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

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Abstract

Amongst the many stimuli orienting the growth of plant roots, of critical importance are the touch signals generated as roots explore the mechanically complex soil environment. However, the molecular mechanisms behind these sensory events remain poorly defined. We report an impaired obstacle-avoiding response of roots in Arabidopsis lacking a heterotrimeric G protein. Obstacle avoidance may utilize a touch-induced release of ATP to the extracellular space. While sequential touch stimulation revealed a strong refractory period for ATP release in response to mechanostimulation in wild-type plants, the refractory period in mutants was attenuated, resulting in extracellular ATP accumulation. We propose that ATP acts as an extracellular signal released by mechanostimulation and that the G-protein complex is needed for fine-tuning this response.

Keywords

Arabidopssi; 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

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elements have been identified. Similarly, although touch sensing relies on Ca^{2+}-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 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 (*gpa1*-3, *gpa1*-4, *agb1*-2) or EMS (*agb1*-9). *Arabidopsis* seeds were surface sterilized then stratified for 2 days. Seeds were sown on ½ X MS media (+ 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 1mM 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 sec. 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 N=kg × 9.8 m/s^2. The force was 198 mN. One standard deviation was estimated to be ~170 mN. Mock touch controls were performed by bringing the indicated probe up to but not touching the seedlings. Constant stimulation was performed by agitation on a rotary shaker. Seedlings grown in the indicated liquid media gently tumbled during rotation. This is a modification of the method used to generate phasic shear stress in animal cells cultures [8] and whole plants [6]. For *in situ* assays of ATP release [9], seedlings were grown on solid medium which was coated on a cover slip as described further below.

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 ½ 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 figures 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 luciferaseCBD solution were mounted in a perfusion chamber on ½ MS media with 1% (w/v) sucrose (1.2% (w/v) type VII agarose) and assay buffer (20 mM Tricine, 2.67 mM $MgSO_4$, 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 [γ^{32}P]ATP as described previously [11] and calculated according to the following formula. $v = k\ [ATP]_{bl}$, where v = the hypothetical rate of ATP hydrolysis at steady state, k = 1/half life (min), $[ATP]_{bl}$ = basal ATP concentrations (1 nM [specifically, 500 pmol in 500 ul]).

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 angle relative to the barrier using Image J software (National Institutes of Health, Bethesda, MD). Roots responding to gravistimulation were prepared, imaged and analyzed in the same manner, except that no cover glass was 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 (AtGPA1, 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 mechanoresponse, 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,Supplemental 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 Supplemental 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 presence of seedlings by using [γ^{32}P]ATP as a tracer, which yielded a rate of hydrolysis of 2.8 fmol/min (supplementary Fig 1A), with a $t_{1/2}$ of 180 mins (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 mechano-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 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 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 3B). 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 apoplast 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 supplemental material Fig 2C, mutant), suggesting that basal release of ATP can obscure or down-regulate touch-induced ATP release by the root cap.

Because mechano-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 *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 G-protein complex did not exhibit the pronounced refractory period to the same extent as wild type roots, suggesting that G-protein-dependent events are required to down-regulate mechano-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 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 concentrations 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 wildtype although the difference was not statistically significant (Supplemental figure 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 wildtype 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/Ca^{2+} 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 [21,22,23,24], and the downstream transduction mechanism, including a further search for purinoceptors [26,27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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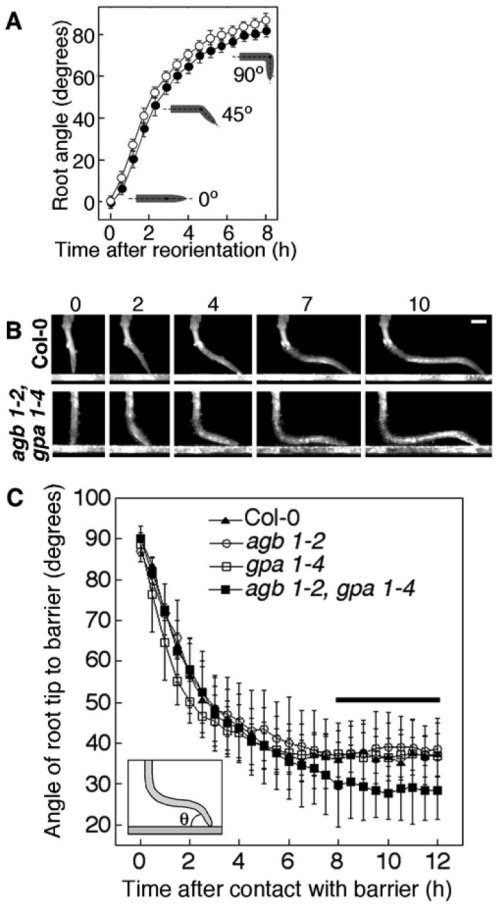

Fig 1. G-protein mutants are altered in obstacle avoidance but not gravitropism

(A) The graviresponse is unchanged in roots lacking a functional G-protein complex. WT (open circles) and *agb1-2*, *gpa1-4* (closed circles) seedlings were grown vertically then gravistimulated by turning the plates 90 degrees. The angle of the root tip relative to vertical was recorded over time (insets). (B) Roots lacking the G-protein complex show aberrant growth responses when presented with a barrier to vertical growth. Time-lapse movies were made of WT and G-protein mutant (*gpa1-4*, *agb1-2*) roots (Supplemental data, movies 1 and 2). Frames from the recording at the indicated times (hr) are shown for both genotypes. Images representative of ≥10 independent experiments. Scale bar = 300 μm. (C) Root tip angles of WT, *agb1-2*, *gpa1-4*, and the double mutant *agb1-2*, *gpa1-4* relative to a horizontal barrier were measured over time. Inset diagram indicates the root tip angle θ that was measured. Although the kinetics of the barrier response appeared different between *gpa1/agb1* and wild-type, there was sufficient variability in the response of the double mutant to necessitate rigorous statistical analysis of the angle of the root tip to the barrier to assess whether any apparent differences were significant. Based on the sampled population (average), the root tip angles of the double mutant are significantly different ($P<0.05$, two-way ANOVA, $n \geq 5$) during the time ranging from 8 to 12 hr after contacting the barrier (as indicated on the graph by a bar).

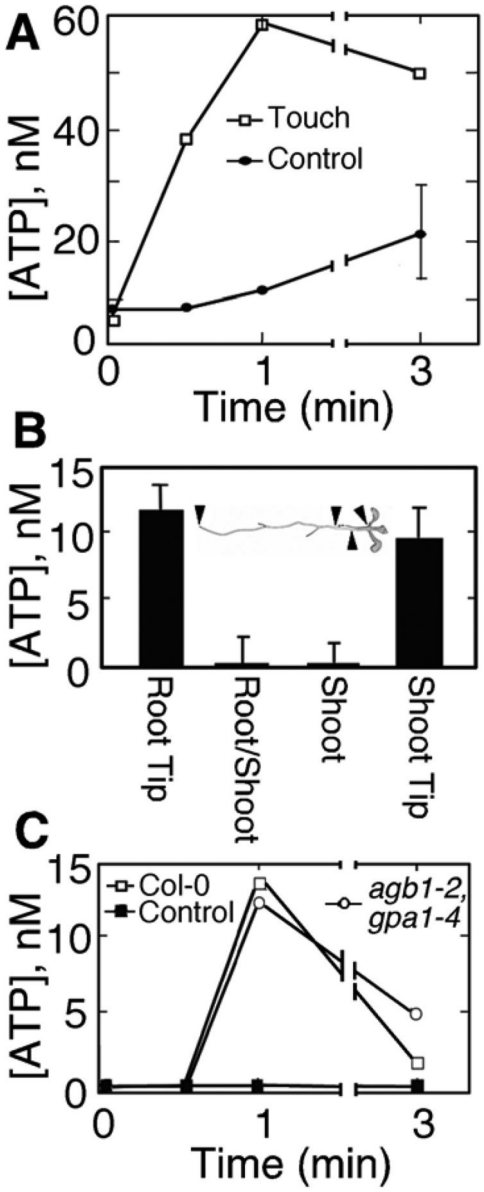

Fig 2. Touch-induced ATP release in Arabidopsis

(A) ATP concentration in the surrounding medium of plants touched by a pipette tip at t = 0 near the root tip (squares) and mock touch controls (circles). (B) ATP concentrations following touch at different positions of the seedling (inset with position touched marked by arrowheads). (C) ATP concentration over time after touch at t = 0 of WT (Col-O) (open squares) and G-protein mutant (*agb1*-2, *gpa1*-4) seedlings (open circles). Mock touch control of WT (closed squares). For these experiments (panels A-C), 1 seedling was placed in each well. Triplicates were performed and the standard error is less than the area of the symbols. These experiments were each repeated at least once.

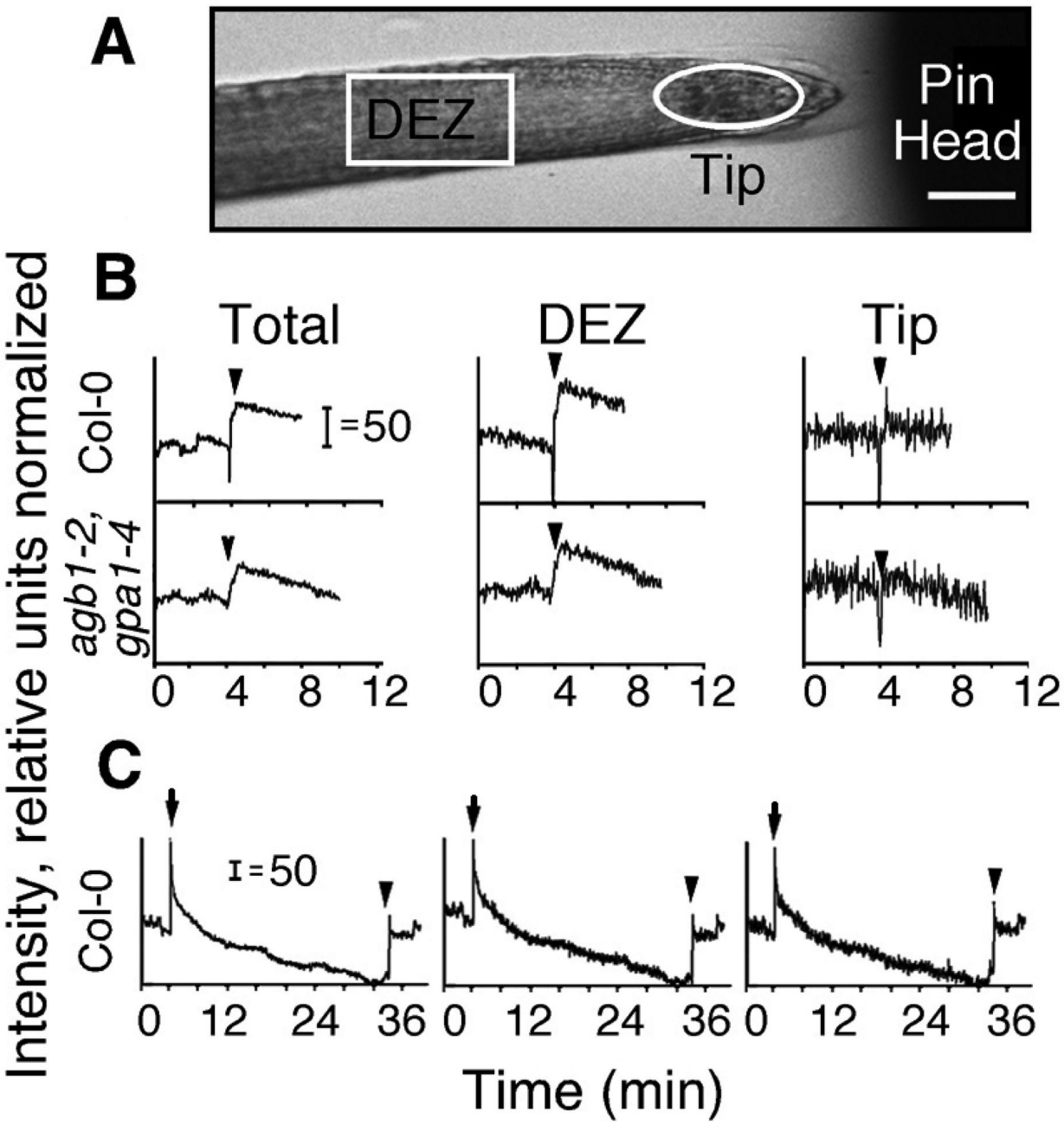

Fig 3. Touch-induced release of ATP visualized using luciferase-CBD

(A) Regions of the root were observed with the CBD-luciferase ATP assay. DEZ, distal elongation zone. (B) ATP, as reported by luciferase luminescence intensity, of both WT and G-protein mutant plants that were touched once using a pinhead (dark area labeled pinhead in panel A) as the stimulation probe (arrowhead marks the time) as described in the Materials and Methods. (C) Luciferase signal due to ATP release following touch (arrowhead) in roots treated with apyrase (arrow) are shown. Arrowhead marks the time when the root was touched. In all cases, values are the average of >5 independent experiments. Scale bar = 50 intensity units.

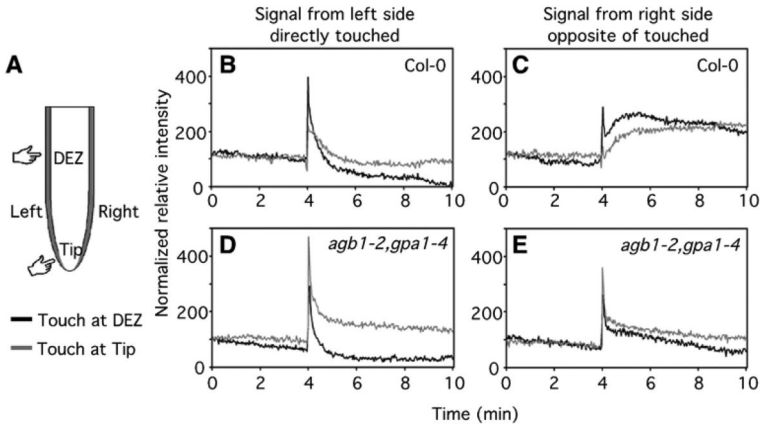

Fig 4. Effect of asymmetrical touch on ATP release

(A) Schematic showing a plant root touched on the left side at the DEZ or the tip. (B-E) ATP changes as indicated by luciferase signal intensity changes in WT on the (B) left and (C) right side and in the G-protein mutant on the (D) left and (E) right side. The area of the root analyzed is highlighted in dark gray in the cartoon of the root at the left. Touch at the tip region is shown in gray and DEZ in black. Each trace represents an average of 5 independent experiments. Typical SEM = 17.97 units. Correlation coefficient for each trace: (B vs. C) ATP on left vs. right when wild type plants are touched at the tip, r= -0.2123389 (not correlated); when touched at the DEZ, r= −0.46342686 (not correlated); (D vs. E) ATP on the right side when mutant plants are touched at the tip, r= 0.639966994 (correlated); when touched at the DEZ, r= 0.952578032 (correlated).

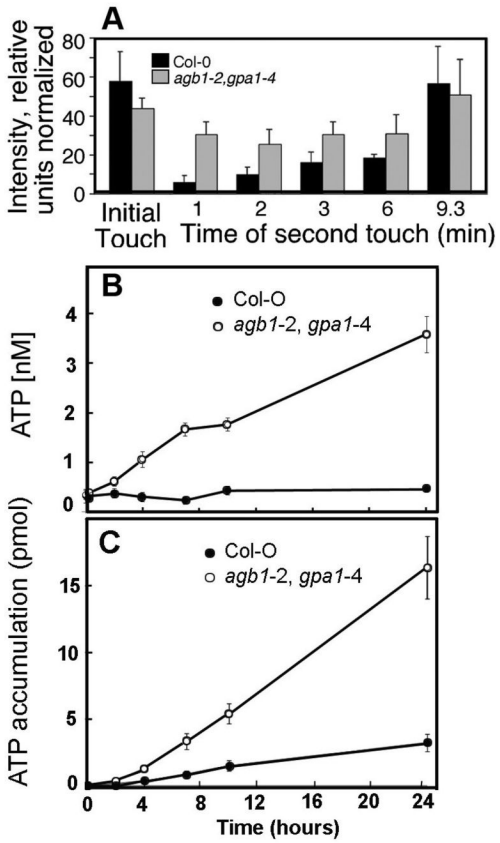

Fig 5. Touch-induced ATP release exhibits a refractory period in WT but not in the G protein mutants

(A) ATP release upon a second touch at the indicated time following initial stimulation from roots of WT and *agb1*-2, *gpa1*-4. Note the reduction of ATP release in WT within 1 min that recovers over 9.3 min whereas the G-protein mutant shows only a slightly reduced response over the equivalent time frame. Averages are from at least 5 independent trials. (B) ATP concentrations in the buffer, and (C) total ATP released from 5-day-old seedlings grown under constant mechano-stimulation (125 rpm). For these experiments (B, C), 2 seedlings were placed in each well as described. Genotypes are indicated.