Putative phosphoglycerate kinase	At1g79550	−1.41
1731	Fructose-bisphosphate aldolase	At3g52930	1.23
1817	Glyceraldehyde-3-phosphate dehydrogenase	At3g04120	1.21
1943	Malate dehydrogenase (EC 1.1.1.3)	At3g47520	1.24
2200	Phosphoglycerate mutase	At2g17280	−1.26
Antioxidative proteins			
2562	Putative GST	At2g30870	−1.29
2575	Putative GST	At1g02930	1.96
2591	Putative GST	At1g02930	3.01
2602	Putative GST	At1g02930	2.98
2605	Putative quinone reductase	At5g54500	1.69
Unclassified			
396	Putative aconitase	At4g26970	1.20
848	Putative ferredoxin–nitrite reductase	At2g15620	1.21
906	Vacuolar ATPase, A subunit	At1g78900	1.21
1200	Tubulin α -3/ α -5 chain	At5g19770	−1.66
2282	Expressed protein	At3g22850	−1.29
2370	ATP-dependent Clp protease, proteolytic subunit	At1g09130	−1.71

^aSpot number on the master gel.^bMunich Information Center for Protein Sequences database accession number.^cPaired average ratio: treatment:control for upregulation, control:treatment for downregulation. A minus sign denotes a decrease in abundance.

of them. First, it could provide its γ -phosphate for phosphorylating a target protein. This notion is supported by the observation that overriding the binding of extracellular ATP to targets by a nonhydrolyzable analog induced death. A simplistic prediction would be that extracellular ATP is required for phosphorylating some extracellular or membrane-bound pro-death substrate that becomes inactivated when phosphorylated. An example of this type of regulation is the phosphorylation-dependent posttranslational modulation of plant nitrate reductase activity. Inhibitory 14-3-3 proteins bind to and inactivate only phosphorylated nitrate reductase but neither bind nor inhibit nonphosphorylated forms of the same protein (Kaiser and Huber, 2000; Lillo et al., 2004). Although the presence of extracellular phosphoproteins has been reported (Chivasa et al., 2002; Ndimba et al., 2003), plant ectoprotein kinases responsible for the phosphorylation have yet to be identified. Second, extracellular ATP could act as a ligand, binding directly to the extracellular domain of a transmembrane receptor similar to animal P2X and P2Y receptors. There is biochemical and electrophysiological evidence for the presence of P2X-type receptors in plants, but none has been identified to date. Third, the full ATP molecule may mediate the binding of two extracellular proteins. For example, the neuronal apoptosis inhibitor protein (NAIP) undergoes a conformational change that enables it to bind to caspase-9 only when there is an ATP molecule in its nucleotide binding domain (Davoodi et al., 2004). Binding of NAIP to caspases prevents default cell death in neurons (Maier et al., 2002).

Results obtained with the nonhydrolyzable ATP analog indicate that ATP cleavage is required for cell viability. The potential role of P2X- or P2Y-type receptor activation and the possibility that ATP functions as an adaptor for binding cognate protein partners during the initiation of signaling cascades to sustain cell viability will require further investigation. The primary target protein/molecule for ATP in the extracellular milieu is not known, and its discovery will lead to the identification of components of this pathway. There is precedence from animal cells for individual extracellular proteins to sustain viability by inhibiting default cell death. For example, neutrophin-3, a secretory protein that binds to cell surface receptors of neuronal cells, is required to sustain their viability, and its gene deletion or blockade by specific antiserum triggers a default programmed cell death response (Zhou and Rush, 1995). Unraveling the mechanism by which extracellular ATP sustains plant cell viability is now the subject of further experimental investigations by our group.

Changes in Protein Abundance in Response to Extracellular ATP Removal

To elucidate the molecular basis for the ability of ATP to sustain plant cell viability, we used the glucose–hexokinase system to deplete extracellular ATP and analyzed attendant changes in the proteome of *Arabidopsis*. We found that removal of ATP in the pericellular space perturbed the steady state levels of a diverse range of cellular proteins in various organelles and numerous biochemical pathways. These changes in intracellular protein abundance in response to extracellular ATP removal suggest

that signals initiated by extracellular ATP perception funnel into numerous intracellular metabolic pathways spanning several cellular compartments. This reinforces, at the molecular level, the finding that cells are dependent on extracellular ATP for viability.

Previous studies of plant programmed cell death responses have identified changes in the expression of genes involved in primary metabolism, molecular chaperones, and other enzymes regulating protein folding, mitochondrial proteins, and antioxidative stress proteins (Desikan et al., 1998; Swidzinski et al., 2002, 2004). *Arabidopsis* microarray data available via the Genevestigator database (<http://www.genevestigator.ethz.ch>) and web browser data-mining interface (Zimmermann et al., 2004) reveal that most of the genes for the proteins identified in this study are differentially expressed in response to a diverse range of death-inducing treatments such as herbicides, toxins, and attack by bacterial and fungal pathogens. This finding suggests that cell death induced by extracellular ATP depletion possibly funnels into a shared core death pathway at some point downstream from the position where ATP deprivation is crucial. The concept of a shared core death machinery into which disparate signaling pathways activated by various pro-death stimuli feed and converge has been proposed before (McCabe and Leaver, 2000). This is similar to the sensory and signaling cascades in the cyanobacterial stress response, in which various stresses have unique components close to the initial stimulus sensing but share common components of the signaling cascades (Marin et al., 2003).

The strongest response was observed in GSTs, a family of stress-responsive proteins whose gene expression can be induced by pathogen attack (Lieberherr et al., 2003) or treatment with pathogen-derived elicitors (Desikan et al., 1998). Wagner et al. (2002) reported that salicylic acid suppresses gene expression of the GST (At2g30870) whose abundance at the protein level decreased in response to extracellular ATP deprivation in this study. Gene expression of the GST protein (At1g02930) upregulated by ATP removal in our study is also activated by endogenous signaling molecules such as H₂O₂, salicylic acid, ethylene, and jasmonic acid (Wagner et al., 2002). This suggests that the cell death activated by the removal of extracellular ATP may potentially be mediated by some of these signaling molecules. Indeed, it is already known that FB1, which triggers death via extracellular ATP removal, induces the accumulation of reactive oxygen species in *Arabidopsis* and that FB1-induced death in the same species requires ethylene, salicylic acid, and jasmonic acid signaling (Asai et al., 2000).

Depletion of Extracellular ATP Mediates Elicitor-Induced Cell Death

Cell death is an essential part of plant development that is regulated by a programmed genetic template and affects single cells, particular cell layers, or entire organs (Buchanan-Wollaston, 1997; Fukuda, 1997; Groover et al., 1997) and is invoked in the hypersensitive response to pathogen attack (Lam et al., 2001). Having established that extracellular ATP is vital to sustain cell viability and that artificially removing it triggers cell death, we

discovered that depletion of extracellular ATP mediates an elicitor-induced cell death response. FB1, a mycotoxin that acts as an elicitor in *Arabidopsis* and triggers defense responses and cell death, induced a heightened hydrolysis of extracellular ATP preceding the breach in plasma membrane integrity and the onset of death. Crucially, exogenous ATP was able to rescue plants treated with the elicitor from death. Elicitor-induced cell death requires an influx of apoplastic Ca²⁺ into the cell, and its chelation can block cell death (Levine et al., 1996). However, the effect of exogenous ATP on FB1-induced death is unlikely the result of chelation of divalent cations because 1 mM solutions of AMP and ADP, which have a higher chelation capacity than 200 μM ATP, failed to abolish cell death when 200 μM ATP blocked it. In cell cultures, prevention of ATP depletion by adding exogenous ATP concurrently with FB1 or correcting the deficit by the addition of exogenous ATP several hours later, up to 40 h, was able to prevent death. When added 48 h after FB1 or later, exogenous ATP was unable to rescue cells from death, implying that commitment to death occurs ~48 h from FB1 addition.

In cell cultures, it is clear that FB1 upregulates an extracellular ATP hydrolytic activity after a lag of ~16 h. This activity does not discriminate between different nucleoside triphosphates and appears not to act on nucleoside monophosphates and diphosphates, as shown by the failure of AMP and ADP to block FB1-induced death, which was abolished by all of the tested nucleoside triphosphates. The observation that a pathogen elicitor stimulates extracellular nucleoside triphosphatase activity is not unprecedented because elicitors have been reported to activate membrane-bound and cell wall-bound ATPase activity (Kiba et al., 1995, 1999). The exact mechanism by which FB1 activates extracellular ATP hydrolysis is not yet defined. However, plant extracellular ATP hydrolysis is performed by ecto-apyrases (Komoszynski and Wojtczak, 1996), cell wall-bound ATPases (Kivilaan et al., 1961; Shiraiishi et al., 1991), and extracellular purple acid phosphatases (Bozzo et al., 2002; Olczak et al., 2003). Perhaps FB1 stimulates the activity of a similar extracellular enzyme, as has been reported for other elicitors. For example, chitin, a pathogen-derived elicitor known to trigger cell death (Tada et al., 2001), has been reported to specifically bind and stimulate the activity of an extracellular apyrase (Etzler et al., 1999). Recently, a fungal pathogen elicitor was reported to stimulate a recombinant plant apyrase *in vitro* (Kawahara et al., 2003). However, the delay between FB1 addition and the activation of extracellular ATP hydrolysis might be an indication that FB1 does not directly activate the enzymes but requires *de novo* synthesis of secondary messengers or, alternatively, that synthesis and secretion of the hydrolytic enzymes occur during the lag period.

Our results allow a rationalization of extracellular ATP, cell death, and the response of plant cells to some pathogen elicitors. Elicitors clearly affect cell viability (Levine et al., 1994) and have been reported to activate enzymes that modulate the level of extracellular ATP (Kiba et al., 1995; Etzler et al., 1999; Kawahara et al., 2003). Further studies on a wide range of elicitors and incompatible host-pathogen combinations are now required to investigate whether or not hypersensitive cell death in general is mediated via extracellular ATP depletion.

METHODS

Plant Material and Growth Conditions

Cell suspension cultures of *Arabidopsis thaliana* var *erecta* were maintained as described previously (May and Leaver, 1993). Maize (*Zea mays*) cell cultures were maintained by weekly 10-fold dilution in fresh Murashige and Skoog (MS) growth medium (Murashige and Skoog, 1962) with 2% (w/v) sucrose and 2 mg/L 2,4-D. Suspension cell cultures of both species were grown either in continuous darkness or with a 16-h photoperiod at 25°C and were used for treatment 3 to 5 d after transfer to fresh medium. Dark-grown cell cultures were used in all experiments except for those involving FB1 treatments, in which light-grown cell cultures were used because FB1-induced cell death in plants is dependent on light (Asai et al., 2000; Stone et al., 2000). Glucose-hexokinase treatments were performed with both light- and dark-grown cell cultures. Tobacco (*Nicotiana tabacum*), Landsberg *erecta* and Columbia ecotypes of *Arabidopsis*, and bean (*Phaseolus vulgaris*) plants for injecting with solutions were grown in soil in a growth cabinet with a 16-h photoperiod at 20°C and 8 h of darkness at 15°C. RH was maintained at 60%, and the photon flux density was 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Plants were used for treatment 5 to 8 weeks after sowing.

Treatments

Enzymes were dissolved in water and filter-sterilized (0.2 μm). Dialysis was performed against water using membranes with a molecular mass cutoff of 8 kD. AMP, ADP, ATP, and AMP-PCP stock solutions in water were neutralized to pH 7.0 using KOH and filter-sterilized. Whole plants were treated by infiltration of the apoplast of a small area or entire leaf on the abaxial surface using a syringe with or without a hypodermic needle. Concentrations used to treat intact plant tissues were 6 mg/mL BSA, 1 mM AMP, 1 mM ADP, 5 mM ATP, 5 mM AMP-PCP, 50 mM glucose, 1 mM glucose-6-phosphate, 0.5 units/ μL apyrase, and 1.85 units/ μL hexokinase. In cell culture experiments, glucose was used at a concentration of 100 mM. Cell cultures were incubated with the appropriate solutions in a final volume of 1.5 mL and a cell density of 5% (w/v). After 72 h, the cultures were diluted to 50 mL in fresh growth medium. Ninety-six hours later, 1-mL aliquots were withdrawn and the packed cell volume was determined as a percentage of the culture volume. A 7 mM stock solution of FB1 was prepared in absolute methanol, and a working solution of 50 μM (diluted in water) was used for treatments at a final concentration of 1 μM , with an equivalent volume of 0.71% methanol being used to treat controls.

Hydroponic Experiments

Floating Plants

Seeds of *Arabidopsis* ecotype Columbia were surface-sterilized as described previously (Topping and Lindsey, 1991) and sown on 7-cm-diameter, 1-cm-thick polyurethane foam discs soaked in MS medium (0.22% [w/v] MS salts, 1% [w/v] sucrose, adjusted to pH 5.7 with KOH/HCl). The discs were placed in sterile phytatrays (Sigma-Aldrich) and incubated under a 16-h-light/8-h-dark cycle at 22°C. One week later, when the seeds had germinated and the roots had penetrated the foam, 30 mL of MS medium was added to the phytatray, which caused the foam discs to float. The trays were transferred to a shaking platform (25 rpm) under the same environmental conditions. During the second week, the roots of the plants had emerged through the other side of the foam and were submerged in the nutrient-rich medium. Exactly 2 weeks after sowing, the plants were treated with water (control), 45 mM glucose, 45 mM glucose + 1 mM glucose-6-phosphate, and 45 mM glucose + 100 units/mL hexokinase.

Submerged Plants

Surface-sterilized *Arabidopsis* seeds were incubated in 5 mL of MS medium in 25-mL conical glass flasks on an orbital shaker (120 rpm) at 22°C in a 16-h photoperiod regime. Five days later, the plants were treated with 1 μM FB1 and 1 μM FB1 mixed with either 1 mM ATP or 1 mM KH_2PO_4 . At 72 h after treatment, the plants were stained with Evans blue dye as described below.

Agar Plate Assays

Surface-sterilized *Arabidopsis* seedlings were plated on 0.4% (w/v) agar-containing MS medium. Five days later, the plants were transferred to agar plates supplemented with 1 μM FB1 or 1 μM FB1 + 1 mM ATP. The plants were photographed 4 weeks later. This experiment was performed using both the Landsberg *erecta* and Columbia ecotypes of *Arabidopsis*.

Microscopy

Roots of *Arabidopsis* treated with glucose or glucose-hexokinase were stained with fluorescein diacetate and examined using a LSM 510 Meta microscope (Carl Zeiss). Autofluorescence was acquired at an excitation wavelength of 543 nm and an emission long pass of 560 nm. The fluorescence of fluorescein diacetate was detected at an excitation wavelength of 488 nm and an emission band pass between 505 and 530 nm. The same tissues were also imaged using transmitted light phase contrast, and all three images were merged.

Cell Death Assay

Determination of cell viability by the MTT assay was performed as described previously (Watts et al., 1989) with minor modifications. An aliquot of 400 μL of 5 mg/mL MTT solution in water was incubated with 5 mL of cell culture for 4 h. A lawn of cells was placed in wells of a microtiter plate and scanned to show the color development.

Evans blue assay for the death (and plasma membrane integrity) of cell cultures was performed by incubating triplicate 100- μL cell aliquots with a final 0.0125% (w/v) Evans blue solution for 20 min. Cell death levels in the treatments were obtained by spectrophotometrically (600 nm) determining the amount of dye taken up by cells and assuming that control cells were 100% viable and cells boiled for 5 min before staining were 0% viable. For hydroponic plants, five plants in 5 mL of 0.04% (w/v) Evans blue solution were vacuum-infiltrated by holding the vacuum for 30 min and then washed three times with water for 4 h each wash.

ATP Measurements

De novo synthesis and secretion of ATP were performed by feeding 100 μCi of [^{32}P]H₃PO₄ to 5 mL of 3-d-old light-grown cell cultures adjusted to a density of 1% (w/v). Cell-free 170 μL of medium was withdrawn 1 h later and acidified with 0.6% trichloroacetic acid. Aliquots of 2 μL were mixed with an equal volume of 50 mM citrate buffer, pH 5.5, and separated by thin layer chromatography (TLC) on silica gel plates with dioxane:ammonium hydroxide:water (6:1:6, v/v/v). To demonstrate the activity of apyrase and hexokinase, cell cultures labeled with a similar amount of [^{32}P]H₃PO₄ for 1 h were treated with 100 units/mL apyrase or 100 mM glucose + 200 units/mL hexokinase for a further 6 h, and medium samples were analyzed by TLC. Apyrase at 20 units/mL was also used in parallel experiments.

To monitor extracellular ATP during FB1 treatment, 5 mL of light-grown cell cultures was adjusted to 1% (w/v) density, treated with 0.014% (v/v) methanol as a control or 1 μM FB1, and spiked with 4.5 μCi [γ - ^{32}P]ATP

16 h later. Cell-free medium aliquots were withdrawn periodically and analyzed by TLC as described above.

In other experiments, medium ATP was measured by the nonradioactive luciferin-luciferase method using the Enlighten ATP assay kit (Promega). Cell-free 100- μ L medium aliquots were mixed with 2 μ L of 50% (w/v) trichloroacetic acid containing 0.0005% (w/v) acid blue 147 and stored at -20°C . For ATP assay, 5 μ L was mixed with 95 μ L of 100 mM Tris-acetate buffer, pH 7.8, and the reaction was started by the addition of 30 μ L of luciferin-luciferase followed by a reading delay of 0.3 s and an integration time of 10 s using the Anthos Lucy 1 luminometer (Labtech International).

Proteomic Analyses

Aliquots of 5 mL each of 3-d-old, dark-grown *Arabidopsis* cell cultures were treated with 100 mM glucose (to serve as controls) or a combination of 100 mM glucose plus 200 units/mL hexokinase. The experiment was performed using five independent biological replicates. Cells were harvested 48 h later, and protein was extracted and analyzed by two-dimensional difference gel electrophoresis as described previously (Ndimba et al., 2005). Gel images were scanned using the Typhoon 9400 scanner (GE Healthcare), and image analysis was performed using the DIA and BVA modules of the DeCyder software (GE Healthcare) as described before (Ndimba et al., 2005). Proteins whose abundance changed (up or down) by a minimum of 20% were excised from silver-stained gels, with a total protein loading of 400 μ g, and identified via mass spectrometry and database (National Center for Biotechnology Information nonredundant database) searches using the methods described by Chivasa et al. (2002).

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