Touch induces ATP release in Arabidopsis roots that is modulated by the heterotrimeric G-protein complex

Ravisha R. Weerasinghe a, Sarah J. Swanson b, Seiko F. Okada c, Michele B. Garrett a, Sung-Yong Kim d, Gary Stacey d, Richard C. Boucher c, Simon Gilroy b,*, Alan M. Jones a,e,*

a Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA
b Department of Botany, University of Wisconsin at Madison, Madison, WI 53706, USA
c Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC 27599, USA
d Department of Plant Microbiology and Pathology, University of Missouri, Columbia, MO 65211, USA
e Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, USA

Article history:
Received 26 May 2009
Revised 2 July 2009
Accepted 7 July 2009
Available online 15 July 2009

Edited by Michael R. Sussman

Keywords:
Arabidopsis
ATP
Heterotrimeric G-protein
Touch desensitization

1. Introduction

Plants show highly adaptive responses to mechanical stimulation including thickening of organs, reduced growth rate, and complex changes in the orientation of organ growth [1,2]. In the root, touch stimulation causes directional-growth responses that are fundamental to obstacle avoidance and navigation through the stimulus-rich soil environment. Although there are many reported mutants in root directional-growth responses, such as gravitropism, these mutants often relate to the hormonal integration of growth [3], and surprisingly few signal transduction elements have been identified. Similarly, although touch sensing relies on Ca2+-dependent signaling [1,2], the identity of the receptors triggering such responses and the role of any intermediate signal transduction events are unknown.

In mammalian cells, it is well-characterized that ATP is released upon mechano-stimulation and acts upon ion-channel types of receptors (P2X receptors) or G-protein coupled receptors (P2Y receptors) to elicit downstream cellular responses [4]. Although extracellular ATP has been proposed to regulate plant stress and wound responses [5,6], it is unknown whether ATP release acts to mediate plant growth responses to mechanical stimulation. G-proteins are key signaling components for mechano-perception in organisms as diverse as humans [4] and fungi [7], in part transducing ATP signals via P2 receptors [4], but any role of G-protein coupled events in plant mechano-transduction also remains to be defined.

We report that touch stimulation as occurs during obstacle-avoiding responses elicits extracellular ATP release from plant roots. Although G-protein mutants exhibit normal touch-induced ATP release, they are impaired in down-regulating ATP release during persistent stimulation and are also impaired in obstacle-avoiding responses. Thus, plants appear to use an ATP-dependent touch response system in which ATP release is fine tuned by the G-protein complex.

2. Materials and methods

2.1. Plant growth and stimulation

The ecotype used here was Columbia (Col). Accession numbers for AGB1 and GPA1 are At4g34460 and At2g26300, respectively. Mutations are null alleles generated either by T-DNA insertion...
Arabidopsis seeds were surface sterilized then stratified for 2 days. Seeds were sown on 1/2 MS medium (+1% sucrose) and grown vertically at 23 °C with 16/8 light/dark cycle for 3 days. For experiments measuring ATP in the medium, seedlings that were grown on solid medium were moved to wells containing assay buffer (fresh medium or 1 mM EDTA with 1% sucrose) and allowed to acclimate for 1 h. Buffer was gently added and the seedlings were stimulated as described for the individual experiments. Stimulation by touch was performed by hand using the indicated probe (pipette tip, outer diameter = 1.0 mm; or pin head, outer diameter = 2.5 mm) by touching for ~0.5 s. The touch force to roots was determined by replicating the touch on an analytical balance and taking the average weight based on displacement. Force was calculated in Newtons by \( F = mg \) where \( m \) is the hypothetical rate of ATP hydrolysis at steady state, \( k = 1/\text{half life (min)} \), [ATP]<sub>b</sub> = basal ATP concentrations (1 nM [specifically, 500 pmol in 500 µl]).

2.2. ATP measurements

Bulk extracellular ATP concentrations were determined by measuring ATP concentrations in sampled buffer [10]. Seedlings were placed in 12-well plates containing 1/2 strength Murashige and Skoog basal salts with macronutrients plus 1% sucrose, stimulated by a single touch or constant rotation (125 rpm), and an aliquot of the buffer was removed at the indicated time points. The number of seedlings per well is indicated in Figs. 2 and 5. To determine rates of ATP release, the buffer for the seedlings was 1 mM EDTA plus 1% sucrose in order to minimize ATP hydrolysis. Collected samples were immediately put on ice and then heated at 98 °C for 2 min to inactivate ecto-ATPases. Sample ATP concentrations were measured by luciferin-luciferase based method in a luminometer (TR717, Applied Biosystems, Bedford, MA). At least 2 replicates were performed for each time point and genotype. Values provided are the mean of these replicates and the error shown is the standard error of the mean. Where appropriate, data were analyzed by one-way analysis of variance with GraphPad InStat software. Statistical significance was defined as \( P < 0.05 \).

The in situ luciferin–luciferase assay was performed as described by Kim et al. [9]. Luciferase fused with a cellulose binding domain (CBD) was used as an extracellular ATP reporter [9]. Plants incubated in luciferase-CBD solution were mounted in a perfusion chamber on 1/2 MS media with 1% (w/v) sucrose (1.2% (w/v) type VII agarose) and assay buffer (20 mM Tricine, 2.67 mM MgSO₄, 0.1 mM EDTA, 2 mM dithiothreitol and 470 mM d-luciferin). Light emission was measured with a Roper Cascade CCD camera at maximum gain. Luciferase expression was verified by immunoblotting. Potato apyrase (Sigma) was made fresh at 1 unit/5 ml. All experiments using luciferase were calibrated at the end by adding a known amount of ATP. Data was processed using IP lab 4.1 (Olympus) and Excel.

Seedling ATP hydrolysis rate was determined by measuring the hydrolysis of \( ^{32} \text{P} \text{ATP} \) as described previously [11] and calculated according to the following formula:

\[
v = k \frac{[\text{ATP}]_b}{[\text{ATP}]_b}.
\]

where \( v \) = the hypothetical rate of ATP hydrolysis at steady state, \( k = 1/\text{half life (min)} \), [ATP]<sub>b</sub> = basal ATP concentrations (1 nM [specifically, 500 pmol in 500 µl]).

2.3. Time-lapse movies of plant root growth

Seedlings were grown on 0.5% (w/v) Phytagel (Sigma, St. Louis, MO) as previously described [12]. To determine the effect of a physical barrier to root growth, a sterile piece of cover glass was inserted into the Phytagel 2–3 mm in front of the growing root. The root was then oriented vertically and imaged using a Proscope camera and software (Bodelin Technologies, Lake Oswego, OR) followed by analysis of growth kinetics using iVision (Biovision Technologies, Exton, PA) and the root tip angles of the double mutant are significantly different (~0.05, two-way ANOVA, \( n \geq 5 \)) during the time ranging from 8 to 12 h after contacting the barrier (as indicated on the graph by a bar).
inserted in front of the growing root and plates were turned 90° prior to imaging.

3. Results and discussion

The Arabidopsis genome contains a single canonical alpha (AtG-PA1, hereafter GPA1) and beta (AGB1) subunit of the heterotrimeric G-protein complex. In agreement with previous growth analyses [13], at 3–4 days after germination, the time we chose to perform the analyses described below, the morphology (not shown), growth rate and gravity response of the primary root in the agb1-2, gpa1-4 double mutant was not significantly different to wild-type (WT, 217 ± 33 μm/min, agb1-2, gpa1-4 241 ± 49 μm/min, P > 0.05, t-test; Fig. 1A). Thus, differences in the mechanical response of this mutant detailed below are unlikely to reflect inherent differences in root development in the mutant. It should be noted that by 7 days after germination, agb1-2, gpa1-4 double mutant roots grow detectably faster than WT [13].

To assess a possible lesion in mechano-response, a glass barrier was inserted in front of the growing root leading to a mechanically-induced avoidance response [14]. The behavior of WT roots is highly reproducible under these conditions, forming two bends as the root tip tracks over the barrier (Fig. 1B, Supplementary movie S1, and [14]). The agb1-2 mutant was previously reported to have a wild-type touch response [15]. However, in contrast to WT, the roots of the agb1-2, gpa1-4 double mutant, where the G-protein complex has been genetically ablated, failed to properly form these bends, i.e. the mutant failed (Fig. 1B) to maintain as large a tip angle as seen in WT (Fig. 1C bracket and Supplementary movie S2). Quantitation of the single gpa1 and agb1 root behavior showed no statistical difference from WT for either mutant in this assay.

In animals, mechanical stimulation commonly triggers release of ATP which then acts on purinoceptors in an autocrine and paracrine manner [4]. Although previous work showed that plants release ATP several minutes after vigorous shaking [6] and mM levels of exogenous ATP induce root curling [16], whether these responses play a role in physiological mechanical sensing is unknown. We, therefore, characterized the spatial and temporal kinetics of touch-induced ATP release in WT and G-protein mutant roots. As shown in Fig. 2A, roots, which had an average force of 198 mN applied locally, rapidly released ATP to nM levels in the surrounding medium. The applied force is well within the range that root tips experience when growing through soil. This force is near a minimum that modulates growth behavior [17], and is far below the force required to induce cell damage. Catabolism of extracellular ATP (e.g. from apyrases) could potentially impact the amounts of ATP measured. Therefore, we tested the catabolism rate of ATP in the assay buffer in the pres-
ence of seedlings by using \( {\gamma}^{32}P \)ATP as a tracer, which yielded a rate of hydrolysis of 2.8 femtomoles/min (Supplementary Fig. 1A), with a \( t_{1/2} \) of 180 min (Supplementary Fig. 1B). At this rate of catabolism, the effect of ATP breakdown on the data in Fig. 2A should be negligible. However, we consider this rate a lower estimate as the microenvironment of the cell wall is probably richer in hydrolytic activity than in the bulk solution. In addition, for plants growing in soil, ecto-nucleotidases from microorganisms would be expected to increase the ATP turnover dramatically.

The intensity and kinetics of this touch-induced ATP release was dependent on the site of mecano-stimulation. The shoot and root apices of the Arabidopsis seedlings release ATP upon stimulation within 1 min, much more rapidly than non-growing regions (Fig. 2B). The mutant lacking the \( \text{G-protein complex} \) released ATP with kinetics and amplitude similar to WT in response to a single touch (Fig. 2C).

We, therefore, used a cell wall-bound ATP sensor with increased spatial and temporal resolution (Fig. 3, [9]) to test whether there were more subtle changes in ATP release in the roots of the \( \text{G-protein mutant} \). The largest detectable touch-induced signal from this sensor occurred in WT at the distal elongation zone, whereas little increase in ATP release was detected at the extreme tip of the root (meristematic and root cap region, Fig. 3A and B). As a control, we conducted the ATP assay without luciferin; no change in light emission was detected after touching (Supplementary Fig. 2A). The sensitivity of our ATP detection system extended to the nM concentrations (Fig. 3, [9]) to test whether there were spatial and temporal resolution (Fig. 3, [9]) to test whether there were more subtle changes in ATP release in the roots of the \( \text{G-protein mutant} \). The largest detectable touch-induced signal from this sensor occurred in WT at the distal elongation zone, whereas little increase in ATP release was detected at the extreme tip of the root (meristematic and root cap region, Fig. 3A and B). As a control, we conducted the ATP assay without luciferin; no change in light emission was detected after touching (Supplementary Fig. 2A). The sensitivity of our ATP detection system extended to the nM range and the amount of luciferin in the assay and luciferase adhering to the cell wall was not detection-limiting (Supplementary Fig. 2B).

As the root tip is known to be sensitive to touch [18], the lack of mechanically-stimulated ATP production in this region (Fig. 3B) was unexpected. The cells of the peripheral root cap are highly specialized for secretion of a range of compounds [19] and so this lack of apparent touch-sensitive ATP release might reflect constitutive secretion of ATP. To test the possibility that high apoplastic ATP in the root cap masks the touch-induced ATP, we pretreated the root with apyrase to reduce the ATP potentially present in the apoplasm prior to the applied touch. Depletion of background ATP revealed that the tip region was able to respond to touch by releasing ATP (Fig. 3C, WT, and Supplementary Fig. 2C, mutant), suggesting that basal release of ATP can obscure or down-regulate touch-induced ATP release by the root cap.

Because mecano-stimulation can lead to a directional-growth response, we determined if the spatial kinetics of ATP release showed asymmetrical distributions consistent with such growth modulation. Roots were touched on one side in either the apex or elongation zone (Fig. 4A), and the change in ATP release was quantified (Fig. 4B–E). When touched on the left side, a transient release of ATP was observed on both the left (Fig. 4B) and the right sides (Fig. 4C), although with different kinetics. The kinetics and spatial dynamics of the touch-induced ATP release were not altered in the \( \text{agb1-2, gpa1-4 mutant} \) (Fig. 4B–E) except for a subtle change in kinetics of the right side response (Fig. 4B–E).

Mechano-responses in plants are also known to show a refractory period [18,20]. Therefore, we measured the ATP release by roots in response to a second touch. Re-touching a WT root prior to 9.3 minutes after the initial stimulus resulted in diminished ATP release relative to that elicited by the initial touch stimulation (Fig. 5A). In contrast, plants lacking the \( \text{G-protein complex} \) did not exhibit the pronounced refractory period to the same extent as wild-type roots, suggesting that \( \text{G-protein-dependent} \) events are required to down-regulate mecano-sensitive ATP release.

To determine long-term ATP release due to repetitive mechanical stimulation, WT and mutant seedlings were subjected to constant gentle agitation over long time periods (24 h). ATP concentrations in the medium surrounding WT seedlings remained stable, whereas ATP concentrations in the \( \text{G-protein mutant} \) increased over time (Fig. 5B). Note that the volume of media for seedlings varied between experiments (e.g., Figs. 2 and 5), and thus ATP concentrations are relative only within each experiment. The actual ATP concentration at root cell surface likely was significantly higher than the value in the sampled bulk medium [10].

ATP concentrations at a given time during gentle agitation are achieved by the balance between ATP release and metabolism. An estimate of the total amount of ATP released is possible by minimizing ATP hydrolysis by including EDTA (1 mM) in the buffer to inhibit nucleotidases. The double mutant exhibited 4–5 times greater rates of ATP release than wild-type (Fig. 5C), suggesting that increase in extracellular ATP concentrations in the mutants are results of increased net ATP release rate rather than reduced
catabolism. ATP release for two alleles of each of the two single mutants were slightly greater than for wild-type although the difference was not statistically significant (Supplementary Fig. S3) suggesting an additive effect of the mutations. Since loss of either the Gα or Gβ subunits disrupts heterotrimer formation, but only slightly changes the rate of ATP release during repetitive mechano-stimulation, the results indicate that an intact heterotrimer does not play a signaling role in touch-induced desensitization of ATP release. Furthermore, the results that loss of both subunits resulted in increased ATP accumulation than wild-type indicate that each activated subunit acts independently and in an additive manner in mediating this response.

There is some evidence in mammalian cells suggesting a role of G-proteins in regulating mechano-sensitive ATP release. For example, hypergravity-induced ATP release from bovine endothelial cells via small G-protein Rho A activation [21]; activation of G-protein-coupled-receptors elicited ATP release that was partially dependent on a Gq/phospholipase C/Ca2+ mobilization pathway [22], and Rho-family GTPases modulation of this ATP release in human astrocytoma cells [23]. Mechano-induced ATP release from living cells is postulated to occur via vesicular exocytosis or via conductive pathways, e.g. connexin or pannexin hemichannels, or maxi-anion channels [24]. Our observations suggest that the inability to transiently desensitize the mechano-induced ATP release in the G-protein mutant resulted in its higher ATP release rates and higher extracellular ATP concentrations during repetitive mechano-stimulation. These observations, coupled with abnormalities in obstacle-avoiding responses of G-protein mutant roots (Fig. 1), suggest that extracellular ATP plays a role in a complex system integrating the growth of the root with its ability to react to obstacles. A desensitization period and subsequent gradient of extracellular ATP following a mechano-stimulus may be necessary for the root to perceive changes in pressures surrounding the growth zone and/or to respond to the changes appropriately. For example, root curling is one manifestation of thigmomorphogenesis and it has been proposed that this touch-dependent response also contains a “reset” function as we show here, although a role for released ATP in root curling has not been shown [25].

Because released ATP likely plays a role in appropriate root growth in response to obstacles, further studies should focus on both ATP release pathways [21–24], and the downstream transduction mechanism, including a further search for purinoceptors [26,27].

Acknowledgements

This work was supported by grants from the NIGMS (GM65989-01) the DOE (DE-FG02-05ER15671), the NSF (MCB-0718202, 0723515) to A.M.J. (MCB 0641288, DBI 03-36738 and DBI 03-01460) to S.G., from the USDA (2005-35319-16192) to G.S., and the Cystic Fibrosis Foundation (R026-CR02) to R.C.B. We acknowledge the support of the NIH NIBIR (P41-EB002025-3A1) to Dr. Richard Superfine that enabled us to determine the force applied to the roots. We thank Drs. Eduardo R. Lazarowski and Gabrielle Monshausen for helpful discussions, Ms. J. Yang, Dr. T. O’Brien, Dr. M. Falvo, and Mr. A. Hamden for technical assistance, Dr. L. Johnson for use of the luminometer, and Dr. C. Ane for assistance in statistical analyses of the root behavior in the obstacle avoidance assay.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.007.

References


