Modulation of Root Skewing in Arabidopsis by Apyrases and Extracellular ATP

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When plant primary roots grow along a tilted surface that is impenetrable, they can undergo a slanted deviation from the direction of gravity called skewing. Skewing is induced by touch stimuli which the roots experience as they grow along the surface. Touch stimuli also induce the release of extracellular ATP (eATP) into the plant’s extracellular matrix, and two apyrases (NTPDases) in Arabidopsis, APY1 and APY2, can help regulate the concentration of eATP. The primary roots of seedlings overexpressing APY1 show less skewing than wild-type plants. Plants suppressed in their expression of APY1 show more skewing than wild-type plants. Correspondingly, chemical inhibition of apyrase activity increased skewing in mutants and wild-type roots. Exogenous application of ATP or ATPγS also increased skewing in wild-type roots, which could be blocked by co-incubation with a purinergic receptor antagonist. These results suggest a model in which gradients of eATP set up by differential touch stimuli along roots help direct skewing in roots growing along an impenetrable surface.

Keywords: Apyrase • Arabidopsis • Extracellular ATP • Root • Skewing.

Abbreviations: CFR, cell file rotation; DMSO, dimethylsulfoxide; eATP, extracellular ATP; HGI, horizontal growth index; MS, Murashige and Skoog; NPA, 1-naphthylphthalamic acid; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RNAi, RNA interference; RT-PCR, real-time PCR.

Introduction

Plant roots not only help anchor the plant and acquire soil resources (water and nutrients), but also perceive environmental cues and change their developmental physiology accordingly. Understanding how roots sense and respond to their environment is crucial to improving the yield of crops grown under today’s increasingly stressed conditions (Lynch 1995). Although it is well documented that plant root growth and development are regulated by both environmental stimuli and internal mechanisms, many details of the signal transduction pathways determining root architecture remain unclear.

Plant roots must sense and respond to a variety of environmental stimuli as they grow through the soil. Gravity and touch represent two of the signals that roots must integrate to elicit the appropriate root growth patterns and root system architecture. Roots of Arabidopsis seedlings have been used as a model for studies of root morphogenesis and gravity response (Smyth 1990, Grabov et al. 2005). When Arabidopsis seedlings grow along a slanted semi-solid medium that is impenetrable, their roots show two directional growth responses, waving and skewing. The waving phenomenon was first reported in Arabidopsis, where roots generated a repeating right-to-left undulation across the vertical growth axis as they were grown along a tilted agar surface, producing a regular sinusoidal pattern (Okada and Shimura 1990). Skewing was initially described as the right-slanted growth of roots in the Ws and Landsberg erecta (Ler) ecotypes of Arabidopsis (Rutherford and Masson 1996).

Initial publications proposed that, although skewing was not directed by gravity, the waving root growth pattern was a response to gravity-induced touch stimuli which the root encountered as it grew along the medium surface, and that the root tip changed growth direction in an obstacle-avoidance trajectory. Subsequently other researchers have proposed a combination of causative factors, including gravity, circumnutation, touch-induced responses and a physical interaction between the root tip and the surface of the growth medium (Thompson and Holbrook 2004, Oliva and Dunand 2007, Migliaccio et al. 2013).

Recent studies performed in microgravity reveal that root waving and skewing patterns do not require a gravity stimulus. In fact, in the microgravity environment of space, Arabidopsis roots show exaggerated skewing (Millar et al. 2011, Paul et al. 2012). These results suggest that it is more likely that root waving and skewing are directed mainly by touch or mechanical stimuli. FERONIA, a receptor-like kinase, was recently shown to play a key role in root skewing and other responses to mechanical stimuli (Shih et al. 2014).

Mechanical stimuli not only have an effect on root waving and skewing, but they also trigger ATP release from plant roots (Weerasinghe et al. 2009). The cells of other plant tissues also release ATP into their extracellular matrix or the growth medium when they are mechanically stimulated (Jeter et al. 2004), or when their plasma membranes are stretch activated as they expand (Kim et al. 2006, Wu et al. 2007, Clark et al. 2010). Extracellular ATP (eATP) can modulate the growth of
plant cells, with lower concentrations promoting growth and higher concentrations inhibiting growth (Clark et al. 2014). Treatment with exogenous ATP also inhibits root gravitropism and induces root curling (Tang et al. 2003). Recently, Haruta and Sussman (2012) found that 2 mM ATP increases root skewing in a pH-dependent manner.

The eATP signal in animals acts upon two types of purinergic receptors (P2X and P2Y) (Khakh and Burnstock 2009). In plants, DORN 1 was recently identified as a plant eATP receptor that is essential for the eATP-induced increase in cytoplasmatic calcium levels and the gene expression changes that occur during plant defense responses (Choi et al. 2014).

The concentration of eATP is regulated in part by an enzyme called apyrase (ecto-nucleoside triphosphate diphosphohydrolase). In plants, apyrases play a pivotal role in controlling growth and development. For example, plants suppressed by RNA interference (RNAi) in their expression of two closely related Arabidopsis apyrase genes, APY1 and APY2, are dwarf and have impaired polar auxin transport (Wu et al. 2007, Liu et al. 2012). They also have defective stomatal functions (Clark et al. 2011), and exhibit major changes in cell wall composition and gene expression (Lim et al. 2014). In one RNAi mutant line (R2-4A) in which APY2 is knocked out and APY1 suppression is induced by estradiol, primary roots are very short, with swollen root tips and a reduced elongation zone (Liu et al. 2012).

One function of APY1 and APY2 appears to be to limit the concentration of eATP, because their suppression leads to increased [eATP] (Lim et al. 2014), and high [eATP] can mimic the effects of apyrase suppression on growth and stomatal function. Several reports indicate that APY1 and APY2 are localized primarily in the Golgi (Chiu et al. 2012, Schiller et al. 2012). These reports still allow the possibility that some fractions of these enzymes function as ecto-apyrases in the plasma membrane, as suggested by the data of Wu et al. (2007). However, to the extent that APY1 and APY2 function exclusively in the Golgi, the most likely way they could control [eATP] from this location would be by limiting the [eATP] in the lumen of Golgi-derived secretory vesicles that ultimately serve as a source of eATP when they fuse with the plasma membrane.

Recent studies indicate that tagged versions of APY1 and APY2 hydrolyze only NDPs, not NTPs (Chiu et al. 2012, Schiller et al. 2012, Massalski et al. 2015), whereas a previous study demonstrated that heterologously expressed versions of these enzymes favored NTPs as their substrate (Steinebrunner et al. 2000). Polyclonal antibodies directed against APY1 inhibit the activity of apyrases released into the medium by pollen tubes growing in culture and raise the [eATP] of the medium (Wu et al. 2007). Genetic suppression of the transcript abundance of APY1 and APY2 also raises the [eATP] (Lim et al. 2014). Post-translational or other structural modifications of apyrases are known to affect their substrate specificity (Knowles 2011), and additional studies will be needed to evaluate the nucleotide targets of native APY1 and APY2 in vivo.

The nucleotidase activity of Golgi-localized apyrases could have effects on growth unrelated to their control of [eATP], as luminal NTP and NDP levels control several aspects of Golgi metabolism, including glycosylation of proteins and lipids, and control of wall polysaccharide levels. All of these functions could influence growth generally and root skewing specifically, independently of whether the apyrases also regulate [eATP].

Despite these previous studies on the functions of APY1 and APY2, the general importance of these enzymes in the response of plant roots to stimuli is not well understood. Here we report that APY1 and, to a lesser extent, APY2 help control root skewing in Arabidopsis, and that application of extracellular nucleotides also affects this directional growth response of roots.

### Results

**Apyrase expression levels alter root skewing**

The apyrase single knockout and overexpressing lines were previously characterized (Steinebrunner et al. 2003, Wu et al. 2007). We confirmed apyrase expression levels using gene-specific primers in real-time PCR (RT-PCR) before using these mutants again in this study (Supplementary Fig. S1). When grown vertically on the surface of Murashige and Skoog (MS) medium that contains 1.0% agar, the primary roots of wild-type seedlings (Ws ecotype) grew with a slight rightward skewing, viewed from the back of the plate through the agar. For quantification of root skewing, we calculated the HGI (horizontal growth index). Higher HGI values indicate more skewing and lower values indicate less skewing. Roots of seedlings overexpressing APY1 grew almost straight down with only slight skewing, and they had a statistically significantly lower HGI value than wild-type roots (Fig. 1A). In contrast, the primary roots of apy1 single knockout (APY1 KO) seedlings (Ws background) exhibited increased rightward skewing and had an HGI that is significantly higher (P < 0.05) than that of wild-type roots. Increasing the agar concentration to 1.5% caused increased skewing of the roots (Fig. 1B).

Because root skewing is affected by gelling polymers (Buer et al. 2000), we also performed skewing experiments with seedlings grown on phytogel (1.0%) as a gelling polymer instead of agar. In contrast to the results on agar, we found that both apy1 and apy2 single knockout roots showed increased skewing compared with wild-type roots when grown on phytogel (Fig. 1B). Roots overexpressing APY1 showed decreased skewing compared with wild-type roots when grown on phytogel, just as we observed for APY1 OE seedlings grown on agar (Fig. 1B).

Plates containing 1% agar were positioned at a 30° angle from the vertical to test the effect tilting the plate had on the skewing response of wild-type and mutant roots. Although tilting the plates 30° caused increased skewing of the roots
for all the genotypes compared with when the roots were grown in a vertical position, the statistically significant differences observed between wild-type seedlings and APY1 KO mutants were the same in both the vertical and tilted positions (Fig. 2). Interestingly, when plates were tilted 30°, approximately 60–70% of the APY1 KO primary roots formed an anticlockwise curling (viewed from the back of the plates) in three replica experiments compared with only 20–25% in the wild type, while the roots overexpressing APY1 showed no curling (Supplementary Fig. S3). When grown on agar surfaces, only seedlings with different levels of APY1 expression showed different levels of skewing compared with the wild type.

To confirm further the function of apyrase in the regulation of root skewing, we examined the behavior of RNAi mutants (R2-4A) in which APY2 is knocked out and APY1 suppression is induced by 4 μM estradiol. Treatment of R2-4A seedlings with estradiol induces 70% suppression of APY1 expression in the null background of APY2 and results in shortened roots with swollen root tips. We observed this phenotype in our skewing experiments with this RNAi line. When the estradiol-treated R2-4A mutants were grown on vertical or tilted agar surfaces, skewing was increased compared with estradiol-treated wild-type roots, as indicated by significantly higher HGI values (Fig. 3). The R2-4A mutant roots that were not induced by estradiol had the same HGI value as Ws wild-type and apy2 single knockout roots (Fig. 3).

**Inhibition of apyrase activity increases root skewing**

We tested whether inhibiting the activity of apyrase affected root skewing of Arabidopsis seedlings. We applied two chemical apyrase inhibitors, apyrase inhibitor 4 [N’-(2-hydroxy-5-methylbenzylidene)-2-(1-naphthyl) acetohydrazide] and NGXT 1913 [3-[N-(4-bromophenyl) sulfamoyl]-N-(3-nitrophenyl) benzamide] (Windsor et al. 2002, Clark et al. 2010). These inhibitors are small, organic molecules selected based on their ability to inhibit soluble potato apyrase (Windsor et al. 2002). Application of 2.5 μg ml apyrase inhibitor 4 induced a statistically significant increase in the HGI value for roots of all genotypes tested except APY1 OE roots in comparison with control roots grown on control plates with the same final concentration of dimethylsulfoxide (DMSO) (0.01%) (Fig. 4A). Application of a lower concentration (1.5 μg ml) of NGXT 1913 induced a statistically significant increase in the HGI value for roots of all genotypes tested except wild-type and APY1 OE roots (Fig. 4B). To confirm further the function of apyrase in the regulation of root skewing, we examined the behavior of Ws and RNAi mutants (R2-4A) in response to apyrase inhibitors. When the estradiol-induced R2-4A mutants were grown on the apyrase inhibitors apyrase inhibitor 4 and NGXT 1913, right skewing was increased compared with estradiol-treated wild-type roots and with non-induced R2-4A mutants, as indicated by significantly higher HGI (Fig. 4C).

**Treatment with ATP and ATPγS increases root skewing**

Exogenously added 4 mM ATP alters skewing of the primary roots of wild-type Arabidopsis seedlings. To test the effects of
compounds that could be derived from ATP hydrolysis, primary root skewing was examined in response to comparable concentrations of ADP, AMP and inorganic phosphate (K₂HPO₄). We found that 4 mM ADP increased skewing of wild-type roots, but to a lesser degree than 4 mM ATP (Fig. 5A). In contrast, treatment with 4 mM AMP or 8 mM phosphate had no effect on primary root skewing (Fig. 5A). Treatment with pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), an antagonist of animal purinoceptors, blocked the skewing caused by 2 mM ATP. There was no effect of 300 μM PPADS on root skewing, but, when combined with 2 mM ATP, the root showed the same level of skewing as the control (Fig. 5B).

In dose–response assays testing root skewing of the primary roots of wild-type and apyrase mutant seedlings of Arabidopsis in response to applied ATP, treatment with 1 mM ATP increased the HGI value for APY1 KO and APY2 OE roots, while treatment with 2 and 3 mM ATP increased HGI values for roots of all the genotypes tested (Fig. 6A). A representative image of the skewing phenotypes of wild-type, APY1 KO and APY1 OE roots with and without 2 mM ATP is shown in Fig. 6B. Even after 1 week, 64–73% of the ATP originally in the medium still remained in the plates (Supplementary Table S1).

Blocking auxin transport with 1-N-naphthylphthalamic acid (NPA) increases root skewing

The inhibition of polar auxin transport by NPA significantly increased root skewing in wild-type and mutant plants (Fig. 9A). However, in APY1 OE plants, the skewing direction was to the left instead of to the right (Fig. 9B).

Discussion

A key finding of our results is that the skewing of the primary roots of Arabidopsis on agar surfaces is controlled mainly by the expression levels of an Arabidopsis apyrase, APY1, and, to a lesser degree, by a second apyrase, APY2, as well as by applied ATP or eATP levels that could be regulated in part by the activity of these enzymes. Although these two apyrases localize
primarily to the Golgi as assayed by tagged protein localization studies (Chiu et al. 2012, Schiller et al. 2012), their functions seem to include controlling the concentration of ATP outside of plant cells (Wu et al. 2007, Lim et al. 2014), possibly by controlling the [eATP] in the lumen of Golgi vesicles that deliver much of the eATP. In this study, the increased skewing phenotype observed when expression of APY1 is suppressed, which raises the [eATP] (Lim et al. 2014), is mimicked by raising the [eATP] with exogenous ATP. This result is consistent with the hypothesis that the effect of APY1 suppression on skewing is related to its effects (direct or indirect) on eATP levels. Although eATP levels can regulate auxin transport, and thus regulate growth, luminal apyrases in the Golgi could regulate growth by mechanisms unrelated to their control of [eATP], as noted in the Introduction.

The primary sequence and transcript tissue distribution of APY1 and APY2 are very similar (Steinebrunner et al. 2000, Wu et al. 2007). Although the straight growth of mutants over-expressing or individually knocked out in these two apyrases is virtually identical, only APY1 significantly modulates skewing responses in Arabidopsis roots growing on agar. These results indicate that the higher HGI values in the R2-4A mutant, which is null for APY2 but suppressed 70% in APY1 expression, is due more to the partial suppression of APY1 than to the knockout of APY2. Taken together, our results indicate that APY1 plays a more important role in regulating root skewing than APY2. These results are consistent with the finding that APY1 had higher message levels than APY2 in roots (Steinebrunner et al. 2000).

As shown in Fig. 4, treatment of the knockout mutants of both APY1 and APY2 with apyrase inhibitors results in an additive effect for skewing. This result implies that in the absence of either APY1 or APY2, the other apyrase is able to substitute for its function in skewing, or that there are other apyrases involved in controlling the skewing response in roots. Relatedly, skewing
in the induced R2-4A roots (which are null for APY2) can be increased by treatment with apyrase inhibitors. This result could be explained by the fact that estradiol induction of this mutant results in only the partial suppression of APY1 (~70%), so the inhibitors can act on and suppress the APY1 that is still present in the induced R2-4A roots, which would further increase the HGI.

We observed differences in the seedlings’ skewing response to treatment with the two apyrase inhibitors, in both wild-type and apyrase transgenic roots. These variations are probably due to differences in the ability of the inhibitors to penetrate to where the apyrases function and their differential ability to inhibit apyrase activity. Both inhibitors only partially inhibit apyrase activity in vitro, so their effects in vivo would only partially decrease or, in some cases, have no significant effect on the roots’ apyrase activity, which would help explain their differential effects on root skewing.

The protein sequence of APY1, but not that of APY2, includes a calmodulin-binding motif (Steinebrunner et al. 2000). To the extent that the touch stimuli encountered by skewing roots activate mechanosensitive channels, these channels would both promote Ca\textsuperscript{2+} uptake (Yamanaka et al. 2010) and release ATP (Jeter et al. 2004, Weerasinghe et al. 2009), which would further promote Ca\textsuperscript{2+} uptake (Demidchik et al. 2003, Jeter et al. 2004, Demidchik et al. 2009, Demidchik et al. 2011). Increased [Ca\textsuperscript{2+}]\textsubscript{cyt} would activate calmodulin, and thus would probably have more of a stimulatory effect on the activity of APY1 than on APY2. This may help explain the stronger role of APY1 in skewing responses.

Thitamadee et al. (2002) and Yuen et al. (2005) noted the relationship between root skewing and epidermal CFR angles. Given these findings, the strong role for APY1 in controlling root skewing prompted an assay of its effect on epidermal cell file rotation. Not surprisingly, significant changes in this parameter were evident in both the APY1 KO knockout and the APY1 OE mutants. As pointed out by Yuen et al. (2005), cortical microtubules in cells of the central elongation zone of roots play a critical role in controlling the angles of epidermal CFR. Taken together, these results imply that APY1 may function in part to regulate the cytoskeleton in root responses. This would be in accord with multiple findings that confirm apyrase association with the cytoskeleton in peas (Shibata et al. 2002, Yoneda et al. 2009). Certainly calcium fluxes initiated by eATP, whose concentration is regulated in part...
by APY1 (Lim et al. 2014), would influence cytoskeletal organization and function (Hepler et al. 2001, Wang et al. 2011). For example, changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ can alter the association of calmodulin with microtubules (Fisher and Cyr 1993), and this association can alter the stability and function of microtubules (Cyr 1991, Fisher et al. 1996). To test further the APY1–cytoskeleton connection, assays of the effects of knocking out or overexpressing APY1 on cytoskeletal organization would be instructive.

Although apy2 knockout roots do not exhibit enhanced skewing on solid agar medium (Fig. 1A), they do on the harder phytagel media (Fig. 1B). The mechanism for this altered response is not clear, but, to speculate, it is possible that root tips encountering a harder surface experience a more intense mechanostimulation, which could result in more ATP released to the external medium. A significant role for APY2 in limiting the [eATP] may become evident only when higher levels of eATP are released into the external medium.

The exact mechanisms that account for waving and skewing growth patterns are still not yet fully understood (Roy and Bassham 2014), but now the prevailing opinion is that touch stimuli resulting from the contact between the medium and the root tip are the major factors that direct these growth patterns. Additionally, recent data would predict that asymmetric auxin distribution would be critical for both waving and skewing phenotypes (Paul et al. 2012, Roux 2012).
apyrase and extracellular ATP on skewing could be due to their effects on auxin transport.

Applied ATP can inhibit root gravitropism and basipetal auxin transport in wild-type Arabidopsis seedlings in a dose-dependent way (Tang et al. 2003, Liu et al. 2012). In this study, treatment with 2 mM ATP increases primary root skewing and induces ant-clockwise curling in APY1 KO seedlings. Blocking apyrase expression, which would result in an increase in [eATP], also inhibits auxin transport in primary roots of Arabidopsis (Liu et al. 2012).

If the knockout of APY1 increases the HGI skewing value in part by reducing auxin transport, one might predict that treating the apy1 mutant with NPA, an auxin transport inhibitor, would further increase the HGI value. The results in Fig. 9A confirm this prediction. Similarly, if the overexpression of APY1 reduces the HGI due in part to its promotion of auxin transport, one might predict that treating the APY1 OE seedlings with NPA would reverse this effect and increase the HGI, and Fig. 9A also confirms this prediction. However, although the skewing of APY1 OE seedlings treated with NPA is increased, this skewing is, unexpectedly, in the opposite direction (leftward instead of rightward), a result revealed only by measuring the skewing angle (Fig. 9B). Although a mechanism that would explain this switch to left-handed skewing is unclear, the data suggest that changes in auxin transport may influence how APY1 expression regulates skewing.

Recently, results from a study using a loss-of-function mutant for an Arabidopsis H⁺-ATPase, aha2, suggested a role for the proton motive force in eATP-induced root skewing (Haruta and Sussman 2012). The root skewing response in the aha2 mutant, which has a reduced proton motive force, was insensitive to ATP and ADP treatment, in contrast to the response in wild-type seedlings. The ATP-induced increase in [Ca²⁺]cyt was also impaired in aha2 mutant seedlings (Haruta and Sussman 2012). Furthermore, when Arabidopsis wild-type seedlings were grown on agar medium that had a pH of 7.5 instead of pH 5.7, ATP treatment no longer induced root skewing (Haruta and Sussman 2012). Similarly, we confirmed that when wild-type and apyrase mutants were grown on pH 7.5 agar, ATP treatment also no longer induced root skewing (data not shown). As suggested by Haruta and Sussman (2012), these results indicate that the pH component of the proton motive force is required for the eATP-induced increase in [Ca²⁺]cyt, which may be needed as an early signaling step in the root skewing response.

Two previously proposed and currently viable hypotheses relevant to the novel data presented here are that root skewing is a slanted growth response to touch resulting from an asymmetry in auxin distribution, and that APY1 and eATP play significant roles in controlling auxin transport. Key experimental tests of these hypotheses would be to determine whether there are any changes in the distribution of auxin in primary roots of apy1 or APY1-overexpressing mutants growing along a tilted impenetrable surface, and to determine whether there is any asymmetry in the release of ATP from skewing roots. Data from these and related experiments would provide initial clues for why altered expression of APY1 leads to altered skewing in primary roots.

Changing the expression levels of APY1 either chemically or genetically alters the skewing of primary roots of Arabidopsis growing on impenetrable semi-solid surfaces. To the extent that this result is related to the ability of APY1 to control both [eATP] and auxin transport, and to the extent that auxin plays a central role in skewing control, these results are consistent with a model in which gradients of eATP set up by differential touch stimuli of skewing roots help direct their slanted growth response.

Co-incubation of the purinoceptor antagonist PPADS blocked the root skewing responses induced by applied ATP and ATP;S. PPADS has routinely been used to study eATP-mediated physiological responses in animal cells. However, PPADS is also documented to bind to and affect the activity of other mammalian proteins, and the target(s) of PPADS activity in plant cells in unknown. Thus additional studies would be needed to determine whether DORN-1 is the receptor for the skewing response mediated by eATP and apyrases, or whether there is another receptor.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col-0) and Wassilewskija (Ws) were used as wild-type controls in this study. Seeds were surface sterilized by soaking them in 70% (v/v) ethanol for 1 min, then 20% (v/v) bleach for 10 min, and then washing them 4–5 times in sterilized water. Sterilized seeds were then cold-stratified for 2–4 d at 4°C.

Seeds were planted on solidified MS medium [4.3 g l⁻¹ Murashige and Skoog salts (Sigma), 0.5% (w/v) MES, 1% (w/v) sucrose and 1.0% agar]. Prepared seeds were planted directly on a cellophane membrane placed upon solidified MS medium under 24 h fluorescent light. For the experiments with the RNAi mutant line R2-4A, Ws is the ecotype background for the mutant line, so the Ws ecotype was used as the control wild type for these experiments. The RNAi construct and generation of the R2-4A line were as previously described (Wu et al. 2007). For estradiol treatment, estradiol was added into MS medium to reach a final concentration of 4 µM. Seeds were sterilized as described by Tang et al. (2003) and were then allowed to stratify at 4°C for at least 3 d. Prepared seeds were planted directly on a cellophane membrane placed upon solidified MS medium [4.3 g l⁻¹ MS salts (Sigma), 0.5% (w/v) MES and 1.0% (w/v) agar], raised to pH 5.7 with 1 M KOH. Plates were wrapped with 3M Micropore, placed in a growth chamber and grown at 23°C under 24 h fluorescent light.

For the experiments with the RNAi mutant line R2-4A, Ws is the wild-type background for the mutant line, so the Ws ecotype was used as the control wild type for these experiments. The RNAi construct and generation of the R2-4A line were as previously described (Wu et al. 2007). For estradiol treatment, estradiol was added into MS medium to reach a final concentration of 4 µM. Seeds were sterilized as described by Tang et al. (2003) and were then allowed to stratify at 4°C for at least 3 d. Prepared seeds were planted directly on a cellophane membrane placed upon solidified MS medium [4.3 g l⁻¹ MS salts (Sigma), 0.5% (w/v) MES and 1.0% (w/v) agar], raised to pH 5.7 with 1 M KOH. Plates were placed upright in a vertical position or at a 45° angle in a culture chamber and grown at 23°C under 24 h fluorescent light for 4 d. After 4 d, the plates were opened and the cellophane membrane with seedlings was transferred to treatment plates with equivalent hardness and angled arrangements. Treatment plates were MS agar plates (as described above) with 4 µM estradiol and with and without 2 mM ATP balanced to agar pH equivalence (5.7). After the transfer, the position of each seedling tip was recorded on the back of the square treatment plates with a thin red permanent marker. Plates were placed back in the growth chamber and seedlings were allowed to grow for an additional 3 d.

Experimental treatments

Experimental solutions were prepared from the following stocks and diluted as required: 50 mM stocks of ATP, ADP and AMP; 100 mM stocks of phosphate; all experimental working solutions were prepared fresh on the day of the experiments and the pH was adjusted to 5.7 with 1 M KOH.
RT-PCR confirmation

The apy1 and apy2 mutants were previously characterized (Steinebrunner et al. 2003). Apyrase RNA levels were determined using gene-specific primers in RT-PCR. Briefly, 7-day-old seedlings were collected and frozen in liquid nitrogen. Whole plants were ground to fine powder using a chilled mortar and pestle. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma) following the manufacturer’s protocol. A 2 μg aliquot of RNA was treated with DNase I (Invitrogen). DNase-treated RNA was then used to synthesize first-strand cDNA using a Superscript II reverse transcriptase kit (Invitrogen) according to the manufacturer’s instruction. A 2.5 μl aliquot of the first-strand cDNA was used as template in 30 cycle real-time PCRs using SYBR Green PCR Master Mix (Applied Biosystems). The following pairs of primers were used separately to amplify each of the cDNA samples: apyrase-specific primers APY 1F (5'-AAA AAC CAC GAG GGA GTT C-3') and APY 1R (5'-TCA CTC CAG CAT TGG EAG TC-3') were used to amplify APY1; APY 2F (5'-GCA TCC CAT TCA GGC C-3') and APY 2R (5'-TCT CCG TAT TTC ACC TTC TTC ACT AAC GT-3') were used to amplify APY2. Actin-specific primers were used as a control for all the RT-PCR experiments. PCR products were run on a 0.8% (w/v) agarose gel.

Quantification of root skewing

NHI Image J was used to determine root length as well as angle of deviation from the gravity vector and the measured length of the idealized root response (L). Excel formula calculations were conducted to obtain l and L, and subsequently the HGI was used to quantify skewing in Arabidopsis roots as described previously (Grabov et al. 2005, Vaughn and Masson 2011). Overall root length was measured from a marker that identified root tip location at the time of transfer to the root abscessa using a free-hand tool and a stylus on a touch screen computer. The horizontal growth index or HGI is the ratio of displacement a root experiences along its x-axis, L divided by L (the root length). This measurement provides a quantified method of defining root skewing. Statistically significant of root skewing was determined by one-way analysis of variance (ANOVA). Significantly different means (P < 0.05, α = 0.05) were separated by Tukey tests (significance level = 0.05). Different letters above the bars in the figures indicate mean values that are statistically significantly different from one another.

Apyrase inhibitor assay

Seeds were sterilized as described before, kept at 4°C for at least 3 d, and then sown on MS medium with or without 2.5 μg/ml apyrase inhibitor 4 or 1.5 μg/ml NGX 1913, which was dissolved in DMSO. The control plates contained the same final concentration of DMSO (0.01%) as the treatment plates. Seedlings were imaged and measured by Image J.

Assay of ATP in growth media of apyrase mutants

The concentration of ATP in the growth media was determined using the ENLITEN ATP Assay system from Promega. Reactions were carried out on 96-well plates, and luminescence was detected using the Mithras LB 940 microplate reader. In each reaction, 70 μl of ENLITEN reagent was added to a 15 μl extraction sample of agar medium. An ATP standard curve from 10⁻⁷ to 10⁻¹⁴ was measured for each plate. In all cases, at least three replicate samples were included in the average.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


