Title: Growth regulation by apyrases: insights from altering their level of expression in yeast, Arabidopsis and soybeans

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Abstract:
Apyrases are NTPDases that remove the terminal phosphate from NTPs and NDPs, but not from NMPs. They have conserved structures and functions in yeast, plants and animals. Among the most studied APYs in plants are those in Arabidopsis (AtAPYs) and peas (PsAPYs), both of which have been shown to play major roles in regulating plant growth and development. Valuable insights on their functional roles have been gained by transgenically altering their transcript abundance, either by constitutively expressing them or by their suppression. This review focuses on studies of transgenic lines of yeast and multiple different plants that revealed insights on the growth-altering functions of plant apyrases in different organisms. APY expression can also be inhibited post-translationally by chemically blocking its enzymatic activity, so this review also briefly covers studies that used inhibitors to suppress APY activity in plants and fungi.

Introduction: Early studies link eATP, ecto-apyrase activity, and growth regulation

Although an early report suggested the possibility that extracellular ATP (eATP) could function as a signaling agent in plants (Udvardy and Farkas, 1973), it has been only within the last two decades that a continuous stream of publications every year has provided definitive evidence for this conclusion. These reports, including critical data on plant eATP receptors (Choi et al., 2014; Pham et al., 2020), raised the question of how plants control the level of extracellular nucleotides. The answer to this question was already evident in animal cells more than 25 years ago, when ecto-apyrases (ecto-NTPDases), which have the lowest K_m among the known ecto-phosphatases that can hydrolyze eATP, were accepted as being critically important in removing NTPs and NDPs from the extracellular matrix (ECM) (Zimmermann, 1994). Apyrase enzymes are highly conserved throughout evolution (Clark et al., 2014), so it was not surprising when multiple reports indicated that ecto-apyrases also played a major nucleotide-hydrolyzing role in the ECMs of diverse plants, such as Arabidopsis (Wu et al., 2007), soybeans (Govindarajulu et al., 2009), potato (Riewe et al., 2008), and poplar (Deng et al., 2015).

Initially the most studied apyrases linked to growth control were the pea enzyme, psNTP9 (hereafter abbreviated PS) and two almost identical apyrases in Arabidopsis, AtAPY1 and AtAPY2. Early evidence indicated that at least some portion of both the pea and Arabidopsis enzymes could be localized in the ECM and could thus qualify as ecto-apyrases (Thomas et al., 1999; Shibata et al., 2002; Wu et al., 2007). Another feature of both PS and AtAPY1 that could involve them in growth control is that both have been shown to bind to calcium-activated calmodulin, a signaling agent that helps regulate diverse growth responses of plants to stimuli (Basu et al., 2021). This binding simulates their enzyme activity, potentially making them more sensitive to calcium (Clark and Roux, 2018). Additionally, calmodulin has a documented role in

nuclear trafficking of proteins including transcription factors (Sweitzer and Hanover, 1996; Hanover et al., 2009), so it could play a role in targeting PS and AtAPY1 to the nucleus.

Early studies also showed that the "ecto" role of apyrases included growth regulation (Thomas et al., 1999; Steinebrunner et al., 2003). How cell wall/extracellular matrix (ECM) NTPDase activity could be linked to growth became clear when dose-response assays showed that low levels of eATP could promote growth and high levels could suppress it (Steinebrunner et al., 2003; Tang et al., 2003). Subsequently, the apyrase-growth connection was found to have a hormonal basis when Tang et al. (2003) found that high [eATP] could inhibit auxin transport, and when subsequent transgenic studies showed that enhanced expression of *AtAPY1* or *AtAPY2* could promote auxin transport while their suppression inhibited this transport (Liu et al., 2012).

The link between eATP, ecto-apyrase activity, and growth extends also to defense responses, in which a key early event after insect or pathogen attacks is the release of cellular ATP into the ECM through broken or permeabilized membranes. This increase in eATP serves as a signal to turn on defense genes (Tanaka and Heil, 2021), but it also induces an increase in apyrase expression, as discussed in Clark et al. (2021). Recent RNA-seq data reported in the SRA data base of the NIH National Library of Medicine (Accession number SRX3943120) indicate that defense-related genes are the ones whose expression is most upregulated in transgenic plants that constitutively expressed AtAPY1. These included the *TGG2* gene (up-regulated 84-fold), which encodes one of the thioglucoside glucohydrolases released from wounds. As reported recently by Gao et al. (2023), these enzymes function as Ricca factors that provoke long-distance electrical waves, which move to distant undamaged sites. There these signals induce defense responses needed to protect against anticipated future attacks from herbivores (Gao et al., 2023). These results suggest that upregulating apyrase expression may be a key signal transduction step induced by eATP to elicit defense responses. This hypothesis could be tested by examining the effects of apyrase suppression on the effectiveness of eATP as a DAMP signal.

The substrate specificity of apyrases can be readily altered by biochemical modifications (Knowles, 2011). Although untagged PS and AtAPY1/2 purified from the nuclei of etiolated seedlings favor ATP as their NTP substrate (Chen et al., 1987; Weeraratne, 2019), tagged AtAPY1 purified from light-grown cells favors ADP as its substrate (Massalski et al., 2015; Chiu et al., 2015). The molecular bases of these differences in substrate specificity remain to be discovered, but it seems likely that native AtAPY1 can function as an ecto-apyrase to limit [eATP], as demonstrated for pollen AtAPY1/2 by Wu et al. (2007).

The "ecto" location of apyrases (i.e., either in the ECM or on the plasma membrane with its active site facing to the ECM) in multiple different plants has been demonstrated (Clark et al., 2021), but GFP-labeled AtAPY1 is localized mainly in Golgi (Chiu et al., 2012; Schiller et al., 2012). However, even in this location it could regulate the [eATP] by limiting the [ATP] in Golgi-derived secretory vesicles that release their contents into the ECM, as discussed in Clark et al. (2021).

Transgenic approach to evaluating apyrase functions

A favored approach plant scientists use to evaluate the function of a protein in any specific plant is to either constitutively express it or genetically knock it out in that plant. However, discovering which of the apyrases in a plant are specifically **ecto**-apyrases is challenging, because most plants have multiple apyrases, most of which function inside the cell. For example, soybean has 13 different apyrases, but so far only GS52 has been identified as an ecto-apyrase (Govindarajulu et al., 2009), and potato has 10 different apyrases, but so far only three of these have been shown to function in the ECM (Riewe et al., 2008). Similarly, not all apyrases in a plant stimulate growth when constitutively expressed, or suppress growth when genetically suppressed. For example, of the 7 apyrases in Arabidopsis, initial transgenic studies favored only AtAPY1 and AtAPY2 as having critically needed roles in growth control (Wu et al., 2007; Wolf et al., 2007; Yang, 2011). So far, the apyrases that have been reported to regulate growth in Arabidopsis, soybeans, potato, peas, and poplar all have the plasma membrane/ECM as one of their major locales, so all could potentially function as ecto-apyrases (Clark et al., 2021). Thus, the ecto-apyrase-eATP-growth regulation link seems to be a consistent theme in plants.

To the extent that only some of the apyrases in a plant play major roles in growth control, it would be valuable to identify these specific apyrases in other plants, especially in crop plants. A relatively rapid assay that could be used for this purpose was illustrated in Wu et al. (2007). In that report, AtAPY1 and AtAPY2 were the only apyrases in Arabidopsis whose expression was strongly correlated with growth rates of tissues in seedlings. That is, they were highly expressed in hypocotyls that are rapidly growing in darkness, but almost absent from the same tissue when its growth was rapidly suppressed by exposure to light. Thus, in the scores of plants for which the genomic sequences are now known, it would be straightforward to design primers to assay which apyrase transcripts in the hypocotyl or epicotyl of a given dicot seedling dramatically changes expression when it transits from darkness into light.

Similarly, for monocots such as maize, the same strategy could be used to identify those apyrases that are highly expressed in the rapidly growing mesocotyl when it is in darkness, but then are rapidly suppressed when its growth rapidly declines upon exposure to light. Of course, the success of this strategy would depend on whether the specific growth-regulatory apyrases in the assayed plant are preferentially expressed in rapidly growing tissues, and it remains to be seen how many other plants follow the pattern seen in Arabidopsis.

Recent studies linking apyrase expression to growth control of plants

Beyond the evidence reviewed by Clark et al. (2021), more recent publications have provided new insights on the apyrase-growth control connection. These reports revealed new ways in which enhancing or diminishing the expression of this enzyme can induce changes in the growth and development of plants, and they all merit further discussion here.

Endophytic bacteria induce AtAPY5 production in Arabidopsis, increase growth

Plants and microorganisms interact in multiple ways, some of which are beneficial for both. Multiple reports have demonstrated that some microorganisms that live in plants can promote their growth and seed yields, and thus can be used as biofertilizers. Endophytic *Bacillus* species that can survive in adverse environments are among the microorganisms most used in this way, and some have been shown to produce metabolites that promote plant growth and increase crop production (Ke et al., 2021).

A recent report demonstrated that endophytic bacteria can influence growth by a mechanism distinct from just producing nutrients for plants (Xu et al., 2022). This report showed that the interaction of the endophytic bacterium *Bacillus aryabhattai* with Arabidopsis and tobacco plants resulted in a statistically significant, two-fold increase in their growth, as measured in both dry and fresh weight. In Arabidopsis, its co-incubation with the endophyte induced it to turn on the transcription of multiple genes that stimulate growth (Xu et al., 2022). The second highest fold-increase in transcript abundance (over two-thousand-fold) was for the transcript encoding AtAPY5. Based on this fact, the authors hypothesized that this apyrase functioned in some key role that influenced growth. However, this role was not very obvious because a previous study had shown that null *atapy5* mutants had no clear phenotype that differed from wild-type plants (Yang, 2011). Interestingly, *AtAPY5* expression was increased almost 5-fold in apyrase 1 overexpressing (*APY1 OE*) seedlings, which showed improved growth when grown on Pi-sufficient, NTP supplemented media (Slocum et al., 2023). Although AtAPY5 may not play an essential role in growth regulation, its enhanced expression could still promote growth.

One hint of how AtAPY5 could have promoted growth of Arabidopsis and tobacco is that it has a Golgi localization and can complement a yeast mutant that is defective in cell wall synthesis (Chiu et al., 2015). Several of the other genes whose transcript abundance was upregulated by the endophyte were known to promote lignin synthesis, a change that would certainly impact plant growth (Liu et al., 2018), so further studies on the role of AtAPY5 in the production of this cell wall component would be warranted.

APYRASE1/2 play a critical role in mediating light-induced growth changes in Arabidopsis

As noted above, when etiolated seedlings emerge from darkness into light, the activation of phytochrome induces major changes in gene expression and in growth. The growth changes differ in different tissues: hypocotyl growth rate drops dramatically and quickly, while that of roots and cotyledon-hook tissue increases somewhat more slowly (Montgomery, 2016). The results of Wu et al. (2007) demonstrated that the strong and rapid decrease in the growth of the hypocotyl occurred with the same rapid kinetics as an equally dramatic decrease in both the transcript and protein levels of AtAPY1 and AtAPY2. This raised the question whether the transcript and protein levels of AtAPY1/2 in the root and hook-cotyledon tissue of seedlings also change when light induces their increased growth during de-etiolation, and, if so, whether these changes were needed for the growth changes. The answers to these questions turned out to be Yes, as detailed in the report of Weeraratne et al. (2022), who used transgenic, transcriptomic, and immunoblot approaches to reach their conclusions.

The growth changes in primary roots induced by red light (R) are not unidirectional, for roots initially grow slower during the first 12 h after irradiation (Correll and Kiss, 2005), but then, at later time points, they grow faster (Kircher and Schopfer, 2012). At the same time points, after 12 h of light AtAPY protein levels decreased, but after 24 h the levels of both *AtAPY* transcripts and protein increased.

Unlike the growth response of roots to light, hook-cotyledon tissue shows a steady, consistent increase in growth, and immunoblot assays showed that the kinetics of AtAPY protein increase in these tissues coincided with their growth increase. However, in this case, *AtAPY* transcript levels did not increase in parallel, indicating that in these aerial tissues R increased AtAPY expression mainly by increasing its translation and/or decreasing its turnover (Weeraratne et al., 2022). Other instances of phytochrome inducing changes in gene expression mainly by controlling translation are known (Paik et al., 2012). Specifically, in etiolated seedlings, Cheng et al. (2021) recently reported that R-induced changes in the level of certain proteins occurred without parallel changes in the transcripts that encode those proteins.

A well-studied morphological change that occurs during R-induced de-etiolation is hook opening. Weeraratne et al. (2022) found that RNAi-mediated suppression of *AtAPY1/2* expression severely inhibited hook opening, while the constitutive expression of either *AtAPY1* or *AtAPY2* could induce this opening even in unirradiated etiolated plants. These results were consistent with the hypothesis that changes in *AtAPY1/2* expression could help mediate the transcriptomic changes known to be required for this tissue- straightening response to occur. These included the upregulation of *SAUR50* (Dong et al., 2019), which, in turn, required the down-regulation of *SAUR17* (Wang et al., 2020). In support of this hypothesis, Weeraratne et al. (2022) found that the over-expression of *AtAPY2* promoted both the up-regulation of *SAUR50* and the down-regulation of *SAUR17* in the hook-cotyledon tissue of etiolated seedlings. Taken together, these results implied that in the sequence of transcriptomic changes that occur when phytochrome induces hook opening, the up-regulation of the genes encoding *AtAPY1* and *AtAPY2*, both of which have promoter elements known to be regulated by phytochrome (Wu et al., 2007), precede the R-induced changes in the *SAUR50* and *SAUR17* genes.

Other transcriptomic changes that impact growth, and that occur in dark-grown seedlings of transgenic Arabidopsis plants in response to enhanced or suppressed expression of AtAPY1/2 included changes in the transcript abundance of three ECM peroxidases, Prx 15, Prx49, and Prx 59. The activity of these peroxidases catalyze cross links in cell walls that decrease their extensibility (Lim et al., 2014). The constitutive expression of *APY2* decreased the transcript level of all three of these peroxidases while the RNAi-mediated suppression of AtAPY1/2 increased the level of the same peroxidases (Weeraratne et al., 2022). These results further confirmed the role of AtAPY1/2 in regulating the expression of genes that control growth.

AtAPY1 expression can help salvage phosphate from extracellular NTPs

In another recent report, which is now under review, but is available as a preprint online, Slocum et al. (2023) investigated whether another function of apyrase could be the salvaging of phosphate (Pi) from extracellular nucleotides, such as those known to be present in the rhizosphere from diverse sources. In their study of *Arabidopsis thaliana* seedlings that constitutively express *apyrase 1* (*APY1 OE),* they found that under growth conditions in which Pi availability was limiting, both wild-type and *APY1 OE* seedlings showed the typical Pi starvation response of having decreased Pi contents and a characteristically-altered root system architecture (RSA). However, when grown on Pi-sufficient media, *APY1 OE* seedlings had higher Pi contents than wild-type seedlings. Moreover, the addition of NTP increased the Pi contents and expanded the RSA of *APY1 OE* but not wild-type seedlings. Consistent with their elevated Pi contents, *APY1 OE* seedlings showed greater repression of phosphate-starvation-response genes relative to wild-type seedlings, and their expanded RSA was correlated with the increased expression of hundreds of growth-related genes, including over 100 involved in regulation of auxin signaling and transport. The authors concluded that APY1 could modulate this auxin response by promoting increased Pi uptake, including some Pi salvaged from extracellular NTPs. The novel data in this report could have potential value for the development of transgenic crops that have higher fertilizer use efficiency and, consequently, higher seed yields. Also, by salvaging more of the Pi applied in fertilizers, those crops overexpressing *AtAPY1* could reduce the environmental pollution due to phosphate run-off from soils.

Are all growth effects of APY1/2 mediated by their NTPDase activities in the plant ECM?

As noted above, multiple studies have shown that by modulating eATP levels, ecto-apyrases could alter the growth rate of plant cells. However, AtAPY1 and AtAPY2 have been localized not only in the ECM, but also in Golgi (Chiu et al., 2012; Schiller et al., 2012) and in nuclei (Weeraratne, 2019), and, as discussed by Veerappa et al. (2019), they could impact growth by their activities in any of these organelles. Massalski et al. (2015) well discussed how the NDPase activity of AtAPY1 in Golgi could impact protein glycosylation, a step typically needed to insert key-growth regulating enzymes (Veit et al., 2018; Chen et al., 2020). Here we will discuss how the NTPDase activity of APYs in nuclei could affect the expression of growth-controlling genes, such as those highlighted by Weeraratne et al. (2022) noted above.

While there is good evidence for the ECM and Golgi locales of different plant apyrases, the best studied APY in peas, psNTP9 (hereafter abbreviated PS), was originally purified from pea nuclei (Chen et al., 1987), and it was localized in this organelle by immunocytochemistry (Tong et al., 1993). The two most highly expressed apyrases in rapidly growing cells of Arabidopsis, AtAPY1 and AtAPY2 (Wu et al., 2007), also co-localize with purified nuclei and have been immunolocalized there *in situ* (Weeraratne, 2019). Both PS and AtAPY1/2 have nuclear localization signals in their primary structure, and both also co-purify with chromatin (Chen et al., 1987; Weeraratne, 2019). Moreover, PS binds to DNA-affinity columns with high specificity (Chen, 1987). These findings raise the obvious possibility that AtAPY1/2 and PS could have major functions in the nucleus as well as in the ECM.

Beyond the most-studied roles of ATP in various nuclear functions (e.g., transcription, epigenetic modifications, pre-RNA splicing), it also plays a key role as a chromatin hydrotrope and in maintaining nuclear phase-phase separations (Wright et al., 2019). Moreover, a recent study found a role for eATP in regulating chromatin dynamics and chromatin binding proteins in Arabidopsis (Matzke et al., 2019). This is an especially relevant finding because both PS and AtAPY1/2 have been demonstrated to be chromatin-associated proteins (Hsieh et al., 2000; Weeraratne, 2019).

Given all these major roles of nuclear [ATP], it would not be surprising to learn that any changes in the nuclear levels of APY in transgenic plants would dramatically alter the regulation of gene expression. Although none of the transgenic studies (either suppression or over-expression) carried out thus far have tried to assay how the altered levels of APY are differently distributed in the nucleus, ECM or Golgi, there is no doubt that some fraction of the APY is in the nucleus, at least in etiolated seedlings. Thus, one could expect that some of the effects of altered expression of AtAPY1 in Arabidopsis on changes in the expression of genes that impact growth, such as those revealed by Lim et al. (2014) and Weeraratne et al. (2022), would be due to changes in its nuclear activities, independent of how it impacted NTP levels in the ECM or Golgi. As discussed more in the next section, enhanced heterologous expression of different APY genes in Arabidopsis and soybeans also results in gene expression changes that would account for the phenotypic changes observed, although, again, which of these changes would be due to the nuclear activity of the heterologously expressed apyrases was not determined.

In parallel with studies of the role of APY in modulating transcriptomic changes, other reports have shown that eATP also regulates changes in gene expression. The Redox Responsive Transcription Factor, RRTF, was identified as being eATP-responsive and important in Arabidopsis growth and defense responses (Dong et al., 2020; Zhu et al., 2020). Additionally, MYC transcription factors and a calmodulin-binding transcription activator (CAMTA3) play important roles in mediating eATP-induced changes in gene expression (Jewell et al., 2019; Jewell and Tanaka, 2019). Thus, APY control of ATP levels in the ECM could also impact nuclear activities.

Two different approaches are currently being used to help resolve which phenotypic and/or gene expression changes induced by altered expression of apyrase are due to which sub-cellular domain of apyrase function. In one, the nuclear functions of apyrase are being clarified both by ChIP assays, to determine with what (if any) regions of DNA APY interacts, and by co-IP and yeast-two hybrid assays to determine with which (if any) chromatin/nuclear proteins APY associates. In another assay, transgenic Arabidopsis plants are being generated that express altered versions of PS, missing either their nuclear localization signal or their signal peptide. These plants can then be studied to determine how they differ from transgenic plants expressing wild-type PS in their phenotype and in which genes they differentially express.

Heterologous expression of APY in bacteria, yeast, and diverse plants further clarifies its functions

The signal peptide and nuclear localization signals of apyrases could, in principle, allow it to enter into ECM or nuclear domains when it is ectopically expressed in organisms other than its native plants. As another valuable approach to gaining a better understanding of the role of apyrases in plant growth and development, multiple laboratories have studied the expression of different apyrases in a heterologous system.

Bacteria and yeast are the simplest heterologous cells in which to express a plant apyrase, and this strategy has been used many times in order to characterize the enzyme activity of different apyrases. Early studies heterologously expressed apyrases in bacteria to study their biochemical properties. Their results showed that the bacterial expression of both AtAPY1 (Steinebrunner et al., 2000) and potato (Wujac et al, 2013) resulted in the recombinant proteins accumulating in inclusion bodies as insoluble proteins, most likely due to their toxic effect on cellular ATP levels or improper folding due to their expression in procaryotic cells. Nonetheless, these apyrases retained strong NTPDase activity after being solubilized and refolded. In a follow-up study, three potato APYs (StAPY4-StAPY6) were co-expressed with heat shock chaperonins in *E. coli,* and using this approach StAPY5 was produced in a soluble, catalytically active form (Porowińska et al., 2014).

More recently, Karim et al. (2023) described a more efficient method to express and purify a potato apyrase from bacteria. They co-expressed the original potato apyrase studied by Handa and Guidotti (1996) (GenBank: U58597.1) with a bacterial disulfide isomerase at low temperatures and obtained low amounts of soluble enzyme. After a two-step purification, the potato apyrase had high levels of activity toward both ATP and GTP.

Expression of plant apyrases in yeast instead of in bacteria has the advantage of allowing for eukaryotic post-translational modifications to the heterologously expressed apyrase. Expression of PS in a yeast phosphate-transport mutant NS219 restored the ability of the mutant to take up phosphate (Thomas et al., 1999). The transgenic yeast grew better under both phosphate-limiting as well as phosphate replete conditions. The mechanism by which this heterologous expression of apyrase allowed the yeast mutant to grow better is still not understood. However, its ectopic expression in Arabidopsis increased phosphate content in both seedlings (Thomas et al., 1999) and mature leaves (Veerappa et al., 2017), so further studies on how the enhanced expression of PS in plants promotes the uptake of this critical nutrient is warranted. Multiple other past studies of the ectopic expression of apyrases in yeast (e.g., potato apyrase expression in the methylotrophic yeast *Pichia pastoris* (Nourizad et al., 2003)), and the expression of all 7 Arabidopsis apyrases in a yeast double mutant that lacked endogenous apyrases (Chiu et al., 2015) further illustrated that plant apyrases can function well in foreign cellular environments.

The ectopic expression of different apyrases in higher plants has also yielded valuable insights. Here we will review three recent papers that illustrate how the heterologous expression of two different apyrases further substantiated their critical role in regulating plant growth and development. Two of these studied the major effects of PS expression in Arabidopsis and

soybeans, and a third report documented the stress-protective effects of expressing a poplar apyrase in Arabidopsis

The ectopic expression of PS in Arabidopsis and soybeans resulted in enhancing the growth of both plants. These effects included an improved root system architecture (RSA), increased fresh and dry weights, and higher seed yield under both normal and drought conditions in (Veeerappa et al., 2019). Multiple independent transgenic Arabidopsis lines were found to have higher root hair density and longer root hairs as well as longer primary roots and more lateral roots under normal growth conditions and during osmotic stress. Detached shoots and leaves from transgenic lines also showed improved water retention during dehydration, and, accordingly, intact plants showed improved growth and survival in response to drought treatment. RNA-seq analyses of Arabidopsis transgenic lines showed differential expression of genes related to these RSA and drought phenotypes.

The expression of PS in a cultivar of the agriculturally important crop, soybean, also resulted in improved traits when these transgenic plants were grown in the greenhouse. These enhancements included improved RSA and increased yield in normal growth and drought conditions. A model was proposed to explain how the phenotypic changes produced by heterologous expression of PS could be impacted by its activity in any of the three potential cellular locales (Golgi, ECM, nuclei) in which this apyrase could function (Veerappa et al., 2019).

In a follow-up study, Sabharwal (2022) found that the improved growth and yield phenotypes observed for three different transgenic Williams 82 lines when they were grown in the greenhouse were also observed in these same lines when they were grown in multiple field trials. Even elite soybean lines constitutively expressing *psNTP9* exhibited these improved growth and yield features. Specifically, in field-grown transgenic soybeans the typical increase in yield ranged from 10-44%. Moreover, the level of increased yield corresponded to the level of PS being expressed.

The *PS*-expressing soybean lines also had leaf traits that would increase their water use efficiency, and would likely contribute to the increased seed yields. These included higher chlorophyll and protein contents, decreased stomatal density, increased cuticle and cell wall thickness in their epidermal cells, and increased trichome density and trichome length.

RNA-seq analyses of transgenic soybean leaves indicated that their altered phenotypes could be explained, in part, by genome-wide gene expression changes induced by the *PS* transgene. A total of 996 genes were differentially expressed. Among them, those associated with fatty acid and wax biosynthesis and cuticle development and nitrogen assimilation genes were upregulated, and those regulating stomatal development were down-regulated. Importantly, Western blot analyses confirmed that the PS protein was localized to both the ECM and nucleus in transgenic soybean plants (Sabharwal et al., 2022). As noted above, PS expression in either of these sub-cellular domains could impact the gene expression changes observed.

In addition to PS, several other plant apyrases have been ectopically expressed in the model plant Arabidopsis in order to gain insights into their function. One such study expressed two apyrases from the drought tolerant *Populus euphratica* tree species, *PeAPY1* and *PeAPY2*, in Arabidopsis (Zhang et a., 2021). This study found that expression of either of these Poplar apyrases resulted in a drought-tolerant phenotype in Arabidopsis seedlings. Just as was observed in *PS*-expressing Arabidopsis (Veerappa et al., 2019), the expression of *PeAPY1* and *PeAPY2* in Arabidopsis resulted in an increased sensitivity to ABA during stomatal closing. The transgenic *PeAPY1-OE* and *PeAPY2-OE* lines also showed enhanced ABA inhibition of light-induced stomatal opening. Additionally, ABA treatment of wild-type seedlings induced upregulation of NADPH-oxidase expression (*AtRBOHD* and *AtRBOHF*), confirming the important role that reactive oxygen species (ROS) plays in ABA regulation of stomatal aperture. Notably, this upregulation was greatly increased in the PeAPY1-OE and PeAPY2-OE transgenic lines compared to wild-type.

The results of Zhang et al. (2021) were consistent with multiple other reports showing that an increase in ROS levels is an important early signaling step in eATP regulation of growth in wild-type plants (Myers et al., 2022), and that changes in the expression level of apyrases regulates growth. They also further confirmed a key role for PeAPY2 in stress responses that was originally reported by (Deng et al., 2015), who showed that *PeAPY2* expression in Arabidopsis allowed for better primary root growth and increased survival to cold-stress treatment.

Apyrase signaling interactions with other proteins

As noted above, the expression of PeAPY2 and other plant apyrases significantly impacts eATP and ABA responses, stomatal functions, and vesicular trafficking, all of which are regulated by Ca^{2+} signaling. It seems likely, then, that transduction events mediated by plant apyrases would intersect with events mediated by Ca^{2+}-regulated proteins in plants, such as calmodulin and annexin. In this regard, it is relevant to note that members of the annexin family of Ca^{2+} -binding proteins have been shown to play important roles in eATP signaling. For example, one of the earliest detectable eATP-induced signaling steps is an increase in the $[Ca^{2+}]_{cyt}$ and annexin 1 facilitates this eATP-induced Ca^{2+} influx in Arabidopsis roots (Mohammad-Sidik et al., 2021). Annexin 2 and annexin 4 also play roles in the eATP- and eADP-induced increases in $[Ca^{2+}]_{cyt}$ in Arabidopsis roots (Mohammad-Sidik, 2019). Interestingly, the eATP-induced calcium signature in roots is diminished by phosphate starvation (Matthus et al., 2019).

In the Slocum et al. (2023) study previously discussed, transcriptomic analyses revealed that growth on Pi-deficient media induced an increase in transcript levels of annexin 1 in both wild-type and *APY1 OE* seedlings This upregulation of annexin 1 expression in response to Pi starvation may occur to help restore normal eATP-mediated calcium signaling in roots. The Slocum et al. (2023) study also found that when seedlings were grown on Pi-sufficient media, NTP supplementation resulted in the down-regulation of annexin 3 and annexin 4 in *APY1 OE* seedlings but not in wild-type seedlings.

Annexins also participate in auxin signaling mediated by apyrases and eATP in Arabidopsis. Annexin 3 helps regulate eATP-induced changes in seedling growth and polar localization of auxin transporters (Xu et al., 2023). Similarly, annexin 1 and annexin 2 play key roles in regulating PIN3 localization and auxin transport during seedling phototropic response (Wang et al., 2022). Further exploring the connections between annexins and apyrases in regulating eATP signaling would be a fertile area of future research.

Apyrase interactions with other proteins would also have important implications for their specific cellular functions. There is a critical need for more research identifying the protein partners that bind to apyrase and that may be required for its functions in different sub-cellular domains. Prior research has implicated some potential interactors, including ROP1-GTPase, which could partner with AtAPY1 in regulating pollen germination and elongation (Li et al., 2020), and a copper amine oxidase, which could interact with apyrases known to affect extracellular ROS levels during fungal attack (Toyoda et al., 2012). However, these initial studies should be confirmed and extended by independent methods such as yeast-two hybrid and co-IP assays.

Apyrase inhibitors also alter the growth rate of plant tissues

Previous sections of this review focused on transgenic approaches (constitutive expression and knockouts) that have been used to gain new insights on the roles of apyrases in regulating plant growth and development. Of course, apyrase expression can also be controlled post-transcriptionally, and a valuable approach to achieve this has been to use chemical inhibitors of apyrase. Windsor et al. (2002, 2003) reported an assay to find and test the effectiveness of such inhibitors. Using a colorimetric assay method, they assayed a library of small chemical compounds and identified several that could strongly inhibit potato APY. These compounds were relatively specific because they had less suppressive effect on the activity of other ATPases such as alkaline and acid phosphatases and luciferases (Windsor et al., 2002). The effectiveness of two of these inhibitors in enhancing the effectiveness of herbicides was demonstrated by increasing the [eATP], which suppressed the ability of ABC transporters to export the herbicides (Windsor et al., 2003), as previously described (Thomas et al., 2000).

These inhibitors were effective in blocking the activity of ecto-apyrases in diverse plants. As summarized in a prior review (Clark et al., 2021), treating Arabidopsis tissues with these inhibitors mimicked the effects of genetically suppressing apyrase expression by increasing the [eATP] of growing cells and decreasing their growth rate (Wu et al., 2007). For example, both high [eATP] and genetic suppression of apyrase expression block auxin transport (Tang et al., 2003; Liu et al., 2012), inhibit the growth of pollen tubes (Wu et al., 2007), and suppress root growth and root skewing (Yang et al., 2015).

Apyrases are highly conserved throughout the evolution of eukaryotes, with at least four primary sequence domains that are highly similar in widely divergent organisms, including, for example, eudicots, ferns, and fungi (Clark et al., 2014). So, it was not surprising when Tripathy et al. (2017) found that the same inhibitors that suppressed growth in Arabidopsis also did so in

pathogenic fungi (Tripathy et al., 2017). This result underscored the value of apyrase inhibitors in enhancing the potency of fungicides, just as they enhanced the potency of herbicides (Windsor et al., 2003). During infections, pathogens and pests improve the effectiveness of their attack on plants by secreting enzymes that lower the concentration of the eATP that plants use to induce their defense responses. For example, the pathogenic bacterium *Pseudomonas syringae* secretes NTPases as it is invading plant tissues. Pathogenic bacteria harboring a mutation that suppresses their ability to secrete these enzymes are unable to lower eATP levels and less able to infect plants (Tanaka and Heil, 2021).

Herbivores such as corn earworm and whitefly larvae secrete saliva containing ATP-hydrolyzing enzymes. At wound sites, these enzymes block the expression of defense associate genes. This is an evolutionarily developed function that pathogens and pests use to suppress the plant defense responses that are dependent on the eATP signal. Similarly, to promote their colonization of roots, symbiotic rhizobia and an endophytic fungus activate plant ectonucleotides and secrete their own nucleotidases that limit [eATP]. Parasitic worms and bloodsucking insects also follow similar mechanisms to regulate the eATP/ADP concentrations (Tanaka and Heil, 2021).

More recently, Paes-Vieira et al. (2021) reported the major role of ecto-apyrases (e-NTPDases) in host-parasite interactions. *Leishmania amazonensis* infects the host cell by changing the immune response of macrophages, and it uses e-NTPDases to suppress the immune defense system of hosts by bringing down the level of ATP and ADP. This enzyme activity also produced AMP that was subsequently converted to adenosine, which then reduced the inflammatory response. During the parasite development, the expression of two genes encoding NTPDases, *ntpd1* and *ntpd2,* is differentially regulated. Promastigotes of *L. amazonensis* that overexpress either the *ntpd2* gene alone, or both *ntpd1* and *ntpd2* genes simultaneously, were more infective to macrophages than controls. Yet, mice that were transfected with parasites overexpressing *ntpd1* and *ntpd2* had fewer lesions than control. As explained by the authors, this contradictory effect of *ntpd1* and *ntpd2*, may be due to high levels of adenosine, and the activity of at least two different ecto-enzymes that hydrolyze nucleotides, e-NTPDase and ecto-5'- nucleotidase. The combined activity of these two enzymes would interfere with the balance of the immune response to promote the pathogen clearance and maintain the host protection. Overall, across multiple kingdoms, eATP evolved beyond just functioning as chemical energy to act as a danger signal that regulates many different cellular processes (Paes-Vieira et al., 2021).

The several APY inhibitors that have been used in plant publications so far (Clark et al., 2021) have amphipathic structures. This would allow them to cross cell membranes and thus inhibit not only ecto-apyrases, but also intracellular apyrases, including those in nuclei. It would be instructive to learn whether the effects of apyrase inhibitors on gene expression, like the effects of high [eATP] on auxin transport and organ growth, are similar to those observed in apyrase null mutants.

Conclusions and Unanswered questions

This review serves as an update to prior reviews, with a focus on those publications related to the regulation of growth by eATP and apyrases that have been published in the last three years. The new work has further confirmed prior evidence, and expanded it by providing even more data consistent with the hypothesis that, in addition to ecto-apyrase activities, the nuclear functions of apyrases could also play a major role in regulating the growth and development of plants.

This review also highlighted unanswered questions that will require more research to answer (see Outstanding Questions). Experimental approaches in progress that could help answer these questions were discussed, but other, untried methodologies could also yield valuable insights. For example, single cell proteomics and transcriptomics of seedling tissues (e.g., Clark et al., 2022) could demark more precisely those specific root or cotyledon cells that are expressing higher levels of apyrase and determine how closely these levels correlate with the growth rate of these cells. New high resolution fluorescence microscopy methods, such as MINSTED nanoscopy (Weber et al., 2023) would be able to determine sub-nuclear APY localizations (nuclear membrane, nucleoplasm, chromatin?). The application of both classical and newly developed methods will undoubtedly provide unexpected and exciting answers to clarify the mechanisms by which apyrases regulate growth in plants.

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