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Conserved Class of Queen Pheromones Stops Social Insect Workers from Reproducing

Annette Van Oystaeyen *et al.*

Science **343**, 287 (2014);
DOI: 10.1126/science.1244899

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value in summer and a minimum value in winter. During the time period covered by our measurements, the GHSZ was at its maximum in June 2011, when it extended to a 360-m water depth. Increasing bottom-water temperatures from June until December were accompanied by a retreat of the GHSZ at the seafloor to more than 410-m water depth. In the subsurface, the GHSZ retreated further until it reached its minimum in March 2012.

The modeling shows that persistent supply of dissolved methane from below the GHSZ in this section of the slope would lead to the formation of hydrate from winter until summer. The newly formed hydrate would dissociate again during the second half of the year and thus augment methane emissions from the seabed both by opening pathways to gas ascending from underneath and by releasing gas from the hydrate phase. The total volume of sediment that was affected by seasonal shifts of the GHSZ amounted to between 3000 and 5000 m^3 per meter of the margin. Assuming a gas hydrate concentration of 5% of the pore space and a porosity of 50%, the seasonal GHSZ has the potential to periodically store and release between 9 and 15 tons of CH_4 per meter of the margin. However, these amounts represent the upper limits of the seasonal buffering capacity, because the latent heat of hydrate kinetics was not included in the simulation. Depending on the concentration and distribution of gas hydrates in the sediment, alternating formation and dissociation would dampen the oscillation of the GHSZ and thus reduce its volume.

Although the modeling shows that seasonal bottom-water temperature variations are capable of modulating the observed gas emissions, we found no direct evidence in the heat flow data that would suggest that the slope sediments experienced decadal-scale warming. The combined data demonstrate that hydrate is playing a fundamental role in modulating gas seeps between 380- and 400-m water depth at the upper limit of the GHSZ, whereas ascending gas would be trapped or deviated up along the base of the GHSZ further seaward. Long-term variations in seepage may exist, but presently available data are insufficient to document annual, decadal, or centennial changes in seepage. Our data suggest that shallow hydrate accumulations are sensitive to bottom-water temperature changes and therefore that significant anthropogenic warming will affect the shallow parts of the hydrate system. This sensitivity demonstrates the need for quantifying the total amount of gas hydrate in the shallowest part of the gas hydrate stability zone if climate feedback mechanisms are to be assessed beyond simple global models (*20*, *21*). Our observations show that methane seepage west off Svalbard has been ongoing for much longer than anthropogenic warming. Therefore, observations of large contemporary emissions reported in other studies cannot be considered proof of accelerating hydrate destabilization, although neither do they prove that catastrophic destabilization is not accelerating.

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Acknowledgments: This manuscript is dedicated to the memory of our beloved colleague and friend Victoria Bertics. We are grateful to K. Bergmann and the officers and crew of R/V *Maria S. Merian* for their help at sea. The German Research Foundation (DFG), the Swiss National Science Foundation, and the Cluster of Excellence "The Future Ocean" supported the project financially. Further support came from the ESONET project (European Seas Observatory NETwork), the PERGAMON project (European Cooperation in Science and Technology), and the Alexander von Humboldt Foundation. Figure 1 was drafted by using Generic Mapping Tools (*22*). Supplementary data are available at http://dx.doi.org/10.1594/PANGAEA.824947.

Supplementary Materials

www.sciencemag.org/content/343/6168/284/suppl/DC1
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23 September 2013; accepted 16 December 2013
Published online 2 January 2014;
10.1126/science.1246298

Conserved Class of Queen Pheromones Stops Social Insect Workers from Reproducing

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A major evolutionary transition to eusociality with reproductive division of labor between queens and workers has arisen independently at least 10 times in the ants, bees, and wasps. Pheromones produced by queens are thought to play a key role in regulating this complex social system, but their evolutionary history remains unknown. Here, we identify the first sterility-inducing queen pheromones in a wasp, bumblebee, and desert ant and synthesize existing data on compounds that characterize female fecundity in 64 species of social insects. Our results show that queen pheromones are strikingly conserved across at least three independent origins of eusociality, with wasps, ants, and some bees all appearing to use nonvolatile, saturated hydrocarbons to advertise fecundity and/or suppress worker reproduction. These results suggest that queen pheromones evolved from conserved signals of solitary ancestors.

Eusocial insects exhibit a remarkable reproductive division of labor between queens and largely sterile workers. Mechanistically, pheromones emitted by the queen are thought to play a key role in suppressing worker reproduction (*1*). However, for most species, the identity of these pheromones remains unknown. In fact, decades of research have resulted in the successful identification of only a few, structurally unrelated, sterility-inducing queen pheromones in the domestic honeybee, *Lasius* ants, and one termite species (*1*–*5*). This paucity of data is unfortunate, because insights into the evolution of queen pheromones could contribute greatly to our understanding of the evolution of sociality.

In the present study, we test whether a structurally related class of queen pheromones might be biologically active across three independently evolved eusocial lineages. We hypothesized that long-chain hydrocarbons, which recently have been found to act as queen pheromones in *Lasius* ants (*3*, *4*) and which are thought to advertise fertility in diverse groups of social insects (*6*–*10*), could play such a role. We searched for sterility-inducing queen pheromones in representative species of wasps, bees, and ants—namely, the common wasp, *Vespula vulgaris*; the buff-tailed bumblebee, *Bombus terrestris*; and the desert ant, *Cataglyphis iberica*. Candidate queen pheromones were selected by comparing the cuticular

chemical profiles of queens and workers and ranking the components according to their queen-caste specificity [for details see (*11*) and table S1]. Subsequently, synthetic samples of several putative queen pheromones were tested for their ability to inhibit worker reproduction in nests from which the mother queen had been removed, after which all workers were dissected and their ovaries classified as undeveloped, developed, or regressed.

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Our results support the hypothesis that, in all three species, long-chain linear and methyl-branched saturated hydrocarbons are used to stop workers from reproducing (Fig. 1 and table S2). This reproductive inhibition occurred via two distinct physiological processes: (i) by preventing workers from activating their ovaries and (ii) by causing secondary oocyte resorption (regression). Live queens have also been shown to induce both of these effects, with queen-induced ovary regression being particularly well documented in bumblebees (table S2). Hence, the application of synthetic pheromones closely mimicked the effect of the presence of a live queen. Three of the same compounds that induced sterility in the common wasp—the linear alkanes n-C_{27} and n-C_{29} and the methylalkane 3-MeC_{29}—also did so in the desert ant (Fig. 1). Furthermore, the sterility-inducing queen pheromones of all three species were structural homologs of the previously identified queen pheromone of *Lasius* ants, 3-MeC_{31} (*5*, *6*), and the fertility signal n-C_{25} isolated from the ant *Aphaenogaster cockerelli* (*8*). The linear alkane n-C_{25} also induced strong ovary regression in the bumblebee, whereas four queen-specific esters had no effect on worker ovary development (Fig. 1). Thus, saturated hydrocarbons appear to act as a conserved class of queen pheromones in bumblebees, ants, and wasps, even though these taxa derive from separate, ancient origins of eusociality (*12*). These results do not necessarily imply that there is perfect conservation at the level of individual compounds. For example, the linear alkane n-C_{29}, which was active in both *Vespula* wasps and *Cataglyphis* ants, is most likely not biologically active in bumblebees, given that this compound is not overproduced in *Bombus terrestris* queens. In addition, we cannot exclude the possibility that other, as-yet-unidentified compounds act in synergy with the pheromones identified here.

To further investigate the extent to which sterility-inducing queen pheromones might be conserved at the level of chemical families across distantly related species, we conducted a systematic review of the chemicals that are overproduced by fertile individuals (mainly queens) relative to infertile individuals (workers) (table S3).

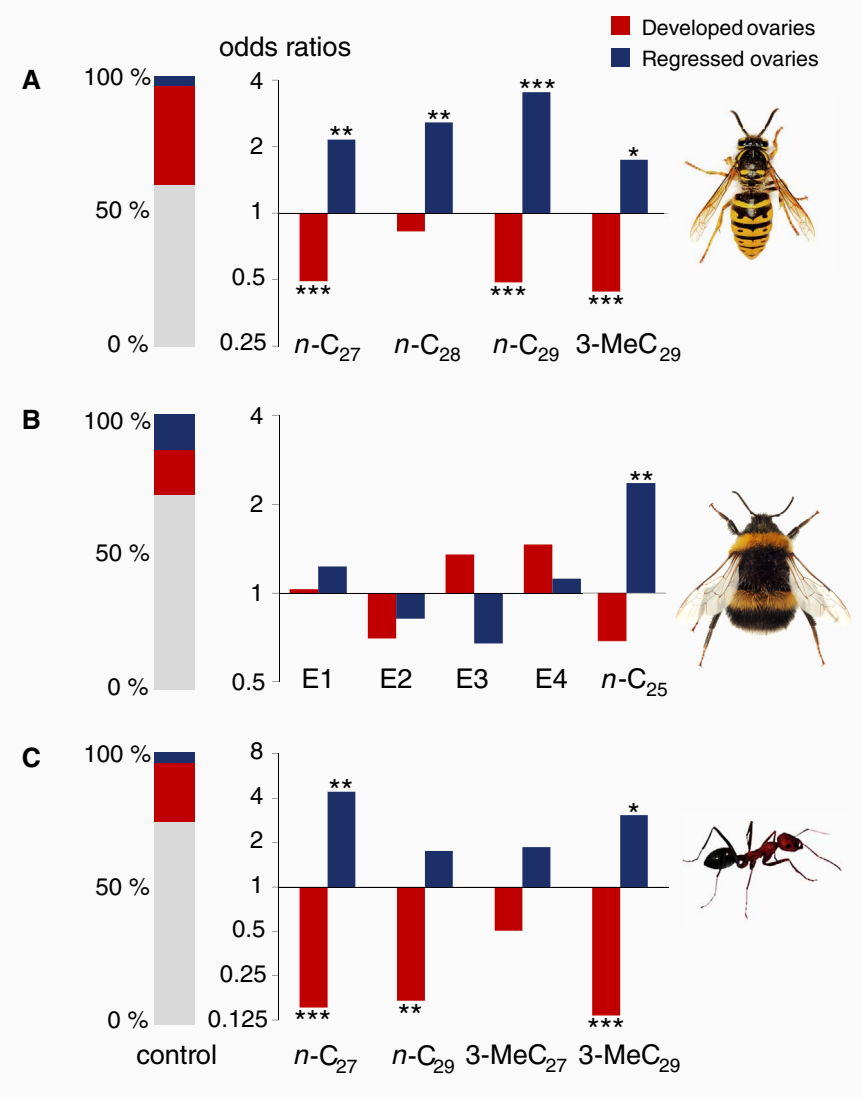

Fig. 1. Queen pheromone bioassays. The results demonstrate that long-chain cuticular hydrocarbons act as a conserved class of sterility-inducing queen pheromones in three independently evolved social insect lineages, represented by the wasp *V. vulgaris* (**A**), the bumblebee *B. terrestris* (**B**), and the ant *C. iberica* (**C**). Treatment of queenless worker groups with the linear alkanes n-C_{27} and n-C_{29} and the methyl alkane 3-MeC_{29} caused a two- to sevenfold reduction in the odds of workers having fully developed ovaries in the common wasp and the Iberian ant (bar charts, red bars) relative to a pentane-treated control (left, stacked bar charts) (significance levels based on binomial mixed models: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; see table S2 for detailed statistics). Furthermore, the probability of workers having regressed ovaries (blue bars) was increased ~two- to four-fold by treating colonies with n-C_{27}, n-C_{28}, n-C_{29}, and 3-MeC_{29} in the common wasp, n-C_{25} in the bumblebee, and n-C_{27} and 3-MeC_{29} in the Iberian ant. Decreased worker ovary development mimicked the effect of the presence of a live queen, as did the increased worker ovary regression observed in the bumblebee (table S2). By contrast, none of four queen-specific esters tested (E1 to E4: eicosyl, docosyl, tetracosyl, and hexacosyl oleate) significantly influenced worker ovary development in the bumblebee. Colony size and the number of males present were included in the model whenever they had a significant effect (table S2).

Based on data from 90 published studies, we reconstructed the evolutionary history of putative fertility or queen signals across 64 hymenopteran species comprising five independent origins of eusociality [ants, halictine bees, corbiculate bees, stenogastrine wasps, and polistine and vespine wasps (*12*)]. This comparative analysis demonstrates that saturated hydrocarbons are the single most common class of chemicals overproduced by queens or fertile individuals (Fig. 2). In fact, our ancestral state reconstruction shows that saturated hydrocarbons were most likely used as fertility

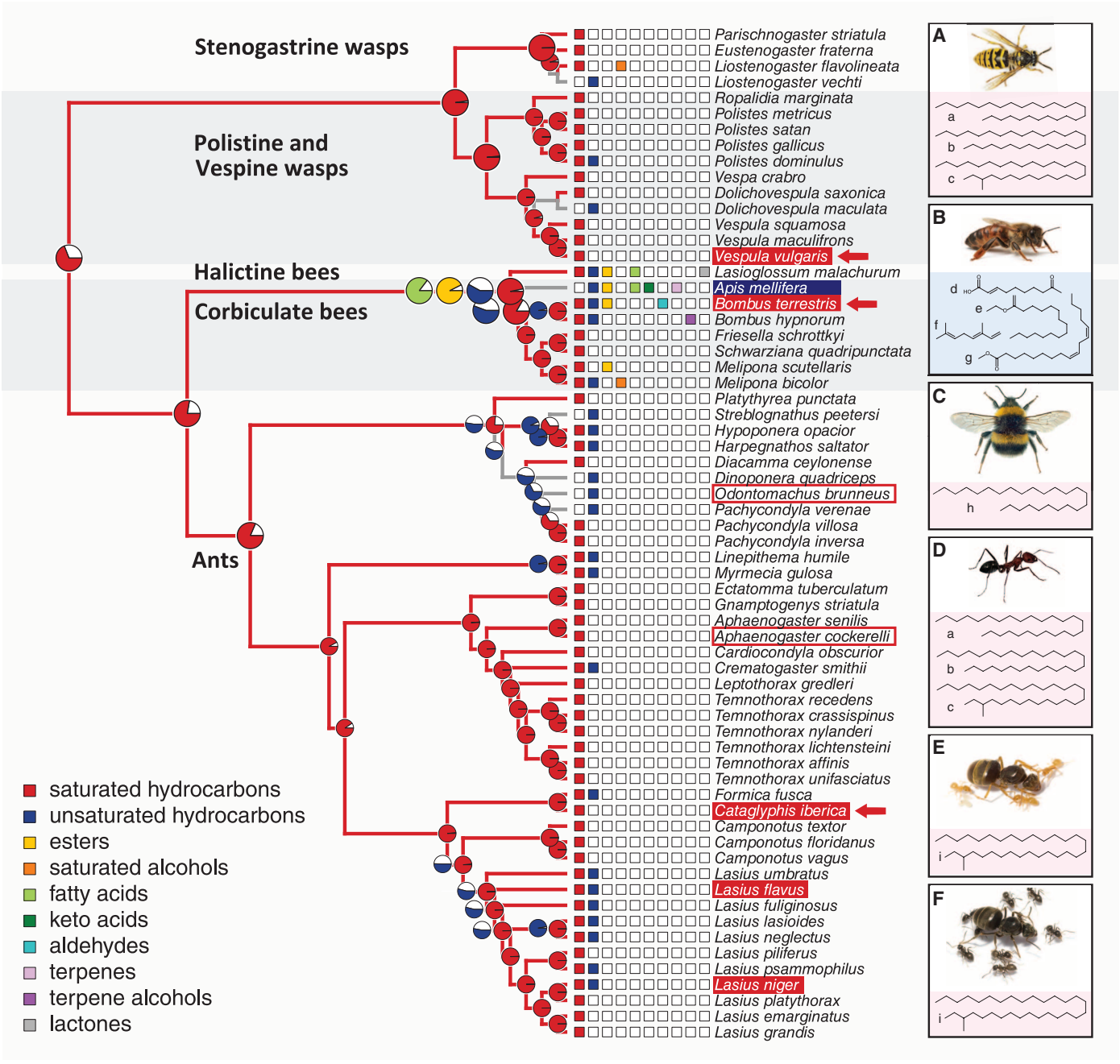

Fig. 2. The evolutionary history of queen and fertility signals across major clades of social hymenopteran insects. Each alternately shaded clade indicates an independent origin of eusociality (*12*). The pie charts show the likelihoods of different compound classes being used as queen or fertility signals as inferred from a maximum likelihood ancestral state reconstruction under a single-rate evolutionary model. Saturated hydrocarbons (linear and methyl-branched alkanes) receive very high support for being used as conserved queen or fertility signals across several independent origins of eusociality (red pie charts, see likelihood values in table S5; branches with likelihood of >50% are highlighted in red). By contrast, the use of other compound classes, such as the keto acid (*E*)-9-oxo-2-decenoic acid (*2*) (dark green) and the esters ethyl palmitate and methyl linoleate (*19*) in *A. mellifera* honeybees (yellow) or the alkene (*Z*)-9-nonacosene in *Odontomachus* ants (*9*) (blue), appear to be either derived or highly taxon-specific (pie charts of maximum likelihood reconstructions of particular compound classes are shown when likelihood values are >50%). Data are based either on the overrepresentation of particular compound classes on queens relative to workers or on direct experimental evidence for specific pheromones being used to suppress worker reproduction (solid highlights indicate direct physiological inhibition; open boxes indicate aggression-mediated inhibition) (table S3). Species tested in the present study are marked with arrows. The main active compounds are shown in the insets (**A**) *V. vulgaris* and (**D**) *C. iberica*: a = heptacosane, b = nonacosane, c = 3-methylnonacosane. (**B**) *A. mellifera*: d = (*E*)-9-oxo-2-decenoic acid, e = ethyl palmitate, f = (*E*)-β-ocimene, g = methyl linoleate. (**C**) *B. terrestris*: h = pentacosane. (**E**) *L. flavus* and (**F**) *L. niger*: i = 3-methylhentriacontane.

cues in the common solitary ancestor of all ants, bees, and wasps, which lived ~145 million years ago (*13*). Furthermore, the use of other compound classes as queen pheromones in all cases appears to be derived (Fig. 2). These secondary changes could be explained by minor biochemical alterations in pheromone biosynthesis. For example, up-regulation of desaturases could result in a shift from the use of saturated to unsaturated hydrocarbons (*14*, *15*). In addition, selection for other, more volatile, compound classes could be favored when queen pheromones serve multiple functions. In the honeybee, for example, the more volatile queen pheromone blend not only stops workers from reproducing but also induces long-range mate attraction and worker retinue behavior (*16*).

In conclusion, the results of our bioassays and comparative analyses demonstrate that structurally related compounds act as queen pheromones across several independent origins of eusociality. This finding implies that social insect queen pheromones likely evolved from preexisting fertility signals directed at males, e.g., in the context of mate choice or mate attraction (*14*, *15*). This theory is supported by the fact that, in both social and solitary insects, specific hydrocarbons are produced as direct or indirect by-products of ovary development and that some of these compounds function as mate attractants in solitary species (*14*, *15*). In the house fly, for example, ovary development results in the release of 20-hydroxyecdysone, which causes the production of the cuticular hydrocarbon sex pheromone (*Z*)-9-tricosene (*14*). Hence, our data provide important new support for the hypothesis that many traits in social insects are derived directly from a preexisting ground plan of solitary ancestors (*17*, *18*).

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Acknowledgments: We thank the Research Foundation Flanders (FWO), KU Leuven Centre of Excellence PF/2010/007 and the Erasmus Mundus Master in Evolutionary Biology (MEME) for funding, X. Cerdá and A. Lenoir for help with collecting the *Cataglyphis* ants, and M. Ayasse and R. Gadagkar for comments. P.d.E. was supported by a European Union Grant (FP7-MC-ERG-2009-256524) and C.A.O. and R.C.O. by scholarships from CNPq-Brazil (201959/2012-7 and 238127/2012-5). We are also grateful to G. Derdelinckx and K. Vestrepen for allowing access to their Shimadzu gas chromatograph– mass spectrometer. Our data are available from the Dryad Repository: http://dx.doi.org/10.5061/dryad.cg4qp.

Supplementary Materials

www.sciencemag.org/content/343/6168/287/suppl/DC1
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19 August 2013; accepted 26 November 2013
10.1126/science.1244899

Identification of a Plant Receptor for Extracellular ATP

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Extracellular adenosine 5′-triphosphate (ATP) is an essential signaling molecule that is perceived in mammals by plasma membrane P2-type purinoceptors. Similar ATP receptors do not exist in plants, although extracellular ATP has been shown to play critical roles in plant growth, development, and stress responses. Here, we identify an ATP-insensitive *Arabidopsis* mutant, *dorn1* (Does not Respond to Nucleotides 1), defective in lectin receptor kinase I.9 (*Arabidopsis* Information Resource accession code At5g60300). DORN1 binds ATP with high affinity (dissociation constant of 45.7 ± 3.1 nanomolar) and is required for ATP-induced calcium response, mitogen-activated protein kinase activation, and gene expression. Ectopic expression of *DORN1* increased the plant response to physical wounding. We propose that DORN1 is essential for perception of extracellular ATP and likely plays a variety of roles in plant stress resistance.

Adenosine 5′-triphosphate (ATP), the universal energy currency in all organisms, also acts as an extracellular signaling molecule (*1*). The first report that ATP could play an extracellular signaling role was viewed with skepticism (*2*). This skepticism disappeared when the plasma membrane receptors for extracellular ATP were identified in mammals (*3*, *4*). These receptors are of two general types: ligand-gated ion channel P2X and G protein–coupled P2Y receptors (*5*, *6*). The various members of these two receptor classes show distinct ligand binding affinity. Spatial and temporal expression patterns of these receptors support their postulated roles in the regulation of a broad range of mammalian physiology, including neurotransmission, muscle contraction, inflammation, and cell growth and death (*5*).

In contrast, relatively little is known about the role of ATP as an extracellular signal in plants, and some skepticism exists as to the role this molecule may play in plants. However, a number of papers have suggested a role for extracellular ATP in plant growth (*7*, *8*), development (*9*, *10*), and stress responses (*11*–*13*). Furthermore, the presence of extracellular ATP was visualized at actively growing root hair tips (*7*). ATP is actively released from plant cells in response to abiotic stresses (*14*), fungal elicitors (*7*, *15*), and mechanical stimuli (*16*). Some plant cellular responses to ATP are similar to those seen in mammals (*17*), for example, cytoplasmic calcium increase, production of reactive oxygen species and nitric oxide, and the role of ecto-apyrases that regulate extracellular ATP homeostasis. However, efforts to identify plant ATP receptors through their genomic sequence homology to animal purinoceptors failed to identify any suitable candidate proteins.

To identify genes involved in extracellular ATP recognition in plants, we performed a forward genetic screen for *Arabidopsis thaliana* mutants impaired in their ability to respond to ATP treatment. Exogenously applied ATP triggers cytoplasmic calcium influx in wild-type *Arabidopsis* seedlings expressing the calcium reporter protein, aequorin (*18*, *19*). We screened 50,000 ethylmethanesulfonate (EMS)–mutagenized seedlings for the ATP-induced calcium response (fig. S1) and identified two mutants lacking a cytoplasmic calcium response to ATP addition (Fig. 1A and fig. S2). As described below, these mutants also failed

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