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Role for apyrases in polar auxin transport in Arabidopsis

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FOOTNOTES

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ABSTRACT

Recent evidence indicates that extracellular nucleotides regulate plant growth. Exogenous ATP has been shown to block auxin transport and gravitropic growth in primary roots of Arabidopsis. Cells limit the concentration of extracellular ATP in part through the activity of ectoapyrases (ecto-nucleoside triphosphate diphosphohydrolases), and two nearly identical Arabidopsis apyrases, APY1 and APY2, appear to share this function. These findings, plus the fact that suppression of APY1 and APY2 blocks growth in Arabidopsis, suggested that the expression of these apyrases could influence auxin transport. This report tests that hypothesis. The polar movement of \[^{3}\text{H}]\text{IAA} \text{ in both hypocotyl sections and primary roots of Arabidopsis seedlings was measured. In both tissues polar auxin transport was significantly reduced in } \text{apy2 null mutants when they were induced by estradiol to suppress the expression of APY1 by RNAi. In the hypocotyl assays the basal halves of APY-suppressed hypocotyls contained considerably lower free IAA levels when compared to wild-type plants, and disrupted auxin transport in the APY-suppressed roots was reflected by their significant morphological abnormalities. When a GFP fluorescence signal encoded by a DR5:GFP construct was measured in primary roots whose apyrase expression was suppressed either genetically or chemically, the roots showed no signal asymmetry following gravistimulation, and both their growth and gravitropic curvature were inhibited. Chemicals that suppress apyrase activity also inhibit gravitropic curvature and, to a lesser extent, growth. Taken together these results indicate that a critical step connecting apyrase suppression to growth suppression is the inhibition of polar auxin transport.}
INTRODUCTION

In both animals and plants, cells release nucleotides into their extracellular matrix, where they function as signaling agents, inducing rapid increases in the concentration of cytosolic calcium that are transduced into downstream changes in cell physiology (Kim et al., 2006; Burnstock, 2007; Roux and Steinebrunner, 2007; Tanaka et al., 2010a; Tanaka et al., 2010b; Demidchik et al., 2011). Prominent among these downstream changes in plants are changes in the growth of cells, including the growth of pollen tubes (Steinebrunner et al., 2003), root hairs (Clark et al., 2010b), and cotton fibers (Clark et al., 2010a). These results suggest the possibility that the signaling changes induced by extracellular nucleotides intersect with signaling changes induced by one or more of the hormones that regulate plant cell growth. Consistent with this possibility, Tang et al. (2003) showed that a concentration of applied nucleotides that inhibited the gravitropic growth of roots could block the transport of the growth hormone auxin, and that this effect could not be attributed to either pH changes or chelation of divalent cations. Correspondingly, Clark et al. (2010a) showed that when the application of nucleotides to cotton ovules growing in culture altered the rate of cotton fiber growth, it also induced the production of ethylene, a hormone known to regulate the growth of cotton fibers.

Given the potency of extracellular nucleotides to regulate cellular activities, it would be important for cells to control the concentration of these nucleotides. In both animals and plants the principal enzymes that limit the build-up of extracellular ATP (eATP) and eADP are ectoapyrases (apyrase, E.C. 3.6.1.5). These enzymes, which are nucleoside triphosphate diphosphohydrolases, are characterized by apyrase-conserved regions (ACRs) whose peptide sequences are highly similar throughout the plant and animal kingdoms (Clark and Roux, 2009). Based on this structural criterion, there are seven apyrases in Arabidopsis (APY1 through APY7), and two of these, APY1 and APY2, share 87% protein sequence identity but are less than 30% similar to the other 5 apyrases. These two apyrases partially complement each other’s function and play central roles in growth control in Arabidopsis, as judged both by genetic and biochemical criteria (Wu et al., 2007; Wolf et al., 2007). Polyclonal antibodies raised to APY1 (Steinebrunner et al., 2000) inhibit the apyrase activity released into the medium of growing pollen tubes, and when these antibodies were added to the culture medium of germinated pollen they both blocked the growth of the pollen and raised the concentration of ATP in the medium (Wu et al., 2007). Similarly, treatment of cultured cotton ovules with antibodies that recognize
cotton fiber apyrase both inhibit the growth of the fibers and increase the concentration of ATP in the medium, further establishing the link between apyrase activity and regulation of the [eATP] in growing tissues (Clark et al., 2010a).

Because wild-type pollen tubes expressing active APY1 or APY2 and cultured cotton fibers with wild-type apyrase activity grow at a normal rate, and because the antibodies inhibit apyrase activity (Wu et al., 2007), the growth inhibition induced by the antibodies further implicated apyrase activity as critical for the growth of these tissues. The antibodies were unlikely to enter the pollen tubes or cotton fibers, so these results also suggested the pollen and cotton apyrases were ectoapyrases. However, these data do not rule out a possible Golgi function for APY1 and APY2 and for the cotton APY(s), as discussed by Wu et al. (2007) and Clark and Roux (2011). In fact, there is strong evidence that APY1 and APY2 are localized in the Golgi and may function there to regulate protein glycosylation and/or affect polysaccharide synthesis (Chiu et al., 2012; Schiller et al., 2012).

Although suppression of APY1/APY2 or of apyrase activity has a dramatic effect on growth, overexpression of APY1 or APY2 has much less of an effect. Constitutive expression of APY1 induces a small but statistically significant increase in the growth of etiolated hypocotyls, while overexpressing APY2 has no effect on this growth (Wu et al., 2007). This is probably because the wild-type levels of apyrase expression are near optimal for growth (Roux and Steinebrunner, 2007).

The double knockout apy1apy2 is sterile because the pollen of this mutant does not germinate (Steinebrunner et al., 2003). However when APY1 is suppressed only ~60% by an inducible RNAi construct in apy2 null mutants, pollen of these mutants will germinate, permitting fertilization and subsequent normal development, although the adult plants of these mutants are dwarf (Wu et al., 2007). Suppression of ectoapyrase activity would be expected to raise the equilibrium concentration of eATP (Wu et al., 2007), and since higher levels of eATP can inhibit auxin transport in roots (Tang et al., 2003), it was reasonable to hypothesize that suppression of apyrase by RNAi could suppress auxin transport. The experiments described in this report test this hypothesis. The results indicate that suppression of APY1/APY2 expression in an inducible RNAi line R2-4A (Wu et al., 2007) results in a significant inhibition of polar auxin transport in Arabidopsis hypocotyls and roots with a concomitant altered distribution of endogenous auxin. Consistent with this result and with the results of Tang et al. (2003),
suppression of APY1/APY2 also blocks the asymmetric distribution of a GFP reporter encoded by a DR5:GFP construct in gravistimulated primary roots of Arabidopsis seedlings, and diminishes the extent of the elongation zone in these roots. These results are consistent with the novel conclusion that inhibition of auxin transport is a key step in the signaling pathway that links the inhibition of apyrase expression to growth inhibition.

RESULTS

APY1 and APY2 play a role in polar auxin transport in hypocotyls and roots

To determine if APY1 and APY2 play a role in auxin transport we assayed polar auxin transport in hypocotyls of loss- and gain-of-function apyrase mutants, including the RNAi line R2-4A in which the expression of APY1 in the apy2 null background can be suppressed by estradiol treatment. First, we tested whether the estradiol-treated R2-4A seedlings grown in the conditions used for the auxin transport assays (6-d growth in the dark followed by 2-d growth in the light), showed the inhibited growth phenotype previously reported (Wu et al., 2007). We observed inhibition of hypocotyl and root growth under these conditions (Fig. 1A). In hypocotyls IAA moves in a single basipetal or rootward polarity, with acropetal or shootward transport at background levels. We assayed basipetal and acropetal transport of [3H]IAA in hypocotyl sections from wild-type (Col-0 and Ws ecotype) and the non-induced R2-4A seedlings, which, without estradiol treatment, is an apy2 single knockout. We found that basipetal auxin transport was not statistically different in all three genotypes, just as the background of acropetal auxin movement (Fig. 1B). The auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) inhibited basipetal auxin transport to the same degree in each of these genotypes. However, we found that suppression of APY1 in induced R2-4A plants resulted in significant inhibition of basipetal auxin transport (Fig 1C). Next we tested polar auxin transport in the single apy1 and apy2 knockout lines as well as in the corresponding overexpressing lines for each of these apyrases. There was no difference observed in IAA transport in the hypocotyl assay between single knockout lines and Ws wild-type (Fig 1D). In contrast the APY1-OE line showed a statistically significant increase in polar auxin transport compared to Ws wild-type (p < 0.05), while the APY2-OE line showed a strong, but marginally insignificant (p < 0.07) increase compared to Ws wild-type (Fig 1D). The APY1-OE line was also more resistant than wild-type plants to the inhibitory effects of 600 µM ATPγS on hypocotyl growth (Supplemental Figure S1). The growth of R2-4A
hypocotyls was also more resistant to treatment with 600 μM ATPγS, possibly because their growth is already inhibited. Basipetal polar auxin transport was inhibited in hypocotyls of the estradiol-induced R2-4A line to the same level that NPA inhibited transport in hypocotyls of the untreated and treated Ws wild-type seedlings.

In order to determine whether the altered hypocotyl-section auxin transport results were reflected in changes in the distribution of endogenous auxin, we measured the levels of free IAA in the shoot apices, apical halves, and basal halves of Arabidopsis hypocotyls from estradiol-treated and untreated whole seedlings from Ws wild-type and the R2-4A plants. Our results showed that there was significantly less free IAA found in the basal half of hypocotyls from the estradiol treated R2-4A line compared to basal halves of hypocotyls from estradiol-treated Ws wild-type (Fig 2A). There was also more free IAA in the shoot apices of estradiol-treated R2-4A seedlings compared to estradiol-treated Ws wild-type apices (p < 0.07). Estradiol treatment had no effect on IAA levels in hypocotyls of Ws wild-type seedlings. The highest level of free IAA was observed in the apices of estradiol-treated R2-4A seedlings, with an average of 6.73 ng/g FW IAA, while the lowest level of free IAA was observed in the basal half of the estradiol-treated R2-4A plants, with an average of 1.13 ng/g FW IAA (Fig. 2B). These results are consistent with those from the hypocotyl section transport assays, suggesting that reduced basipetal auxin transport leads to a reduction of free IAA in the lower portion of the tissue and increased IAA in the upper portion of the plant when APY1 is suppressed in the R2-4A line.

Since polar auxin transport is inhibited in hypocotyls of induced R2-4A plants, we also measured IAA transport in induced R2-4A roots, as extracellular ATP has already been implicated in regulation of root basipetal IAA transport (Tang et al., 2003). In contrast to hypocotyls, IAA moves in two polarities in roots in two distinct transport streams, with basipetal (or shootward) transport occurring in the epidermal cells in the root tip and linked to root gravitropism and elongation (Rashotte et al. 2000). Acropetal or rootward IAA transport occurs in the central cylinder and is linked to control of lateral root formation (Reed et al. 1998). IAA transport values are reported as percent of transport in wild-type. Basipetal IAA transport was significantly reduced in induced R2-4A (p < 0.04) (Fig. 3). In contrast, acropetal IAA transport was not altered in this RNAi line. This finding is consistent with other regulatory strategies, such as reversible protein phosphorylation, that show differential regulation of these two polarities of root IAA transport (Rashotte et al. 2001; Sukumar et al. 2009).
examined the effect of the induction of this RNAi construct on root growth and development, and auxin induced gene expression in this tissue, focusing on root apical development and growth and gravitropism, as these processes are linked to basipetal IAA transport.

**Suppression of APY1 expression in apy2 DR5:GFP plants induces altered GFP expression and morphological changes in roots**

Both hypocotyl and root growth are inhibited in the estradiol-treated R2-4A seedlings, but primary root length is more dramatically affected in this line. We tested whether the reduced root growth was accompanied by altered auxin induced gene expression using a DR5:GFP reporter. The transgenic DR5:GFP plants were crossed with the R2-4A RNAi mutant line to introduce DR5:GFP into the RNAi mutant. We performed a time-course treatment of the R2-4A seedlings with estradiol and used confocal microscopy to monitor the GFP signal, thereby evaluating the endogenous auxin response in roots by this indirect method. Treatment with estradiol for 1 d or 2 d did not induce changes in the GFP signal or in morphology of the Ws wild-type or R2-4A root (Supplemental Fig. S2). However, after 3 d of estradiol treatment, when R2-4A roots begin exhibiting reduced APY1 expression (Supplemental Fig. S3) and reduced root growth, changes in the GFP signal begin to appear (Fig 4A-D). The GFP signal is reduced in the columella and the lateral root cap and epidermal cells at the root tip. After 4 d of estradiol treatment the typical pattern of GFP distribution in Ws wild-type primary roots is even more disrupted in the mutant, with more of the GFP signal observed in root cortex cells associated with the distal root vasculature and epidermal cells in the zone of the root where root hairs begin differentiating (Fig. 4E-H). These results suggest that the suppression of apyrases mimics the effects of treating wild-type seedlings with high concentrations of ATP, and that one mode by which apyrase suppression can inhibit root growth is by disrupting the normal pattern of auxin transport.

The light microscope images of estradiol-treated R2-4A roots indicated that root morphology was greatly altered as apyrase expression was suppressed. In order to better examine the structure and determine the specific regions of the root affected in the R2-4A line, scanning electron microscopy was performed on Ws wild-type roots and mutant roots with suppressed apyrase expression after treatment with estradiol for 6 d. In the mutants, many differentiated root hairs, which are a mark of the maturation zone, were observed extending all the way from...
the root-shoot junction to near the meristematic zone just basal to the root cap (Fig. 5). The mutant roots also showed a lack of a well-defined meristematic zone, a greatly reduced zone of elongation, as well as a larger diameter near the tip than Ws wild-type seedlings. When DR5:GFP R2-4A seedlings are grown on estradiol for 6 d, the GFP fluorescence accumulates in most cells near the root tip, and the auxin maximum is not apparent in the quiescent zone, in contrast to the strong maximum seen in Ws wild-type roots (Supplemental Fig. S4).

**Root gravitropic response is altered by genetic suppression of apyrase expression and chemical inhibition of apyrase activity**

In R2-4A roots that were not treated with estradiol there was asymmetry of GFP fluorescence, similar to that observed in DR5:GFP Ws wild-type roots (data not shown). In contrast to DR5:GFP seedlings that have normal APY1 and/or APY2 expression, there is no asymmetry of GFP fluorescence in RNAi-suppressed roots of DR5:GFP-expressing seedlings after gravistimulation (Fig. 6A, B). A similar result was observed in wild-type DR5:GFP seedlings when they were treated with 800 µM ATPγS or with apyrase inhibitor NGXT1913; i.e., in these seedlings also there is no asymmetry of GFP signal after gravistimulation (Fig. 6C-F). These results suggest that apyrases and extracellular nucleotides may play a role during root gravitropism, so we gravistimulated both RNAi-suppressed plants and Ws wild-type plants treated with a chemical inhibitor of apyrase inhibitor, NGXT1913 (Windsor et al., 2002). The growth of estradiol-induced R2-4A roots is significantly decreased in comparison to Ws wild-type roots (Wu et al., 2007), and this growth inhibition would be expected to also inhibit gravitropic growth, which is what we observed (data not shown). Treatment with the apyrase inhibitor NGXT1913 (Windsor et al., 2002) also decreased both the gravitropic angle and elongation growth of Ws roots reoriented relative to the gravity vector, although its effect on gravitropism was significantly greater than its effect on growth (Supplemental Fig. S5). A similar result (data not shown) was obtained with another apyrase inhibitor, NGXT191 (Windsor et al., 2003). These results are consistent with the inference that apyrase activity also contributes to the lateral transport of auxin that is needed for the gravitropic response.

We also tested the effects of NGXT1913 on the growth of wild-type and R2-4A hypocotyls treated with estradiol. Inhibitor treatment reduced the growth of wild-type hypocotyls by a statistically significant 13% (p < 0.05), but it inhibited the growth of R2-4A mutants
significantly less (6.5%; p < 0.05), which would be expected since the mutants already have suppressed growth even without NGXT1913 treatment. These hypocotyl results were the average of 4 biological repeats, with n ≥ 30 for each repeat.

Both cell elongation and mitosis are inhibited when apyrase expression is suppressed in primary roots

After R2-4A plants were treated with estradiol there was a reduction in the overall length of their primary roots (Table I) due to a combination of factors. The overall dimensions of the quiescent center in both Ws wild-type and R2-4A plants were approximately the same (data not shown), but in R2-4A plants the length of the mitotic zone (MZ) and the elongation zone (EZ) were shorter than in Ws plants (Table I). Measurement of cell lengths in these zones showed R2-4A plants had much less uniform cell sizes than Ws wild-type plants, a difference that was quantified by showing that a higher percentage of cells in R2-4A plants were smaller than half the mean diameter compared to Ws wild-type plants (Supplemental Table I). Linear counts of cells showed there were fewer cells in both the mitotic and elongation zone of R2-4A plants than Ws wild-type plants (Table II). When taken as a whole, the data revealed that the reduction in length of R2-4A roots was due to both fewer cells in the MZ and EZ, and less expansion in the EZ.

Growth-inhibiting levels of applied nucleotides do not alter the localization of PIN1, PIN2, AUX1, or ABCB19 transporters

Dose-response assays of the effect of applied ATPγS on the growth of etiolated hypocotyls and light-grown roots revealed that concentrations between 500 and 800 µM were needed to have a significant inhibitory effect (Butterfield, 2006; Roux et al., 2000; Tang and Roux, unpublished). However, 800 µM ATPγS treatment does not alter the distribution of either PIN1 or ABCB19, which participate in acropetal IAA transport, or PIN2 and AUX1, which participate in basipetal IAA transport, in light-grown roots (Supplemental Figs. S6 and S7). Additionally, ATPγS treatment significantly inhibits the elongation growth of primary roots in pin2 and aux1 mutants just as it does in wild-type seedlings (data not shown).

Gravity response of Col-0 roots is inhibited by applied ATP
The original experiments on the effects of applied ATP on root gravitropic growth were carried out in the Ws ecotype (Tang et al., 2003). To test whether ATP–induced growth effects could be seen in Col-0, the original tests of ATP effects on root growth and gravitropism, were repeated in Col-0 seedlings. The results indicated that in this ecotype 1 mM ATP could significantly inhibit root gravitropism without significantly inhibiting growth (Supplemental Table S2), whereas in Ws 3 mM was the lowest concentration that had inhibitory effects (Tang et al., 2003). In Col-0, as in Ws, 2 mM and 5 mM ATP significantly inhibited gravitropism, but in Col-0, unlike in Ws, these higher concentrations also had a significant inhibitory effect on root growth, although the growth effects (< 2-fold) were much smaller than the curvature effect (> 4.5-fold). In the Col-0 tests, the pH in ATP-containing media remained at or above 5.0, a pH that by itself does not inhibit gravitropic growth. Overall the data indicated that the gravitropic growth of Col-0 ecotype roots was more sensitive to the inhibitory effects of applied ATP than Ws roots.

DISCUSSION
In etiolated hypocotyls, pollen tubes, cotton fibers, and root hairs low concentrations of applied nucleotides (ATPγS or ADPβS) promote growth, and higher concentrations inhibit growth, so there appears to be an optimal [eATP] for growth (Wu et al, 2007; Roux and Steinebrunner, 2007; Reichler et al., 2009; Clark et al., 2010a, 2010b). These dose-response results are qualitatively similar to the bimodal dose-response curves obtained using auxin (Mulkey et al., 1982) or ethylene (Pierek et al., 2006). Studies in Arabidopsis indicate that APY1 and APY2 may function in part as ectoapyrases to regulate eATP levels outside the cell (Wu et al., 2007). Both are abundant in rapidly growing tissues and appear to be needed to maintain optimal eATP levels for normal plant growth (Wu et al., 2007; Wolf et al., 2007). There is recent evidence that APY1 and APY2 localize to Golgi where they could function to regulate protein glycosylation and/or polysaccharide levels (Dunkley et al., 2004; Riewe et al., 2008; Chiu et al., 2012; Parsons et al., 2012; Schiller et al., 2012).

Given the complex interaction of signaling pathways, the steps that lead from eATP to growth control are likely to intersect at some point with transduction steps induced by hormones. Consistent with this idea, applied nucleotides disrupt basipetal auxin transport in maize and
Arabidopsis roots (Tang et al., 2003). This finding led us to hypothesize that suppressing the expression of \textit{APY1} and \textit{APY2} could affect auxin transport in Arabidopsis seedlings. We tested this idea directly by examining whether plants of the RNAi line R2-4A that are suppressed in the expression of \textit{APY1} in the background of \textit{apy2} null knockout would show disrupted auxin transport. We demonstrate that both in hypocotyls and roots reduction in apyrase expression using RNAi leads to reduction in basipetal auxin transport in these tissues in which growth is altered by apyrase suppression. Our data suggest that the growth suppression by \textit{APY} mRNAi is at least partially due to auxin transport inhibition.

Correspondingly, suppression of \textit{APY1/APY2} expression results in an altered GFP reporter pattern in \textit{DR5:GFP}-expressing roots, consistent with there being altered auxin distribution (or sensitivity) in the roots of these mutants. Estradiol-treated R2-4A seedlings also have reduced lateral root formation (Wu et al., 2007) and root acropetal IAA transport. This is true also for mutants in genes encoding auxin transport proteins (Marchant et al., 2002; Benkova et al., 2003; Lewis et al., 2011) and in wild-type plants treated with inhibitors of polar auxin transport (Reed et al., 1998; Casimiro et al., 2001).

One mechanism by which \textit{APY1/APY2} suppression could block auxin transport would be by suppressing the transcript abundance of genes that are involved in auxin transport. Polar auxin transport in plants is well characterized and is mediated by several protein families, \textit{AUXIN RESISTANT1/LIKE AUXIN1 (AUX1/LAX)} influx carriers, \textit{PIN-FORMED (PIN)} efflux facilitators and \textit{ATP binding Cassette subfamily B (ABCB)} transporters (Peer et al., 2011). To test this hypothesis we have used microarray and qRT-PCR to examine potential expression changes for \textit{AUX1}, \textit{LAX3}, \textit{ABCB1}, \textit{ABCB4}, \textit{ABCB19}, \textit{PIN1}, \textit{PIN2}, \textit{PIN3}, \textit{PIN4}, \textit{PIN5}, \textit{PIN6}, \textit{PIN7} and \textit{PIN8}. We compared message levels for these genes in both dark- and light-grown R2-4A and wild-type seedlings and found that although there were major changes in expression of many genes there were no significant differences in the message levels of any of these auxin transporters/carriers (data not shown; Wu, Yao and Roux, manuscript in preparation). However, as noted below, these results do not rule out the possibility that apyrase suppression could alter the post-transcriptional activity of auxin transporters.

Rahman et al. (2007) characterized the inhibitory effects of applying IAA or auxin analogs as well as chemical inhibitors of auxin transport on root growth. This study, which used treatment concentrations that decreased root growth rates by 50%, showed that these compounds...
could be separated into two groups based on their mode of growth inhibition. Indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), and tri-iodobenzoic acid (TIBA) inhibit root growth by decreasing the size of the elongation zone while 2,4-dichlorophenoxy-acetic acid (2,4-D) and naphthylphthalamic acid (NPA) inhibit root growth mainly by decreasing the rate of cell production in the meristem. We analyzed morphological changes in root development by performing a time course evaluation of the effects of estradiol treatment in the R2-4A RNAi line. Our results indicated that apyrase suppression both altered the rate of cell expansion in the elongation zone and disrupted normal patterns of cell division. Thus the decrease in root length should be attributed to diminished rates of both cell elongation and mitosis, which is an expected result of auxin transport inhibition. The swelling and epidermal cell blebbing also observed in primary roots would suggest an alteration in cell wall integrity, which could be induced by 1-aminocyclopropane-1-carboxylic acid (ACC; Tsang et al., 2011) or ethylene, which both affect and are affected by auxin transport changes in roots (Muday et al., 2012).

Prolonged treatment of wild-type roots with high concentrations of eATP or apyrase inhibitors does not result in the same severe root morphological abnormalities observed in estradiol-treated R24A roots (data not shown). Additionally, there are rapid gene expression changes observed in estradiol-treated R2-4A roots, and recent data indicate that APY1 and APY2 localize to and function in the Golgi (Dunkley et al., 2004; Chiu et al., 2012; Parsons et al., 2012; Schiller et al., 2012). Thus even though suppressing APY1 and APY2 expression increases eATP levels and disrupts basipetal auxin transport it seems probable that the R2-4A root phenotype may be due to effects of suppressing apyrase expression that are not directly related to an increase in the eATP concentration.

Applied nucleotides induce a rapid increase in the transcript abundance of genes encoding ACC synthase 6 and ethylene response factor 4 in Arabidopsis (Jeter et al., 2004; Song et al., 2006). Correspondingly, higher levels of applied nucleotides can induce ethylene production in the cotton fibers of cultured ovules, and the ACC synthesis inhibitor aminovinylglycine (AVG) can block the effects of applied ATPγS and ADPβS on the growth of fibers (Clark et al., 2010a), hypocotyls (Roux and Steinebrunner, 2007), and root hairs (data not shown). Thus it would be expected that in addition to auxin-mediated effects on root growth and development there might also be a role for ACC and/or ethylene in the R2-4A root phenotype. In fact, many of the root-tip anatomical changes described in Tables I, II, and Supplemental Table I,
and in Figure 4 can be influenced by ethylene (Ma et al., 2003). We also found that application of 1 µM AVG to estradiol-treated R2-4A seedlings had a slight but statistically significant promotive effect on their root growth (data not shown). The interaction between auxin and ethylene in root growth and development is complex, with these two hormones acting synergistically and antagonistically in different processes in the root (Muday et al. 2012). Furthermore, alterations in auxin transport and auxin levels induce ethylene production in the root elongation zone, while increased ethylene results in increased auxin levels in the root cortex and quiescent center (Bennett and Scheres, 2010).

Chemical inhibition of apyrase activity by application of apyrase inhibitors results in growth inhibition of pollen (Wu et al., 2007) and cotton fibers (Clark et al., 2010a). Based on the altered auxin transport phenotype observed in the R2-4A line, where expression of APY1 and APY2 are genetically suppressed, one might predict that application of apyrase inhibitors could also affect polar auxin transport. Our results show that the apyrase inhibitor NGXT1913 does indeed suppress the gravitropic response of roots, but, unlike NPA, it does not block polar auxin transport in hypocotyl sections (data not shown). This may reflect its better penetration into roots than into cuticle-covered hypocotyls, or its limited disruption of only the lateral transport of auxin needed for gravitropism, rather than of the basipetal polar transport measured in the hypocotyl assays.

As discussed earlier, ABCB19 (MDR1) and other ABCB proteins have been shown to facilitate auxin transport (Lewis et al., 2009; Titapiwatanakun et al., 2009). Thomas et al. (2000) described a potential role for ectoapyrases and eATP in regulating the transport activity of ABCB19. The molecular mechanism by which ABCB proteins transport the efflux of compounds is poorly understood, and, although hydrolysis of cytoplasmic ATP is required for their activity, it is also possible that a gradient of ATP from inside to outside the cell is needed for their proper function. In agreement with this idea Lee et al. (2011) have shown that expression of an ABCA1 protein in animal cells results in increased levels of eATP in these cells and that changes in [eATP] regulate ABCA1 transport activity. To the extent that [eATP] could influence ABCB transport activity in plant cells, this could be a plausible mechanism by which inhibition of ectoapyrase activity, which would raise the [eATP], inhibits auxin transport.

Applied nucleotides induce nitric oxide (NO) production in plant cells (Foresi et al., 2007; Torres et al., 2008; Wu and Wu, 2008; Reichler et al., 2009; Clark et al., 2010b). Thus
another possible mechanism by which ectoapyrases and eATP levels could regulate auxin signaling is by inducing increased nitric oxide (NO) levels. The two best documented mechanisms by which NO can affect plant growth and development are by increasing the level of the cGMP signal and by nitrosylation of the cysteine residues of key enzymes, a reversible post-translational modification that can alter protein function. A recent report found that the TIR1 auxin receptor can be nitrosylated and that its nitrosylation regulates its interaction with AUX/IAA proteins, thereby controlling their degradation (Terrile et al., 2011). Additionally, Fernandez-Marcos et al. (2011) showed that high levels of NO inhibit PIN1-dependent auxin transport in Arabidopsis roots. Speculatively, the increased NO production induced by apyrase suppression could result in nitrosylation and inhibition of one or more of the proteins that drive auxin transport. This would provide a mechanistic basis for understanding the link between the suppression of APY1/APY2 and the inhibition of polar auxin transport.

MATERIALS AND METHODS

Polar auxin transport measurement in Arabidopsis hypocotyl sections

Seedlings of Arabidopsis ecotype Ws, RNAi line R2-4A (apy2 Ws transformed with APY1 mRNAi), apy1 single knockout, apy2 single knockout, APY1 overexpressor (OE), or APY2 OE were grown in darkness for 6 d and transferred to continuous cool white fluorescent light (80 μmol m⁻² s⁻¹) for 2 d. The assay was performed as described in Liu et al. (2011) with slight modifications. Briefly, agar donor blocks containing 10⁻⁷ M [³H]IAA were placed in contact with the apical end of 6 mm hypocotyl sections, an agar receiver block was placed on the basal end of each section, and transport was allowed to occur for 3 h. Agar blocks (1.5%, 2 mm x 2 mm) were separated from the agar plates by pieces of plastic wrap. Sections were kept upside-down in a humid chamber during the transport period. Each section was then split into apical and basal halves, and radioactivity was determined in each half and in the receiver blocks. Data are expressed as dpm in the receiver block plus the basal portion of the tissue as a % of total dpm in the tissue plus the receiver blocks (Basipetal). Diffusion controls were run with the orientation of the tissue section reversed (Acropetal). Polar Transport is defined as the % of total dpm in the basal portion of the tissue plus the receiver block at the end of the transport period. RNAi was induced by inclusion of 4 μM estradiol in the growth medium.
Measurement of Shootward and Rootward Auxin Transport in Arabidopsis Roots

The assays of shootward (basipetal) and rootward (acropetal) auxin transport in wild type and apyrase RNAi line R2-4A primary roots were performed as described in Lewis and Muday (2009). For both assays, wild-type and R2-4A seedlings were grown under continuous cool white fluorescent light for 4 d at 25 ºC on MS media to ensure roots were at least 10 mm; then transferred to media containing 4 µM estradiol for 4 d. A droplet of agar (5 µl containing 0.05% MES, 1.25% agar, and 100 nM \(^{3}\)H]IAA (American Radiolabeled Chemicals) was applied so that it was just touching the root tip for shootward assays or at the root shoot junction for rootward assays. During the assay, plants were placed under yellow-filtered light to prevent the breakdown of IAA that occurs under white light. For the shootward assays, after 5 h a 5 mm segment located 2 mm from the root tip was excised. For the rootward assay, a 5 mm segment at the root tip was excised after 18 h. Radioactivity was quantified by scintillation counting. The reported values are the average and SE of auxin transport calculated relative to wild type in each assay and represent several independent trials in which values from more than 42 individuals of each genotype were pooled for basipetal transport assays and more than 60 individuals were pooled for acropetal transport assays.

Determination of Free IAA Distribution in Arabidopsis Seedlings

Seedlings of Arabidopsis ecotype Ws and RNAi line R2-4A were grown in darkness for 6 d and transferred to continuous cool white fluorescent light (80 µmol m\(^{-2}\)s\(^{-1}\)) for 2 d. The harvesting, dissection, extraction, and assay of the plant material were performed exactly as described in Barkawi et al. (2010), using a SPE 215 liquid handling system (Gilson) (Liu et al. 2011). Levels of free IAA were measured in the apices and apical and basal halves of Arabidopsis hypocotyls, using isotope dilution Gas Chromatography-Selected Ion Monitoring-Mass Spectrometry (GC-SIM-MS; Barkawi et al. 2010).

Generation of DR5:GFP RNAi lines

To generate the DR5:GFP RNAi lines, the R2-4A line was crossed with a transgenic line expressing DR5:GFP. The F1 generation plants were selected on MS medium containing 20 µg/ml hygromycin. The plants that could grow on hygromycin plates were heterozygotes of
Apy2, RNAi gene, and DR5-GFP gene, but homozygotes of Apy1. The seeds harvested from the F1 generation plants were planted on agar in media containing 4 μM estradiol in 150 mm petri dishes. After 6 d, light-grown seedlings that showed decreased root growth were selected and transferred to soil. The seeds collected from the F2 generation were used to screen for homozygotes of the RNAi mutant expressing DR5:GFP.

**Confocal Microscopy**

Seedlings treated with estradiol for a different number of days were originally germinated and grown on MS plates. Both DR5:GFP R2-4A and DR5:GFP WT seedlings were transferred to plates containing 4 μM estradiol on day 2 or day 3, as noted in the legend to Figure 3. Fluorescence images were captured at day 6 by a Leica SP2 AOBS confocal microscope with the filters set at 488/509 nm excitation/emission. For root gravitropic response, seedlings were grown in light for 5 d and then rotated 90°. Images were obtained after 5 h horizontal growth, and the confocal microscope noted above captured fluorescence images of the GFP signals.

**Scanning Electron Microscopy**

Fresh six-d-old seedlings were fixed in 0.1 M phosphate buffer solution (pH 5.7) containing 1% paraformaldehyde and 2% glutaraldehyde. Samples were fixed under vacuum for 30 min and incubated at 4° C for 12-24 h. Fixed seedlings were washed three times in phosphate buffered saline (pH 7.4), followed by two times in distilled water for 10 min. Samples were dehydrated at room temperature in an ethanol series for 15 min at each step as follows: 15%, 30%, 50%, 70%, 80%, 90%, 95%, 2x absolute ethanol. Dehydrated specimens were further dried by critical point drying and mounted onto the stubs using double-sided mounting tapes. Dry seedlings were sputter coated with gold immediately after critical point drying. Images were captured by a Philips EM 515 scanning electron microscope operating at an accelerating voltage of 14.7 kV.

**Apyrase inhibitor assays**

Seeds (Ws) were sterilized as described by Tang et al. (2003), kept at 4 C for at least three d, then sown on MS media with or without apyrase inhibitor NGXT1913 (Windsor et al., 2002) or NGXT191 (Windsor et al., 2003), which was dissolved in DMSO. The control plates contained the same final concentration of DMSO (0.01%) as the treatment plates. Seedlings were grown in
darkness for 3 d on vertically oriented plates, then the plates were moved into continuous white light, reoriented 90 degrees, and the seedlings were grown an additional 18 h. The growth temperature throughout was 23 C. Seedlings were imaged before being reoriented and 18 h later, and Image J was used to measure changes in the angle of curvature and overall growth between these two time points.

**Anatomical studies**

Ws wild-type (WT) and R2-4A plants were grown for three, four, five, and six d on plates containing 4 μM estradiol, and the primary roots tips were examined by light microscopy. The quiescent center was measured as the distance in μm or number of cells from the first cell interior to the apical epidermal layer to the first cell adjacent to the mitotic zone. The mitotic zone was measured from the first cell adjacent to the quiescent center to the first visibly elongating cell. The elongation zone was measured from the first visibly elongating cell to the first epidermal cell with a visible root hair bud. The zone of differentiation was determined to begin at the first epidermal cell with a visible root hair bud.

**Root gravitropic assays in Columbia ecotype**

Col-0 seedlings (6-d-old) were transferred to plates with and without ATP and roots were assayed for growth and curvature 24 h after gravistimulation as described by Tang et al. (2003) in their Table I. The media pH for all plates was adjusted to ~5.0.

**SUPPLEMENTAL DATA**

The following materials are available in the online version of this article:

**Supplemental Figure S1.**

Treatment with 600 μM ATPγS differentially inhibits the etiolated hypocotyl growth of wild-type and mutant seedlings. A, Hypocotyl growth of seedlings constitutively expressing APY1 (APY1-OE line) is suppressed less by 600 μM ATPγS than is hypocotyl growth of wild-type seedlings. B, Hypocotyl growth of R24A seedlings induced by estradiol (E) to suppress APY expression is suppressed less by 600 μM ATPγS than is hypocotyl growth of wild-type seedlings treated with estradiol.
Supplemental Figure S2.
Treatment of R2-4A seedlings with estradiol for 1 or 2 days does not significantly alter the expression of the auxin response reporter DR5:GFP in these mutant roots compared to estradiol-treated Ws wild-type roots. Confocal phase and DR5:GFP fluorescence images of primary roots of Arabidopsis seedlings grown for 6 d in the light. Panels A, C, E, G are roots of R2-4A treated with estradiol for 1 d (A, C) or 2 d (E, G). Panels B, D, F, H are Ws wild-type roots treated with estradiol for 1 d (B, D) or 2 d (F, H). These results are representative of 10 or more biological repeats. Scale bars = 100 µm.

Supplemental Figure S3.
A, Time course of loss of APY1 transcripts during continuous treatment of R2-4A mutants with estradiol inducer of RNAi construct. Significant reduction of APY1 transcript abundance is not evident until 2.75 d of continuous treatment, beginning with seed sowing. The equal transcript abundance of APY1 in WT and induced R2-4A seedlings shown at 2.5 d was also observed at 0.5 d, and 1.1 d of estradiol treatment (data not shown). B, Actin loading control.

Supplemental Figure S4.
6-d-old R2-4A primary roots expressing a DR5:GFP construct uninduced (A, B) or induced by estradiol (C, D) to suppress APY1. Panels A, C are confocal images showing GFP signal; Panels B, D, are confocal images of GFP signal overlaid on bright field images.

Supplemental Figure S5.
Chemical inhibition of apyrase activity blocks gravitropic growth of primary roots of Arabidopsis. A, Treatment of 3-d-old seedlings with 7.5 µg/mL NGXT 1913 inhibits root gravitropic curvature. B, Treatment of 3-d-old seedlings with 7.5 µg/mL NGXT 1913 reduces the root growth rate, but this effect is not statistically significant (p = 0.332). These data are the average of two independent experiments. Standard error values are marked by vertical bars. Significant differences between samples are indicated by asterisks (Student’s t test; * P < 0.05; n = 16 for control, n = 18 for NGXT 1913).
**Supplemental Figure S6.**
Distribution of neither PIN1:GFP nor ABCB19:GFP is altered in Arabidopsis primary roots by treatment with 800 µM ATPγS. Confocal images of primary roots of 5-d-old seedlings expressing ABCB19:GFP before and after treatment with 800 µM ATPγS (A, B). Confocal images of primary roots of 5-d-old seedlings expressing PIN1:GFP before and after treatment with 800 µM ATPγS (C, D). * Arrows indicate the polarity of PIN1:GFP.

**Supplemental Figure S7.**
Distribution of neither PIN2:GFP nor AUX1:YFP is altered in Arabidopsis primary roots by treatment with 800 µM ATPγS. Confocal images of primary roots of 5-d-old seedlings expressing PIN2:GFP before and after treatment with 800 µM ATPγS (A, B). Confocal images of primary roots of 5-d-old seedlings expressing AUX1:YFP before and after treatment with 800 µM ATPγS (C, D). * Arrows indicate the polarity of PIN2:GFP and AUX1:YFP, respectively.

**Supplemental Table S1.** Suppression of apyrase increases the size diversity of cells in the mitotic and elongation zones, as measured by the % of cells that have a diameter less than half the average diameter of all cells measured in the population.

**Supplemental Table S2.** Gravity response of Arabidopsis Col-0 roots in the absence (control) or presence of ATP 24 h after reorientation. Each value is the average and SE of 10 separate plants, and the P values for growth and gravity responses were determined by Student's t test.

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FIGURE LEGENDS

Figure 1. Polar auxin transport is inhibited in Arabidopsis hypocotyls after induction of apyrase RNAi by estradiol and promoted in hypocotyls from seedlings overexpressing APY1 and APY2. A, Estradiol-treated RNAi R2-4A seedlings grown for 6-d in the dark and then transferred to light for 2-d show inhibited growth compared to Ws, non-induced RNAi R2-4A, and estradiol-treated Ws seedlings. B, Auxin transport is similar in non-induced genotypes and there is no difference in the ability of NPA to inhibit basipetal auxin transport among non-induced genotypes. C, Basipetal auxin transport is greatly reduced after suppression of APY2 by induction of mRNAi with estradiol treatment. D, Basipetal auxin transport is promoted in APY1-OE and APY2-OE hypocotyls but is unaffected in the apy1 and apy2 single knockout lines. NPA inhibits basipetal auxin transport in hypocotyls of all genotypes but to a lesser degree in hypocotyls of the estradiol-treated RNAi R2-4A line. Standard error values are marked by vertical bars. In panel B, n = 10 for all groups; in panels C and D, n = 10 for basipetal groups, and n = 5 for acropetal and NPA groups. These results are representative of 3 or more biological repeats. Statistically evaluated differences between samples are indicated by asterisks (Student’s t test; ** P < 0.05, * P < 0.07).

Figure 2. Free IAA levels are increased in the shoot apices and decreased in the basal halves of hypocotyls of Arabidopsis seedlings after induction of apyrase RNAi by estradiol. Free IAA levels were significantly decreased in the basal half of R2-4A (+estradiol) hypocotyls compared to the basal halves of R2-4A (-estradiol) or Ws wild-type (+/-estradiol) hypocotyls, and the increase of free IAA found in the apices of R2-4A (+estradiol) hypocotyls was only marginally insignificant. Free IAA in the apical half of hypocotyls showed no difference among the groups analyzed. Panel A is a graphical representation of the data shown in panel B. Standard error values are marked by vertical bars. These results are representative of 3 or more biological repeats. Statistically evaluated differences between samples are indicated by asterisks (Student’s t test; ** P < 0.002, * P < 0.07, n = 3).

Figure 3. Polar auxin transport is inhibited in primary roots of Arabidopsis after induction of apyrase RNAi by estradiol. IAA transport was measured in both the basipetal (shootward) and acropetal (rootward) directions in 8 day old seedlings, 4 days after transfer to estradiol. Basipetal
IAA transport was reduced in R2-4A roots when induced by estradiol. The average and SE are reported for greater than 42 seedlings in the basipetal assay and greater than 60 seedlings in the acropetal assay and are a summary of 3 biological repeats. Basipetal IAA transport levels in wild-type averaged 4.2 fmoles and acropetal IAA transport values in wild-type averaged 8.8 fmoles. Statistically significant differences between wild-type and the RNAi line are indicated by an asterisk (Student’s t test; * P < 0.05).

**Figure 4.** Suppression of *APY1* in the *apy2* mutant alters the morphology of roots and expression of the auxin response reporter *DR5:GFP*. Shown are primary roots of Arabidopsis seedlings grown for 6 d in the light. Panels A, C, E, G are roots of *DR5:GFP* R2-4A treated for 3 d (A, C) or 4 d (E, G) with the estradiol inducer. Panels B, D, F, H are *DR5:GFP* Ws roots treated with estradiol for 3 d (B, D) or 4 d (F, H). These results are representative of 10 or more biological repeats. Scale bars = 100 µm.

**Figure 5.** Scanning electron microscopy images of 6-d-old estradiol-treated Ws wild-type and RNAi R2-4A mutant seedlings showing root swelling and ectopic root hair development. A, enlarged apical zone of Ws wild-type root. B, enlarged apical zone of R2-4A root. These results are representative of 3 or more biological repeats. Scale bar = 200 µm.

**Figure 6.** Fluorescent GFP signal in horizontally-positioned primary roots of A, wild-type (WT) and B, R2-4A plants expressing *DR5:GFP*. Both WT and R2-4A plants were treated with estradiol, which induced the suppression of apyrase expression by RNAi in the R2-4A mutant. GFP signal in horizontally-positioned primary roots of wild-type plants expressing *DR5:GFP* before and after treatment with 800 µM ATPγS (C, D), or with 7.5 µg/mL NGXT1913 (E, F). Arrow indicates lower flank of primary root, where the *GFP* signal is evident in the epidermal cells of the WT roots, but not in the R2-4A roots and not in treated roots. These results are representative of ten or more biological repeats. Signal assayed 5 h after roots moved to the horizontal position. Scale bars = 50 µm.
Tables

Table I. Comparison of the lengths\(^a\) of the root tip zone, mitotic zone, and elongation zone in wild-type (WSWT) and R2-4A plants after treatment with estradiol, which suppresses apyrase expression in the R2-4A plants.

| Treated\(^e\) | Root Tip\(^b\) | Mitotic Zone\(^c\) | Elongation Zone\(^d\) |
|--------------|----------------|-------------------|---------------------|
|              | WSWT | R2-4A | WSWT  | R2-4A | WSWT | R2-4A |
| 3 days       | 413.4| 322.6\(^f\) | 145.7 | 93.1\(^f\) | 233.1| 198.5 |
| 4 days       | 405.1| 254.2\(^f\) | 143.6 | 93.1\(^f\) | 227.8| 130.7\(^f\) |
| 5 days       | 386.7| 220.4\(^f\) | 153.8 | 78.4\(^f\) | 203.7| 111.9\(^f\) |
| 6 days       | 519.7| 190.8\(^f\) | 168.0 | 53.5\(^f\) | 315.2| 107.7\(^f\) |

\(^a\)All values shown are length values given in µm, and are the average lengths measured in 7 to 10 different plants each day of treatment.

\(^b\)Measured from cap apex to the beginning of the zone of differentiation.

\(^c\)Measured from the first cell adjacent to the quiescent center to the first visibly elongating cell.

\(^d\)Measured from the first visibly elongating cell to the first epidermal cell with a visible root hair bud.

\(^e\)Days the wild-type and R2-4A mutants were treated with estradiol.

\(^f\)Value is significantly different from the WSWT value (p < 0.05).
Table II. Suppression of apyrase by estradiol-induced RNAi in R2-4A plants reduces the average cell number measured in the mitotic and elongation zone of primary roots.

| Treated<sup>b</sup> | WSWT | R2-4A<sup>c</sup> | WSWT | R2-4A<sup>c</sup> |
|---------------------|------|-------------------|------|-------------------|
| 3 days              | 24.0 | 14.8              | 11.5 | 9.1               |
| 4 days              | 20.9 | 13.9              | 14.7 | 8.6<sup>c</sup>   |
| 5 days              | 21.8 | 10.3<sup>c</sup>  | 14.0 | 6.5<sup>c</sup>   |
| 6 days              | 22.5 | 8.8<sup>c</sup>   | 17.9 | 10.8<sup>c</sup>  |

<sup>a</sup>Zones defined as in Table I. All values are the average number of cells in the zone measured in 5 to 10 different plants.

<sup>b</sup>Days the wild-type and R2-4A mutants were treated with estradiol.

<sup>c</sup>Value is significantly different from the WSWT value (p < 0.05).
**Figure A**

Free IAA Content (ng/g FW) for different genotypes and treatments:

- **Wild Type (-Estradiol)**
  - Apices: 5.41 ng/g FW (SE: 0.25)
  - Apical half: 4.24 ng/g FW (SE: 0.27)
  - Basal half: 2.32 ng/g FW (SE: 0.11)

- **R2-4A (-Estradiol)**
  - Apices: 5.07 ng/g FW (SE: 0.43)
  - Apical half: 4.49 ng/g FW (SE: 0.60)
  - Basal half: 2.58 ng/g FW (SE: 0.14)

- **Wild Type (+Estradiol)**
  - Apices: 5.43 ng/g FW (SE: 0.34)
  - Apical half: 4.27 ng/g FW (SE: 0.36)
  - Basal half: 2.85 ng/g FW (SE: 0.21)

- **R2-4A (+Estradiol)**
  - Apices: 6.23 ng/g FW (SE: 0.43)
  - Apical half: 3.48 ng/g FW (SE: 0.28)
  - Basal half: 1.13 ng/g FW (SE: 0.09)
