Soil-borne fungi alter the apoplastic purinergic signaling in plants by deregulating the homeostasis of extracellular ATP and its metabolite adenosine

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ABSTRACT
Purinergic signaling activated by extracellular nucleotides and their derivative nucleosides trigger sophisticated signaling networks. The outcome of these pathways determine the capacity of the organism to survive under challenging conditions. Both extracellular ATP (eATP) and Adenosine (eAdo) act as primary messengers in mammals, essential for immunosuppressive responses. Despite the clear role of eATP as a plant damage-associated molecular pattern, the function of its nucleoside, eAdo, and of the eAdo/eATP balance in plant stress response remain to be fully elucidated. This is particularly relevant in the context of plant-microbe interaction, where the intruder manipulates the extracellular matrix. Here, we identify Ado as a main molecule secreted by the vascular fungus Fusarium oxysporum. We show that eAdo modulates the plant’s susceptibility to fungal colonization by altering the eATP-mediated apoplastic pH homeostasis, an essential physiological player during the infection of this pathogen. Our work indicates that plant pathogens actively imbalance the apoplastic eAdo/eATP levels as a virulence mechanism.

ONE SENTENCE SUMMARY
The apoplastic Adenosine/ATP balance is a messenger for plant defense and can be manipulated by the fungal pathogen F. oxysporum.

INTRODUCTION
Adenosine-5'-triphosphate (ATP) constitutes the energy currency of all living organisms and is the driving force of many cellular processes. In addition, it fulfills a broad range of tasks in signaling mechanisms once it leaves the cytosol and becomes extracellular (eATP). In plants, eATP contributes to root hair growth, gravitropism, cell death, and to response to abiotic and biotic stresses (1–4). ATP reaches the apoplast through transporters (5, 6) and secretory vesicles (7). Since eATP is involved in a broad selection of signaling processes, tight controlling mechanisms are required to regulate its concentration. These comprise apoplast facing apyrases and purple acid phosphatases that hydrolyze eATP to adenosine monophosphate (AMP) (8, 9). AMP is subsequently hydrolyzed by 5' nucleotidases (5'NT) to extracellular adenosine (eAdo) (8) that is either taken up into to cytoplasm by the EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 3 (ENT3) (10), or further processed by the extracellular protein NUCLEOSIDE HYDROLASE 3 (NSH3) (11). NSH3 removes the sugar moiety of eAdo and generates adenine (Ade), which is transported into the cytoplasm by a purine permease transporter (PUP) (12).

Mechanical wounding of the plasma membrane leads to a high release of ATP to the apoplast (3), increasing eATP concentration up to 80 nM (13), which is sufficient to activate the purinoreceptor DOES NOT RESPOND TO NUCLEOTIDES 1 (P2K1/DORN1) also known as the LecRK-I.9 (lectin receptor kinase I.9) (Kd ~ 46 nM; (4)). Perception of eATP by DORN1/LecRK-I.9 induces cellular responses including increase of cytoplasmic Ca2+ concentrations and of reactive oxygen species (ROS), activation of MAPK cascades by phosphorylation, and transcriptional reprogramming (14). Indeed, 60% of genes differentially regulated after application of exogenous ATP are differentially expressed during wounding processes (4). The role of eATP as a damage-associated molecular pattern (DAMP) is supported by the susceptibility of dornl mutants to different pathogens and their lower response to a beneficial endophyte (15–17).

In mammals, Ado is also recognized as a primary messenger, being a key signal of immunosuppressive responses after being perceived by G-protein-coupled receptors (18, 19). In plants, Arabidopsis ent3nsh3 double mutant, affected in the Ado/ATP ratio at the apoplast, showed increased susceptibility to the necrotroph ascomycete Botrytis cinerea, connected to an attenuation of the upregulation of defense related genes (20, 21). In addition, the beneficial root fungal endophyte Serendipita indica secretes ecto-5'-nucleotidases (E5’NT) that, like plant 5’NTs, are capable of hydrolyzing eATP and thereby shifting the
equilibrium in the apoplast from eATP to eAdo (16). Together, these data suggest that the eATP/eAdo balance is relevant for fungal infection and that microbes might manipulate the apoplastic Ado levels in its favor, as they do with the apoplastic pH (pH_{apo}) (22–24). However, the roles of eAdo and of the eATP/eAdo equilibrium in plant defense remain poorly understood.

_Fusarium oxysporum_ (Fo) is one of the plant pathogenic fungi whose capacity to induce pH_{apo} changes is best studied (23, 24). As a microbe that mainly grows and advances through the apoplast, it represents an excellent model system to study plant-microbe molecular communication in this plant region. We thus made use of an elicitor mix, a crude mycelia extract of a lyophilized Arabidopsis pathogen Fo5176 suspension. This extract led to rapid and local alterations of the pH_{apo} and cellulose synthesis machinery similar to those observed by the fungal hyphae (24). Through fractionation followed by HPLC purification of the elicitor mix, we identified Ado as an abundant and active molecule in the Fo5176 elicitor mix. Our data indicate that Fo5176 increases the levels of eAdo in the apoplast to facilitate its growth in the host. Genetic, transcriptomic, and live-cell high-resolution microscopy approaches revealed that Ado alters ATP-induced plant defense responses.

RESULTS

**Fo5176 secretes Ado that seems to counteract eATP-induced plant defense**

We recently showed that a Fo5176 elicitor mix regulates the growth-defense balance in plants (24). To identify the molecules in the elicitor mix involved in this response, we performed a bioassay-guided fractionation, using a C18 solid phase cartridge with a 10% step gradient of a water:methanol solvent system followed by HPLC on a semiprep C18 column. This approach yielded a purified active component that we identified as adenosine (Ado) by standard 1D and 2D NMR (Table 1) and high-resolution mass spectrometry. Comparison of its retention time and mass data to a pure commercial standard, confirmed that Ado is a main component of elicitor mixes generated from _in vitro_-grown Fo5176 (Figure 1A). We then asked whether the fungus secretes this potential new elicitor during plant infection. As the plant or fungal origin of the extracellular Ado (eAdo) present in the host apoplast cannot be distinguished, we tested the expression of genes required for hydrolysis and secretion of eAdo, ENT and 5’NT, respectively, in the host and the intruder during their interaction. Both FoENT and Fo5’NT were significantly upregulated during root colonization (Figure 1B and C), while the expression of the Arabidopsis’ homologs was not altered by the presence of the fungus (Figure 1-Figure supplement 2). These data are supported by the identification of Fo5’NT protein (g8638) in the secretome of Fo5176-infected roots of hydroponically-grown Col-0 plants (25), indicating that Fo5176 might indeed secrete Ado to the apoplast while colonizing plant roots.

Considering the biochemical relation between Ado and ATP and the reported role of eATP in plant immunity (2, 14, 26), we tested the putative influence of eAdo on ATP-induced plant defense. Thus, we first investigated if the plant response to Fo5176 is eATP-dependent by exposing the plants to different concentrations of ATP (10 µM to 500 µM) while infected by Fo5176, as described previously (24, 27). Indeed, 300 - 500 µM ATP significantly reduced Fo5176 vascular colonization (Figure 1-Figure supplement 3A). To assess the effect of eAdo on ATP-induced plant defense, we exposed the roots to 500 µM ATP and Ado in equimolar to doubled concentrations of ATP (Figure 1-Figure supplement 3B). Plants treated with 1 mM Ado and 500 µM eATP were indistinguishable from control plants regarding vascular penetrations by Fo5176 (Figure 1D and S2B). Ado on its own did not have any detectable effect on fungal vascular penetration under our experimental conditions, indicating that Ado plays an important role in the plant eATP signaling regulation (Figure 1D). Importantly, eATP-induced root and fungal growth inhibition was not recovered by the addition of Ado (Figure 1E-G). These results indicate that the plant response to Ado is ATP-dependent and implicate a mechanism in which eAdo interferes with eATP-induced plant defense responses that is not based on plant- or fungal-growth retardation.

Plants impaired in ATP sensing or with high eAdo/eATP levels are more susceptible to Fo5176
To further test the role of Fo-secreted Ado (Figure 1A-C) interfering with eATP during plant-pathogen interaction, we aimed at creating fungal mutants lacking FoE5'NT and FoENT. Although more than 165 potential transformants showed successful insertion of the resistance cassette into the fungal genome, none of them were knock-out mutants of the target genes, i.e. the cassette was inserted off-target. This indicates the importance of these genes for fungal viability and the very possible lethality of FoΔE5'NT and FoΔENT mutants. As manipulating the fungal eAdo levels was not successful, we addressed the influence of eAdo/eATP on plant-pathogen interactions from the plant’s side using an Arabidopsis mutant altered in this ratio (ent3nsh3(21)) or eATP sensing (dorn1)(4). Double mutant ent3nsh3 plants showed an increased susceptibility to fungal colonization, while the single mutant ent3 was not significantly affected in its response to the pathogen (Figure 2A). Lack of DORN1 caused an increased fungal vascular penetration rate (Figure 2A), underlining the role of eATP as a DAMP in Arabidopsis-Fo5176 interaction and confirming previous data (26). Compared to Fo5176-treated WT, dorn1 and ent3 plants showed significantly increased primary root growth over time, while ent3nsh3 did not differ substantially from WT, despite its higher infection numbers (Figure 2B and C). These data indicate that the anticipated elevated apoplastic Ado/ATP ratio in enth3nsh3 (21) might have a main role in enhancing plant colonization by Fo5176. To test this hypothesis, we measured both soluble Ado and ATP levels in the media of hydroponically-grown plants in control and Fo5176-infected conditions, as proxy for the levels of those molecules in the apoplast. As expected, the growth media of infected ent3nsh3 plants showed significantly elevated Ado levels in comparison to mock treatments, which were not observed in WT or dorn1 plants (Figure 2-Figure supplement 2). In addition, we observed significantly lower amounts of ATP in ent3nsh3 mock-media compared to all other tested genotypes (Figure 2-Figure supplement 2), indicating that this mutant has a higher eAdo/eATP ratio than WT in control conditions that is preserved upon Fo5176 infection due to the increase on eAdo (Figure 2D).

eAdo increases the rapid ATP-induced transient cytosolic Ca^{2+} peak

To molecularly characterize the high susceptibility of dorn1 and ent3nsh3 to Fo5176, we explored earlier cellular immune responses, starting with the eATP-induced cytosolic Ca^{2+} (cytoCa^{2+}) peak (4). Employing the ratiometric cytoCa^{2+} sensor R-GECO1-mTurquoise (28), we first determined the minimal ATP concentration that led to a consistent increase of intracellular Ca^{2+} in the meristematic and elongation zone of Arabidopsis WT roots. 10 µM ATP were enough to consistently induce a cytoCa^{2+} peak (Figure 3A and B), as previously reported (29). After introgressing R-GECO1-mTurquoise into both mutant lines, we found that addition of ATP to ent3nsh3 led to a Ca^{2+} spike in the first 3 min, 1.5 times higher than that detected in WT, which decays to WT levels, indicating that an increased eAdo/eATP proportion might modulate rapid eATP-induced responses (Figure 3A-D). Consistent with the role of DORN1 as the main eATP receptor, we detected no cytoCa^{2+} peak in dorn1 upon ATP treatment (Figure 3E and F). Next, we investigated if the external addition of Ado can interfere with this signaling process by testing various Ado concentrations (Figure 3-Figure supplement 2A-D). While Ado did not induce any changes in the cytoCa^{2+} levels up to a concentration of 200 µM, we detected that Ado enhanced the eATP-induced cytoCa^{2+} spike transiently when the ATP:Ado ratio was at least 1:5 (Figure 3B). The cytoCa^{2+} spike did not further increase in response to higher eATP:eAdo ratios (Figure 3-Figure supplement 2A-D). Accordingly, the high eATP-induced cytoCa^{2+} peak observed in ent3nsh3 did not further increase by adding Ado (Figure 3C and D; Figure 3-Figure supplement 2E-G). These data indicate that chemical or genetic enhancement of eAdo/eATP rapidly increases the eATP-induced transient cytoCa^{2+} peak up to a certain eATP:eAdo concentration ratio. Moreover, we observed that eAdo could not alter the lack of response of dorn1 to ATP (Figure 3E and F; Figure 3-Figure supplement 2H-I), confirming that the plant response to Ado is ATP-dependent.

eAdo alters the ATP-induced apoplast alkalization

Exogenous application of ATP induces apoplast alkalization, as part of the fast plant response to DAMPs (30, 31). Hence, we investigated if, as we observed for the cytoCa^{2+} peak, eAdo also influences the ATP-dependent apoplastic pH (pH_{apo}) changes. By imaging the ratiometric pH_{apo} sensor SYP122-pHusion (24)
in WT roots, we confirmed that the apoplast alkalizes in response to the same ATP concentration required to induce a cytosolic Ca\(^{2+}\) peak (10 µM; Figure 4A-Figure supplement 2A), which we used concurrently for all further experiments. Analogous to the effect on cytosolic Ca\(^{2+}\) levels, Ado did not affect the pH\(_{\text{apo}}\) on its own even at concentrations of 200 µM, while it altered the plant response when combined with eATP starting at 1:5 eATP:eAdo ratio (Figure 4 and S5). eAdo concentrations up to 50 µM counteracted the eATP-induced apoplastic alkalization, while 200 µM eAdo enhanced the eATP-dependent pH\(_{\text{apo}}\) peak (Figure 4A and B, Figure 4-Figure supplement 2A). On the other hand, ent3nsh3 mutants showed comparable pH\(_{\text{apo}}\) response to ATP as observed in WT roots, a response that was not altered by the Ado treatment (Figure 4C and D, S5B). These results indicate that the high eAdo/eATP ratio in ent3nsh3 apoplast cannot alter the plant response to eATP regarding pH\(_{\text{apo}}\) changes but block the effect of exogenous Ado. In this context it has to be highlighted that ent3nsh3 mutants already show an elevated apoplastic pH under physiological conditions (pH = 6.00) in comparison to WT (pH = 5.54) (Figure 4-Figure supplement 2D). Unexpectedly, dorn1 responded to eATP with a slight, but significant, pH\(_{\text{apo}}\) decrease that was restored to control levels by eAdo (Figure 4E and F; S5C). Our data indicate that ATP induces a DORN1-independent apoplastic acidification, which seems to be counteracted by eAdo. Moreover, dorn1 roots also showed a more alkaline apoplast than WT under control conditions, as detected in ent3nsh3 (Figure 4-Figure supplement 2D), hinting at a disturbed proton homeostasis in both mutants.

The expression of Arabidopsis defense genes in response to Fo5176 is eATP/eAdo-dependent

To further investigate the influence of Fo5176 on the activation of eATP/eAdo-dependent plant immune responses, we measured the expression of four defense-related genes upon Fo5176 infection. In agreement with the function of eATP as DAMP, DORN1 expression increased in WT infected-roots but was significantly downregulated in ent3nsh3 mutant plants upon Fo5176 colonization (Figure 5). The expression of three genes previously reported to be activated in Fo5176-infected Arabidopsis roots; WRKY45, WRKY53, and At1g51890 (23–25); followed a similar pattern as they were all upregulated in response to Fo5176 in WT plants but not in dorn1 or ent3nsh3 mutants (Figure 5). These data confirm that Fo5176 induces a eATP/eAdo-dependent plant immune response that might explain the high susceptibility of dorn1 and ent3nsh3 mutants to the fungus.

DISCUSSION

Ado is known as a key extracellular mediator of the animal immune response and its molecular activity in relation with eATP is increasingly recognized (19, 32). However, the knowledge of its role in plant-microbe interaction is very scarce. In this work, we show that an increased apoplastic Ado/ATP ratio enhanced plant susceptibility to the soil-borne pathogen Fo5176 and that the fungus benefits from this effect by modifying its metabolism in planta to raise the Ado concentration in the apoplast.

We identify Ado as a main elicitor of Fo when grown in vitro (Figure 1A). The transcriptional upregulation of the fungal but not of the plant eAdo producing molecular machinery during infection and fungal secretion of Fo5′NT during root colonization indicates that Fo exudes this molecule when colonizing roots (Figure 1B and C, S1, Figure 6). Therefore, our data expand the current knowledge on apoplastic effectors secreted by plant fungal pathogens (23, 33, 34). The presence of Ado in the media during Fo colonization of Arabidopsis roots did not alter the host-microbe interaction on its own, but blocked the ATP-induced plant resistance, while not affecting fungal growth (Figure 1D-G, S2). Hence, we deduce that eAdo interferes with the ATP-induced plant immune system activation upon fungal infection. Genetically encoded plant sensors for cytosolic Ca\(^{2+}\) levels and apoplastic pH confirmed that 10µM ATP induces pattern-triggered immunity in Arabidopsis roots, as we could detect an immediate cytosolic Ca\(^{2+}\) peak and apoplastic alkalization in response to this molecule (Figure 3A-B and 4A-B). Importantly, the application of 50µM Ado further enhanced the transient but not the sustained Ca\(^{2+}\) influx response elicited by ATP, while Ado alone did not induce any plant response different from the control treatment (Figure 3A and B). A comparable mechanism was already discovered in oviductal ciliated cells...
in which adenosine itself is inactive but increases ATP induced calcium influx through activation of protein kinase A (35). The same Ado concentration efficiently blocked the ATP-induced alkalization of the root apoplast, while Ado levels above a certain threshold significantly increased the ATP effect on apoplastic pH without any effect on the sustained Ca\(^{2+}\) influx (Figure 4A and B, S4). As apoplastic alkalization has been reported to be required for Fo pathogenesis (23, 24), our data indicate that eAdo hinders ATP-induced plant resistance above a certain eATP/eAdo ratio by boosting the ATP-induced apoplastic alkalization (Figure 6). Our short-term response data suggest that the rapid apoplastic alkalization generated by eATP is not a main contributor to plant defense against Fo5176, as (1) eAdo boosts this plant response potentially leading to increased fungal virulence (Fig 1D and 4B), and (2) eATP also generates a pH\(_{apo}\) peak in the Fo5172 susceptible mutant ent3nsh3, similar to WT plants. It was previously reported that eATP induces defense-related gene expression independently of its effect on media pH (Jewell et al., 2019). However, more research is needed to clarify the relationship between pH changes and the defense mechanism triggered by eATP, and MAMPs/DAMPs/elicitors in general, in the process of an infection. The elevated pH\(_{apo}\) of dornl under mock conditions might indicate a positive regulatory function of signal transducers downstream of DORN1 on H\(^{+}\)-ATPases (Figure 3-Figure supplement 2D). The reason for an ATP-dependent decrease in pH\(_{apo}\) in dornl mutants remains to be elucidated, although another target of eATP might be involved in that response.

Infection assays revealed an increased susceptibility of ent3nsh3 mutants compared to WT whereas ent3 single mutants were not significantly different from WT (Figure 2A). We did not detect higher Ado levels in the media in contact with ent3nsh3 roots compared to WT and dornl lines (Figure 2D), confirming previous data showing similar eAdo levels in ent3nsh3 and WT roots grown hydroponically (21). Our results, though, indicate that the ent3nsh3 mutant has a higher eAdo/eATP ratio in control media than WT plants, as it secretes comparable levels of Ado but less ATP to the media in mock conditions (Figure 2D and S3). This enhanced eAdo/eATP ratio might explain the constitutive upregulation of DORN1 in ent3nsh3 compared to WT (Figure 5). A potential higher activity of DORN1 in ent3nsh3 under mock conditions can explain its increased response to ATP regarding cytosolic Ca\(^{2+}\) peak and pH\(_{apo}\) (Fig 3C-D, 4C-D). However, during Fo5176 infection, those high DORN1 expression levels detected in mock ent3nsh3 drop, most probably as a result of the lack of its capacity to respond to Fo5176 infection, similar to that observed in the dornl mutant (Fig 5). Thus, we conclude that a constitutively high eAdo/eATP ratio reduces plant resistance to Fo, an hypothesis substantiated by experiments in which WT plants showed similar responses when exposed to these molecules (Figure 1D). Furthermore, deficient defense responses on the transcriptional level in the ent3nsh3 mutants corroborated this idea (Figure 5). The high cytoCa\(^{2+}\) peaks detected in ent3nsh3 in response to ATP, equivalent to what we observed in WT plants upon ATP+Ado, suggest a sufficient enrichment of eAdo/eATP in the mutant to respond to ATP (Figure 3B and D). These high ATP-induced transient cytoCa\(^{2+}\) levels are not enough to activate a proper defense mechanism in ent3nsh3, in agreement with previously reported data (Figure 5, (36)). On the other hand, ent3nsh3 pH\(_{apo}\) changes in response to ATP were similar to those observed in WT, but the response was not changed by the addition of Ado, which altered the alkalization peak in control plants (Figure 4A-D). This could be explained by the conflicting published data about ENT3 being a proton symporter while transporting Ado (10, 37–39). Since Ado requires the presence of ATP to alter the pH\(_{apo}\) in WT plants, it is conceivable that the perception of ATP by DORN1 is required to initiate essential phosphorylation of ENT3 prior to transport, as reported for an ENT-family member in mammals (40). In addition, since we still detected a pH\(_{apo}\) increase in ent3nsh3 mutants in response to ATP, we hypothesize that there is another proton-distribution-modifying component involved. Considering the alkaline apoplast detected in ent3nsh3 under mock conditions (Figure 4-Figure supplement 2D) (41, 42), we anticipate that a plasma-membrane localized H\(^{+}\)-ATPase might be negatively controlled by eAdo. This constitutive high pH\(_{apo}\) measured in ent3nsh3 might explain its different response to ATP + 200 μM Ado compared to WT since the proton deficiency in the apoplast restricts the plant’s ability to further increase pH\(_{apo}\) (Figure 4B and D; S5A and B). It also has to be taken into account that the prevalence of DORN1 in ent3nsh3 mutants is higher compared to WT (Figure 5), which could enable an enhanced induction of downstream signals like
transient cytosolic Ca\(^{2+}\) peak (Figure 3D). The constitutively high apoplastic eAdo/eATP ratio and pH\(_{apo}\)
detected in ent3nsh3 concurs with an enhanced eATP-dependent pH\(_{apo}\) increase and could explain the
higher susceptibility of this mutant to Fo5176 (Figure 6). We anticipate similar functions in response to
other pathogens, based on the reported positive role of DORN1/P2K1 in plant resistance to the soilborne
fungal pathogen Rhizoctonia solani (26).

Our data suggest that Ado could act as an antagonist and compete with ATP over the DORN1 receptor.
However, this hypothesis was discarded by Choi et al. (4) who showed no competitive inhibition of the
DORN1 receptor by Ado. It can, however, not be fully excluded that Ado acts as a non-competitive or
allosteric antagonist of ATP at the DORN1 receptor. It is also possible that eAdo may directly regulate
apoplastic enzymes, e.g., ecto-apyrase/E-NTPDase, E5'NT, or another phosphatase, thereby indirectly
controlling eATP homeostasis. A third option to explain the eAdo influence on ATP-mediated plant
responses is the existence of an eAdo receptor whose activation interferes with the ATP-induced cascade
(Figure 6). Importantly, maximum cytosolic Ca\(^{2+}\) concentrations as well as pH\(_{apo}\) peaks were detected 100
s after simultaneous application of ATP and Ado in all plant genotypes, including the dorn1 mutant.
Therefore we expect eAdo to prompt its effect at the plasma membrane level, like eATP (Figure 3B and D),
and suggest the existence of a dedicated plant Ado receptor as described in animals. Indeed, G protein
coupled receptors are reported to bind and sense adenosine in mammals and yeast (19, 43). However,
considering that AtDORN1 is not directly homologous to its mammalian counterpart (44), an Ado-
receptor analogous to the mammalian purinergic G protein coupled receptor class is unlikely. Further
research is necessary to clarify the mechanism of eAdo perception and activity in plant response to ATP.

MATERIALS AND METHODS

Plant material and growth conditions

All Arabidopsis thaliana lines were in Col-0 background. The pH\(_{apo}\) sensor line pub10::SYP122-pHusion,
the calcium sensor line pub10::R-GECO1-mTurquoise, dorn1-3, ent3-1 and ent3nsh3 were published
previously (4, 21, 24, 28). Seedlings throughout all experiments were grown upright on solid, non-
buffered half MS media (pH 5.75) at 24 °C with a photoperiod of 16 h for the indicated timeframes.

Fungal material and growth conditions

Fusarium oxysporum Fo5176 and Fusarium oxysporum Fo5176 pSIX::GFP were used throughout this
study. Strain culture and storage were performed as described earlier (45). Fo5176 was grown in liquid
half potato dextrose broth (PDB) at 27 °C for 5 days in the dark. Spores were collected by filtering the
suspension through miracloth, centrifuging the filtrate at 3500 rcf, discarding the supernatant and
resuspending the spores in dsH\(_2\)O.

Fungal elicitor mix preparation, and fractionation, and molecule identification

Fungal elicitor mix was prepared as published previously (24, 46) and separated via enrichment using
Discovery DSC-C18 (2 g) columns (Merck) with a H\(_2\)O/MeOH gradient from 100 % to 0 % H\(_2\)O in 10 %
steps. Fractions were bioassayed and active fractions purified to individual components via an Agilent
1100 HPLC using Zorbax SB-C18 (9.4 x 150mm) semi-prep column in a linear gradient of H\(_2\)O/MeOH
and flow rate of 5 mL/min. Individual peaks were assayed for activity. The pure active compound was
characterized by standard 1D and 2D NMR experiments performed at the NMR Service of the Laboratory
of Organic Chemistry at ETH Zürich. All experiments were performed using d6-DMSO in a 600 MHz
Bruker NMR equipped with a 5mm probe. Data was analyzed using MestreNova 8.1 software (Mestrelab
Research, Spain). LC-MS data was obtained on an Agilent 6400 LC-qTOF in scanning positive mode to
produce a single signal with an m/z of 268.1044 (C10H13N5O4 calc. 268.1046 ± 1.72 ppm) and identical
retention time to an external standard of adenosine.

Fungal transformation
PCR and complementary primers (Supplementary File 1) were used to generate two DNA fragments with overlapping ends (47). A resistance cassette containing the neomycin phosphotransferase (npTII) cloned between the A. nidulans gdpA promoter and the trpC terminator (48) was used to generate two DNA fragments promoting the homologous recombination in fungal protoplasts. Protoplasts were produced as described previously (49) and their transformation done as reported by (50).

**In-vitro growth assay of Fo5176**

Freshly harvested Fo5176 spores were diluted to $10^4$ spores/mL and 15 µL of it distributed on solid half MS plates containing 1 mM Ado, 0.5 mM ATP, or both, or none (control). After four days under the plant growth conditions described above (“Plant material and growth conditions”), the colony diameters were measured using FIJI (51).

**Plant plate infection assays**

Plate infection assays were performed as described earlier (24, 27). Ado and ATP treatment plates were generated by mixing hand-warm half MS media, 0.9 % agar, with the specific amount of stock solution. Root growth was measured using FIJI (51).

**Hydroponic infection assay**

Hydroponic infection assays were performed as previously described (52). 30 seeds were grown on a foam floating on 50 mL liquid ½ MS media, pH 5.75, 1 % sucrose. After seven days the media was replaced by ½ MS without sucrose. Samples supposed to be infected were inoculated with $5 \times 10^6$ Fo5176 spores. After the indicated days post transfer to spore-containing media, roots and fungal hyphae were harvested for subsequent expression analysis and the media was filtered. For ATP quantification media was flash frozen in liquid nitrogen, for Ado quantification it was freeze dried.

**Media ATP quantification**

ATP levels in media from hydroponic infection experiments of hydroponically-grown plants were analyzed using the ATP Colorimetric/Fluorometric Assay Kit (Sigma, USA) and an Infinite M1000 plate reader (Tecan, Switzerland). Assays were done as described in the manual and ATP was detected fluorescently. All samples and standards were measured in duplicates.

**Media Ado quantification**

Freeze-dried media samples from hydroponic infection experiments were resuspended in 4 mL MilliQ water. The resulting mixture was loaded onto a 100 mg Discovery DSC-18 column (Supelco, USA). The column was eluted with 1 mL MilliQ water, 1 mL 70 % MilliQ water with MeOH and finally 100 % MeOH. The resulting aqueous elution was analyzed in positive mode using an Agilent 1200 Infinity II UPLC separation system coupled to an Agilent 6550 iFunnel qTOF mass spectrometer (Agilent, USA). Compounds were separated by injecting 5 µL of sample onto a Zorbax Eclipse Plus C8 RRHD UPLC column (2.1x100 mm, 1.8 µm) held at 50°C and eluting with a linear water:acetonitrile (both modified with 0.1 % formic acid) gradient, 99 % water to 99 % acetonitrile. Mass spectral data was acquired in positive mode with an electrospray ionization source and scanning a mass range of 100-2000 m/z. Quantification was done by integrating the m/z values corresponding to Ado in MassHunter Quantitative Analysis Software and compared to a standard curve generated at the time of sample measurements.

**In-vitro growth assay of Fo5176**

Freshly harvested spores were diluted to $10^4$ spores/mL and 15 µL of it distributed on solid half MS plates containing 1 mM Ado, 0.5 mM ATP or both. After four days under plant growth conditions the colony diameters were measured using FIJI.

**Gene expression analysis by real-time quantitative PCR**

Freeze-dried fungal and plant material from plate infection assays respectively hydroponics was ground to powder using glass beads and a TissueLyser II (Quiagen, Netherlands). Total RNA was extracted using GENEzol™ reagent (Geneaid, Taiwan) following the manufacturer’s protocol. 1 µg of RNA was used to generate first strand cDNA using the Maxima™ First Strand cDNA Synthesis-Kit (Thermo Scientific,
USA) following the manufacturer’s instructions. To amplify corresponding cDNA sequences primers (Supplementary File 1.) (4, 23, 24, 53–55) were used along with Fast SYBR Green Master Mix (Thermo Scientific, USA) under following cycle conditions: 95°C for 3 min, 40 cycles of 94°C for 10 s, 58°C for 15 s and 72°C for 10 s. Two technical replicates were performed for each reaction and the reference genes AtGAPDH and F0/0 were amplified on each plate for normalization. Relative expression was analyzed using the 2^ΔCt method (56).

Ratiometric pHapo sensor imaging

Experiments were carried out as described earlier (24). A Leica TCS SP8-AOBS (Leica Microsystems, Germany) confocal laser scanning microscope equipped with a Leica 10× 0.3NA HC PL Fluotar Ph1 objective or a Leica Stellaris 8 equipped with a Leica HC PL APO CS2 10x/0.40 DRY were used. pHusion was excited and detected simultaneously (Excitation: GFP 488 nm, mRFP 561 nm; Detection: GFP between 500 and 545 nm; mRFP between 600 and 640 nm). Five-day old A. thaliana seedlings expressing the sensor SYF2-pHusion grown on ½ MS + 1 % sucrose were transferred to imaging chambers as described previously (57) but placed on top of 1 % agarose cushions. Subsequently, the chamber was filled with ½ MS, pH 5.75. Images were collected as XYt series for 15 min with a time frame of 30 s. Image settings were kept identical throughout the experiments for each reporter line. After a recovery time of 15 minutes the experiment was started by acquiring ten images of the seedling’ roots without treatment to create a baseline of averaged relative signal. Roots were imaged from the tip including their elongation zone. The different treatments were applied in a volume of 100 µL after 300 s.

ΔF:F values were calculated according to following formula: ΔF:F = \frac{relative \ signal - baseline}{baseline}. Maximal amplitudes of ΔF:F signals were obtained by averaging the maximal ΔF:F signals of all curves. To collect standard curves for the pHapo ratiometric sensor a set of nine buffers from pH 4.8 to pH 8.0 were used. Each buffer was based on 50 mM ammonium acetate. Buffer pH 4.8 comprised additionally 22 mM citric acid, 27 mM trisodium citrate. pH was adjusted with 0.01 M HCl. Buffers pH 5.2 to pH 6.4 contained 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), buffers pH 6.8 to pH 8.0 were composed of 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES). 1 M Bis-Tris propane was used to adjust the pH values of buffers pH 5.2 to pH 8.0. Six to eight seedlings per buffer were incubated for 15 min and imaged after transfer to microscope slides.

Ratiometric cytoCa²⁺ sensor imaging

Imaging was done as described for the pHapo sensor (24) with slight modifications. Five-day old A. thaliana seedlings expressing the reporter R-GECO1-mTurquoise (28) were grown on ½ MS, pH 5.75, 1 % sucrose. mTurquoise was excited with 405 nm and detected between 460 to 520 nm, R-GECO1 was excited with 561 nm and detected between 580 and 640 nm. Imaging time frame was set to 20 s. Corrective flat field images for 405 nm were acquired by using 7-Diethylamino-4-methylcoumarin (Sigma D87759-5G, 50 mg/mL in DMSO). Relative signal was calculated by dividing mean gray values of the R-GECO1 channel by the mean gray values of the mTurquoise channel. ΔF:F values and Maximal amplitudes of ΔF:F signals were calculated as described for the pHapo sensor.

Statistical analyses

All statistical analyses were performed using Prism 9. Statistical methods and the resulting P-values are defined in the corresponding figure legends. Outlier tests were performed on datasets with . If the automatically detected fluorescent ratios of the genetic pH or Ca²⁺ sensors were measured to be outside of the standard curve range, they were excluded from the analysis. Such cases could always be allocated to severe drift of analyzed roots in the analysis chamber.

SUPPLEMENTARY MATERIAL

Figure 1-Figure supplement 2. Plant ENT3 and NSH3 expression do not change in response to Fo5176.
Figure 1-Figure supplement 3. ATP-induced plant defense is counteracted by doubled concentration of eAdo.
Figure 2-Figure supplement 2. Ado and ATP levels in the media are altered by Fo5176 infection
Figure 3-Figure supplement 2. Extracellular adenosine increases extracellular ATP induced DORN1 mediated Ca\(^{2+}\) influx
Figure 4-Figure supplement 2. Extracellular adenosine accumulation and absent extracellular ATP receptor DORN1 elevate apoplastic pH.

Supplementary File 1. Primers used in this study
Supplementary File 2. Statistical analysis of Fo5176 pSIX1::GFP root vascular penetrations in the indicated Arabidopsis genotypes.

FILE SOURCE DATA
Figure 1-source data 1. Enhanced apoplastic Ado counteracts eATP-induced reduction of fungal penetration on root vasculature.
Figure 2-source data 1. Increased extracellular Adenosine levels enhance fungal penetration rates
Figure 3-source data 1. eAdo enhances eATP-induced DORN1-mediated cytosolic Ca\(^{2+}\) peak.
Figure 4-source data 1. eAdo alters eATP-induced DORN1-mediated apoplast alkalization
Figure 5-source data 1. Accumulation of extracellular Ado impedes DORN1-mediated gene defense upregulation
Figure 1-Figure supplement 2-source data 1. Plant ENT3 and NSH3 expression do not change in response to Fo5176.
Figure 1-Figure supplement 3-source data 1. ATP-induced plant defense is counteracted by doubled concentration of eAdo.
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REFERENCES AND NOTES
9. S. Deng, J. Sun, R. Zhao, M. Ding, Y. Zhang, Y. Sun, W. Wang, Y. Tan, D. Liu, X. Ma, P. Hou, M.
Cellular Ca2+ Signals Generate Defined pH Signatures in Plants.


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**FIGURE LEGENDS**

Figure 1: Enhanced apoplastic Ado counteracts eATP-induced reduction of fungal penetration on root vasculature.
(A) Overlaid LC-MS extracted ion chromatograms of blank (red), adenosine (green), plant and fungus (blue). Overlaid LC-MS extracted ion chromatogram of blank (black), 250 ng adenosine (blue), fungi (red), plant (gray), plant and fungi (green) (B) and (C) Fo5'NT (B) and FoENT (C) expression relative to FoTub in hydroponically-grown Arabidopsis roots at various days post treatment (dpt) with Fo spores. Values are mean ± SEM, N ≥ 20, Welch’s unpaired t-test: (B) 1 dpt vs. 4 dpt: ** P-value ≤ 0.05, 1 dpt vs. 6 dpt: *P-value ≤ 0.01; (C) 1 dpt vs. 6 dpt: * P-value ≤ 0.05. (D) Cumulative Fo5176 pSIX1::GFP root vascular penetrations in wild-type (Col-0) seedlings at different days post-transfer to plates containing ½ MS (control) and 1mM Ado and/or 0.5mM ATP. Values are mean ± SEM, N ≥ 52 from three independent experiments. RM two-way ANOVA with Tukey post-hoc test on control vs. 0.5 mM ATP: P ≤ 0.001 (treatment), P ≤ 0.001 (time), P ≤ 0.0001 (treatment x time). Significant differences compared to control (black asterisk) and 500 µM ATP (blue asterisks) at 7 dpt are indicated on the graph (Tukey test); statistics of remaining time points are summarized in Supplementary File 2A. (E) Representative images of Col-0 seedlings at 7 dpt to mock (left) or Fo5176 pSIX1::GFP (right) plates. Scale bar = 1 cm. (F) Root growth of plants as shown in (E) at different days post transfer to mock or Fo5176 pSIX1::GFP-containing plates. Values are mean ± SEM, N ≥ 52 roots from three independent experiments, RM two-way ANOVA P (treatment, time, treatment x time): control vs 500 µM ATP (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); control vs 500 µM ATP + 1 mM Ado (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); control infected vs 500 µM ATP infected (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); control infected vs 500 µM ATP + 1 mM Ado-infected (≤ 0.0001, ≤ 0.0001, ≤ 0.0001). (G) Colony diameters of Fo5176 grown for 4 days on plates containing ½ MS (control) and 1mM Ado and/or 0.5mM ATP. Values are mean ± SEM, N = 4, Welch’s unpaired t-test control vs 0.5 mM ATP: **** P-value ≤ 0.0001; control vs. 0.5 mM ATP + 1 mM Ado: **** P-value ≤ 0.0001. Scale bar = 1 cm.

Figure 2: Increased extracellular Adenosine levels enhance fungal penetration rates

(A) Cumulative Fo5176 pSIX1::GFP root vascular penetrations in wild-type (WT; Col-0), dorn1, ent3, and ent3nsh3 seedlings at different days post-transfer (dpt) to spore-containing plates. Values are mean ± SEM, N ≥ 94 from three independent experiments. RM two-way ANOVA P (treatment, time, treatment x time) on WT vs. dorn1 (≤ 0.05, ≤ 0.0001, ≤ 0.05); WT vs. ent3nsh3 (≤ 0.0075, ≤ 0.0001, ≤ 0.0061). Significant differences compared to WT plants at 7 dpt are indicated on the graph (Tukey test); statistics of remaining time points are summarized in Supplementary File 2B. (B) Representative images of 8-day old mock or Fo5176 pSIX1::GFP infected plants as indicated in (A) at 7 days post-transfer to plates containing Fo5176 pSIX::GFP spores. Scale bar = 1 cm. (C) Root growth of plants indicated in (B) at different days post transfer to mock or Fo5176 pSIX1::GFP-containing plates. Values are mean ± SEM, N ≥ 79 from three independent experiments, RM two-way ANOVA P (genotype, time, genotype x time): WT vs. dorn1 (≤ 0.01, ≤ 0.0001, ≤ 0.01); WT vs. ent3 (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); WT vs. ent3nsh3 (≤ 0.0001, ≤ 0.0001, ≤ 0.05); WT infected vs. dorn1 infected (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); WT infected vs. ent3nsh3 infected (≤ 0.0001, ≤ 0.0001, ≤ 0.0001). (D) Ado/ATP ratio content in media from 10-days-old hydroponically-grown wild-type (WT; Col-0), dorn1, and ent3nsh3 seedlings at 4 days after transfer to media with (+Fo5176) and without (Mock) Fo5176 spores, and in media where Fo5176 was growing alone for 4 days (Fo5176). Values are mean ± SEM, N ≥ 3 biological replicates, Welch’s unpaired t-test in respect to their mock (black) or among genotypes (blue): * P-value ≤ 0.05.

Figure 3: eAdo enhances eATP-induced DORN1-mediated cytosolic Ca²⁺ peak.

(A), (C), and (E) Representative images of five-days-old wild-type (WT; Col-0; A), ent3nsh3 (C) and dorn1 (E) roots expressing the cytoCa²⁺ sensor, R-GECO1-mTurquoise -180 s before and 100 s after being exposed to ATP (upper panels) or ATP+Ado (bottom panels). Heatmaps indicate signal intensity (arbitrary units). Scale bar = 125 µm. (B), (D), and (F) cytoCa²⁺ in roots as in (A), (C), and (E) represented as normalized fluorescence intensity changes (ΔF:F) of R-GECO1: mTurquoise. Imaging started 180 s before either ATP or ATP+Ado was added (0 min; arrow head). Values are means ± SEM, N ≥ 18 from three independent experiments. RM two-way ANOVA P (treatment, time, treatment x time): (B) control vs. 10 µM ATP (≤ 0.0001, ≤ 0.01, ≤ 0.0001); control vs. 10 µM ATP + 50 µM Ado (≤ 0.0001,
Figure 4: eAdo alters eATP-induced DORN1-mediated apoplast alkalization

(A), (B), (C), (D), (E), and (F) Apoplastic pH over time in roots expressing the pH
sensor SYP122-
phusion represented as the relative signal compared to the averaged baseline recorded prior to treatments
(ΔF:F). Imaging started 270 s before either ATP or ATP+Ado was added (0 s; arrow head). Values are
mean ± SEM; N ≥ 12 seedlings from three independent experiments. RM two-way ANOVA. P
(treatment, time, treatment x time) on (A) control vs. 10 μM ATP (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); 10 μM
ATP vs. 10 μM ATP + 50 μM Ado (≥ 0.05, ≤ 0.0001, ≤ 0.05); (B) 200 μM Ado vs. 10 μM ATP + 200
μM Ado (≤ 0.05, ≤ 0.001, ≤ 0.0001); 10 μM ATP vs. 10 μM ATP + 200 μM Ado (≤ 0.01, ≤ 0.01, ≤ 0.0001);
200 μM Ado vs. 10 μM ATP (≤ 0.05, ≤ 0.01, ≤ 0.0001); (C) control vs. 10 μM ATP (≤ 0.05, ≤ 0.0001, ≤ 0.0001);
(D) 200 μM Ado vs. 10 μM ATP + 50 μM Ado (≤ 0.05, ≤ 0.0001, ≤ 0.0001); control vs. 10 μM ATP + 50 μM
ATP (≤ 0.0001, ≤ 0.0001, ≤ 0.0001); (E) control vs. 10 μM ATP + 50 μM Ado (≤ 0.05, ≤ 0.0001, ≤ 0.0001); ATP vs. 10 μM
ATP + 50 μM Ado (≤ 0.01, ≤ 0.001, ≤ 0.0001); (D) 200 μM Ado vs. 10 μM ATP (≤ 0.05, ≤ 0.0001, ≤ 0.0001);
200 μM Ado vs. 10 μM ATP + 200 μM Ado (≥ 0.05, ≤ 0.0001, ≤ 0.0001); 10 μM ATP vs. 200
μM Ado + 10 μM ATP; (E) control vs. 10 μM ATP (≤ 0.001, ≤ 0.0001, ≤ 0.0001); (F) 200 μM Ado vs. 10
μM ATP (≥ 0.05, ≤ 0.001, ≤ 0.01).

Figure 5: Accumulation of extracellular Ado impedes DORN1-mediated gene defense upregulation

DONRI, WRKY45, WRKY53, and At1g51890 expression relative to AtGAPDH in WT (Col-0), dorn1, or
ent3nsh3 roots 4 days post-treatment with Fo5176 spores or with control media (Mock). Values are mean
± SEM, N ≥ 3 biological replicates, Welch’s unpaired t-test within each genotype in respect to their mock
(black) or among genotypes (blue); * P-value ≤ 0.05, ** P-value ≤ 0.01.

Figure 6: Scheme of the fungal-induced downregulation of the extracellular ATP and Adenosine
homeostasis to increase its virulence. Previously published results (adapted from (16)) showed that ATP
is released into the apoplast by wounding, active transport and exocytosis. Apyrases (APY) and purple
acid phosphatases (PAP) degrade ATP to AMP, which is further processed by 5’ nucleotidases (5’NT)
and nucleoside hydrolases (NSH) to adenosine (Ado) and adenine (Ade). Equilibrative nucleotide
transporters (ENT) and purine permease transporters (PUP) mediate take up of Ado and Ade into the
cytoplasm. ATP is perceived by the purinergic receptor DORN1, which triggers Ca²⁺ influx. Subsequently calcium dependent downstream signaling re-programming is initiated. First contact with F.
oxysporum 5176 (Fo5176) elicits phosphorylation of Arabidopsis H+-ATPases (AHAs) and accordingly
the apoplast acidifies (24). In this work, we show that during root colonization (red symbols), Fo5176
upregulates expression of eATP hydrolyzing ecto-5’ nucleotidase (Fo5’NT) and Ado transporter (FoENT)
and secretes more Fo5’NT, increasing the extracellular Ado (eAdo) levels. eAdo interferes with ATP-
induced plant immune system activation above a certain eAdo/eATP ratio by boosting ATP-induced
apoplast alkalization and thereby enhancing fungal virulence. Similar results were obtained in the
ent3nsh3 mutant where we detected higher eAdo/eATP levels compared to WT plants. Accordingly, this
plant mutant is more susceptible to Fo5176 than WT plants and we observed a lack of upregulation of
Fo5176-induced defense genes. Our data suggest that eAdo is perceived via a hitherto undiscovered
dedicated plant receptor.
SUPPLEMENTARY MATERIALS. LEGENDS.

Figure 1-Figure supplement 1: NMR data of Ado
The chemical shifts of ^1^H and ^13^C found in Ado. Blanks are heteroatoms in the main chain. Selected COSY and HMBC correlations are included to demonstrate linkage.

Figure 1-Figure supplement 2: Plant ENT3 and NSH3 expression do not change in response to Fo5176.
ENT3 and NSH3 expression relative to AtGAPDH in hydroponically-grown Arabidopsis roots at the respective days post treatment with Fo5176 spores or with control media (Mock). Values are mean ± SEM, N ≥ 3.

Figure 1-Figure supplement 3: ATP-induced plant defense is counteracted by doubled concentration of eAdo. Cumulative Fo5176 pSIX1::GFP vascular penetrations per root in 8-day-old Col-0 plants at different days post transfer to plates with fungal spores alone (control), or with ATP (A) or ATP+Ado (B). Values are mean ± SEM, N ≥ 63 from three independent experiments. RM two-way ANOVA P (genotype, time, genotype x time) on (A) control vs. 300 µM ATP (≤ 0.05, ≤ 0.0001, ≤ 0.05); control vs. 500 µM ATP (≤ 0.05, ≤ 0.0001, ≤ 0.0001); (B) control vs. 500 µM ATP (≤ 0.01, ≤ 0.0001, ≤ 0.0001); control vs. 500 µM ATP + 500 µM Ado (≤ 0.01, ≤ 0.0001, ≤ 0.001). Significant differences compared to control at 7 dpt are indicated on the graph (Tukey test); statistics of remaining time points are summarized in Supplementary File 2C.

Figure 2-Figure supplement 2: Ado and ATP levels in the media are altered by Fo5176 infection. Ado (left panel) and ATP (right panel) content in the media from 10-days old hydroponically grown wildtype (WT, Col-0), dorn1 and ent3nsh3 seedlings at four days after transfer to media with and without Fo5176 spores and media of four days Fo5176 growth alone. Values are mean ± SEM, N ≥ 3 biological replicates, Welch’s unpaired t-test in respect to their mock (black) or among genotypes (blue): * P-value ≤ 0.05.

Figure 3-Figure supplement 2: Extracellular adenosine increases extracellular ATP induced DORN1 mediated Ca^{2+} influx. Five-day-old RGECO-mTurquoise fluorometric calcium reporter line seedlings were imaged in ½ MS (–180 to 0 s). At 0 min, either ATP or ATP+Ado at the indicated concentrations were added ΔF:F represents the relative signal compared to the averaged baseline recorded prior to application (arrow). RM two-way ANOVA P (treatment, time, treatment x time): (A) control vs. 10 µM ATP (≤ 0.0001, ≤ 0.01, ≤ 0.0001); control vs. 10 µM ATP + 10 µM Ado (≤ 0.0001, ≤ 0.01, ≤ 0.0001); (B) 100 µM Ado vs. 10 µM ATP (0.0001, ≤ 0.05, ≤ 0.0001); 100 µM Ado vs. 10 µM ATP + 100 µM Ado (≤ 0.0001, ≤ 0.05, ≤ 0.0001); 10 µM ATP vs. 10 µM ATP + 100 µM Ado (≥ 0.05, ≤ 0.0001, ≤ 0.0001); (C) 200 µM Ado vs. 10 µM ATP (≤ 0.0001, ≤ 0.01, ≤ 0.0001); 200 µM Ado vs. 10 µM ATP + 200 µM Ado (≥ 0.05, ≤ 0.0001, ≤ 0.0001); (E) 200 µM Ado vs. 10 µM ATP (≤ 0.0001, ≤ 0.01, ≤ 0.0001); 200 µM Ado vs. 10 µM ATP + 200 µM Ado (≥ 0.05, ≤ 0.0001, ≤ 0.0001); (F) control vs. 10 µM ATP (< 0.0001, ≤ 0.01, ≤ 0.0001); (G) control vs. 10 µM ATP + 10 µM Ado (≤ 0.0001, ≤ 0.01, ≤ 0.0001); Maximum amplitudes (D), (G) correspond to (A) - (C) and Figure 3 (B) (WT) respectively (E), (F) and Figure 3 (D) (ent3nsh3), N ≥ 13 from three independent experiments. Welch’s unpaired t-test in respect to control (black) or to ATP alone (blue): * P-value ≤ 0.05, ** P-value ≤ 0.01.

Figure 4-Figure supplement 2: Extracellular adenosine accumulation and absent extracellular ATP receptor DORN1 elevate apoplastic pH. Five-day-old fluorometric SYP122-pHusion pH reporter lines were imaged in ½ MS pH 5.75 (~270 to 0 s) with SP8 microscope. At 0 sec, either ATP or ATP+Ado at the indicated concentrations were added. (A), (B) and (C) correspond to Figure 4 (B), (C), (E), (F), (H), (I) and display the averaged maximal amplitude of each curve. (D) apoplastic pH was determined using standard curves. Values are mean ± SEM, N ≥ 16 from three independent experiments, Welch’s unpaired
t-test in respect to control or wild-type (black) or to ATP alone (blue): * $P$-value $\leq 0.05$, ** $P$-value $\leq 0.01$ **** $P$-value $\leq 0.0001$. 
**A** ESI-EIC (268.2)

**B**

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**C**

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**D**

Average penetration/root

- Control
- 1 mM Ado
- 0.5 mM ATP
- 0.5 mM ATP+1 mM Ado

**E**

Mock

Fo5176 pSIX1::GFP

**F**

Mock

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**G**

Colony ø (mm)

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H+
wound
Ca2+ Ca2+
transport
exocytosis
ATP
fungal cytoplasm
plant cytoplasm
apoplast

pH_{apo}
ATP
AMP
Ado
AMP
Ado

5'NT
NSH
ent3nsh3

DORN1 KinaseTM

defense genes expression

plant cytoplasm
**Figure 1-Figure supplement 1: NMR data of Ado**

The chemical shifts of $^1$H and $^{13}$C found in Ado. Blanks are heteroatoms in the main chain. Selected COSY and HMBC correlations are included to demonstrate linkage.

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Average penetrations/root

A: control
- 10 µM ATP
- 100 µM ATP
- 300 µM ATP
- 500 µM ATP

B: control
- 500 µM ATP
- 500 µM ATP + 500 µM Ado
- 500 µM ATP + 750 µM Ado
- 500 µM ATP + 1 mM Ado

Days post transfer

* **