Published in final edited form as:

Nature. 2008 January 10; 451(7175): 207–210. doi:10.1038/nature06478.

Abscisic acid controls calcium-dependent egress and development in *Toxoplasma gondii*

Kisaburo Nagamune¹, Leslie M. Hicks², Blima Fux¹, Fabien Brossier¹, Eduardo N. Chini³, and L. David Sibley¹

¹Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO, 63110

²Donald Danforth Plant Science Center, 975 N. Warson Rd., St. Louis, MO, 63132

³Department of Anesthesiology, Mayo Clinic and Foundation, Rochester, MN, 55905

Abstract

Calcium controls a number of critical events including motility, secretion, cell invasion, and egress by protozoan parasites ¹. Compared to animal ² and plant cells ³, the molecular mechanisms that govern calcium signaling in parasites are poorly understood. Here we demonstrate that the production of the phytohormone abscisic acid (ABA) controls calcium signaling within the apicomplexan parasite *Toxoplasma gondii*, an important human pathogen. In plants, ABA controls a number of important events including environmental stress responses, embryo development, and seed dormancy ^{4,5}. ABA induces production of the second-messenger cyclic ADP ribose (cADPR), which controls release of intracellular calcium stores in plants ⁶. cADPR also controls intracellular calcium release in the protozoan parasite *T. gondii* ^{7,8}; however, previous studies have not revealed the molecular basis of this pathway⁹. Addition of exogenous ABA induced formation of cADPR in *T. gondii*, stimulated calcium-dependent protein secretion, and induced parasite egress from the infected host cell in a density-dependent manner. Production of endogenous ABA within the parasite was confirmed by HPLC purification and GC-MS analysis. Selective disruption of ABA synthesis by the inhibitor fluridone (FLU) delayed egress and induced development of the slow-growing, dormant cyst stage of the parasite. Thus, ABA-mediated calcium signaling controls the decision between lytic and chronic stage growth, a developmental switch that is central in pathogenesis and transmission. The pathway for ABA production was likely acquired with an algal endosymbiont that was retained as a non-photosynthetic plastid known as the apicoplast. The plant-like nature of this pathway may be exploited therapeutically as shown by the ability of a specific inhibitor of ABA synthesis to prevent toxoplasmosis in the mouse model.

Calcium-mediated secretion in *T. gondii* controls both motility and cell invasion and previous studies have demonstrated that these processes utilize the second messenger cADPR, yet the signals triggering this pathway remain unresolved ^{7,8}. In plants ⁶, hydra ¹⁰, and sponges ¹¹, ABA stimulates release of intracellular calcium through elevation of the cyclic nucleotide cADPR. Addition of exogenous ABA proved to be a potent agonist of secretion in *T. gondii* as shown by the release of the protein MIC2, a parasite adhesin that is discharged into the

Correspondence and requests for materials should be addressed to L.D.S. (sibley@wustl.edu).

Supplementary Information accompanies the paper on www.nature.com/nature.

Author contributions:

K.N. performed the majority of experiments on the affects of ABA and FLU on the parasite, L.M.H. performed the MS studies, B.F. performed the animal studies, F.B. contributed to the analysis of ABA genes, E.N.C. performed the measurements of cADPR, L.D.S. supervised the project and wrote the manuscript with input from all the authors.

supernatant in response to increases in intracellular calcium (Fig. 1A). Induction of MIC2 secretion by ABA was highly specific to (+) - ABA and was not induced by (-) - ABA, the precursor β -carotene, or retinoic acid (Fig. 1B). Treatment with ABA lead to a dose-dependent increase in the second messenger cADPR in *T. gondii*, suggesting ABA may be a natural agonist for calcium signaling in parasites (Fig. 1C). Finally, chelation of intracellular calcium in the parasite blocked secretion induced by ABA, confirming that it acts through release of an intracellular calcium pool (Fig. 1D). Collectively these results indicate that ABA in *T. gondii* controls release of calcium from intracellular stores through production of cADPR, thus paralleling the pathway seen in plants⁶, and early metazoans^{10,11}.

Consistent with its physiological effects, ABA was detected in lysates of *T. gondii* by direct biochemical analyses. Extracts of *T. gondii* were purified by HPLC and analyzed by gas chromatography to detect the methyl ester of ABA (methyl-ABA), which migrated with the same retention time (8 min) as the authentic methyl-ABA standard (Fig. 2A). Mass spectrometric analysis of *T. gondii* samples yielded the expected product of m/z 190 as well as peaks at m/z 91 and 162, characteristic of cis-ABA, and m/z 91 and 134, characteristic of trans-ABA, as described previously¹² (Fig. 2B and Supplementary Fig. 1). The slightly noisier spectrum obtained from *T. gondii* is consistent with the lower level of ABA found in the sample, nonetheless its signature unambiguously matches ABA. Similar levels of ABA were detected in parasite extracts using a highly specific ELISA kit (Fig. 2C). To confirm that ABA was indeed synthesized by the parasite, we used the herbicide fluridone (FLU) an inhibitor that specifically blocks phytoene desaturase activity, and hence prevents synthesis of ABA by the indirect pathway that predominates in plants⁵. Treatment with FLU reduced the level of ABA in parasites by more than 4-fold (Fig. 2C). Interestingly, when the level of ABA was monitored during one cycle of intracellular growth, it remained relatively constant during the first 30–36 hrs and then spiked shortly before egress of parasites from the infected host cell, which begins around 40–44 hrs (Fig. 2D).

Toxoplasma is an obligate intracellular pathogen and following invasion, the parasite replicates by binary fission within a vacuole, ultimately leaving the depleted host cell by active egress. Intracellular calcium is central for several of these steps, leading us to examine whether ABA-dependent calcium signaling was essential for growth *in vitro*. Treatment of parasite cultures with increasing concentrations of the ABA inhibitor FLU, lead to inhibition of parasite growth ($EC_{50} \sim 15 \mu\text{M}$ (Fig. 3A)). Surprisingly, FLU treatment did not block invasion or intracellular replication, but instead inhibited exit of mature parasites from the infected cell. While parasites had naturally egressed by ~ 48 hrs, those treated with $15 \mu\text{M}$ FLU were delayed, and those treated with $50 \mu\text{M}$ FLU were completely blocked out to 84 hrs (Fig. 3B), after which the integrity of the monolayer declined (data not shown). Previous studies have implicated calcium signaling in parasite egress from infected cells^{13,14} and the rapid increase in ABA concentrations in the parasite just prior to egress (Fig. 2D), suggested this molecule may be the natural signal for activating exit. Consistent with this, when exogenous ABA was added to late-stage vacuoles, it induced premature egress (Fig. 3C). Finally, ABA was able to overcome the inhibition of egress caused by treatment with FLU (Fig. 3D), consistent with it being able to bypass the metabolic block in biosynthesis. While the responses to exogenously added ABA are somewhat modest, this may reflect the relatively low permeability of the charged anion that exists at physiological pH (pK_a 4.8), and the fact that it has to cross several membranes to reach the parasite. Whether ABA produced by the parasite accumulates within the parasite or is exported to the parasite-containing vacuole is unknown and the mechanism(s) whereby ABA is sensed remain to be elucidated. Nonetheless, these results indicate that ABA is a natural agonist for host cell egress and that in the absence of its production, parasites remain quiescent within the host cell.

Toxoplasma undergoes two fundamentally different forms of growth within host cells: 1) the lytic cycle described above, which rapidly amplifies parasite numbers, and 2) differentiation into semi-dormant cysts that are long-lived. These adaptations serve different roles during infection, the first leading to active dissemination and the second resulting in a life-long chronic infection. The decision to undergo this developmental switch is poorly understood, but is thought to be a response to stress. In FLU-treated cultures, parasites that failed to egress from the host cell, commenced to develop into tissue cysts, as shown by staining with the cyst-wall specific lectin *Dolichos biflorus* (DBL) (Fig. 4A). Tissue cysts produced by treatment with FLU were positive for several stage-specific cyst markers including DBL, the cyst antigens CST1¹⁶, and BAG1¹⁷ (Fig. 4B). Ultrastructural examination of the cysts formed by FLU treatment revealed hallmark features of parasites within cysts including a posterior positioned nucleus, amylopectin granules, dense rhoptries, and a convoluted cyst wall (Fig. 4C). FLU treatment also enhanced the response of parasites to alkaline pH stress (Supplemental Fig. 2), a common experimental method used to induce differentiation¹⁵.

In addition to blocking egress *in vitro*, FLU treatment was also able to prevent lethal infection in laboratory mice, which provide an excellent model for toxoplasmosis (Fig. 4D). Imaging of parasite dissemination *in vivo* using a luciferase expressing stain revealed that FLU treatment greatly reduced parasite burdens during *in vivo* infection in the mouse (Fig. 4E, Supplementary Fig. 3), which led to decreased cyst formation in the CNS during chronic infection (Fig. 4F). Fluridone is a registered herbicide that has low toxicity to mammalian cells, owing to the absence of this biosynthetic pathway in animals. Our results, combined with a previous patent indicating that it is also effective against malaria¹⁸, suggest that this selectivity may be exploited for development of improved anti-parasitic drugs.

ABA synthesis in plants proceeds from isoprenoids using an indirect pathway that flows through β -carotene in the plastid⁵. *T. gondii* utilizes a non-mevalonate pathway for isoprenoid biosynthesis¹⁹, which occurs in the apicoplast, a remnant organelle derived from an algal endosymbiont²⁰. The sensitivity of *T. gondii* to fluridone indicates that it contains an indirect pathway for ABA production, which depends on the plastid, rather than the direct pathway found in fungi⁵, thus providing an additional explanation for the essential nature of the apicoplast²⁰. While the *T. gondii* genome (<http://ToxoDB.org>) contains several candidates with similarity to ABA synthesis genes in higher plants (i.e. *ABA1*, *ABA2*, and *ABA3*)⁵, phylogenetic and domain analyses do not provide unambiguous assignments of these orthologues (data not shown). This divergence is not unexpected given that the plant-like nature of apicomplexans is based on acquisition of an early branching algal endosymbiont²¹. While algae have also been shown to make ABA^{22,23} and respond to this hormone²⁴, the precise pathways and genes involved in ABA production by algae are not established. Future studies aimed at defining the pathway for production of ABA in *T. gondii* may provide further insight into the role of this hormone outside of higher plants.

Our findings demonstrate the conservation of a common plant-like, calcium-signaling pathway in the protozoan parasite *T. gondii*. Production of ABA is a density-dependent signal that influences the decision for lytic growth vs. dormant development in *T. gondii*. ABA has recently been shown to regulate gene expression by altering mRNA translation²⁵, and to activate G-protein coupled receptor signaling²⁶ in plants, indicating that it has additional important functions beyond calcium signaling. These ABA-response genes are conserved in *T. gondii*, and the related parasite *Plasmodium* (<http://ApiDB.org>), where they may play analogous roles in ABA-mediated signaling and development. Disruption of ABA-mediated signaling in parasites offers a promising new target for development of improved interventions aimed at combating infection with apicomplexan parasites.

Methods Summary

Growth and egress assays

Intracellular parasite growth was monitored using β -galactosidase activity⁷, or by microscopic examination determination of the number of parasites per vacuole^{13,15}. Egress was monitored by the % of intact vacuoles after cultured for defined intervals with and without FLU, followed by treatment with ABA.

Secretion assay

Calcium-dependent secretion was monitored by release of the microneme protein 2 (MIC2)^{7,27}, with β -galactosidase as a cell-loading and non-specific lysis control.

ABA purification and analysis

ABA was extracted from parasites, purified by HPLC, converted to methyl-ABA²⁸, and analyzed by tandem mass spectrometry using an Agilent Technologies GC-MS instrument to detect the diagnostic fragments¹².

ABA detection by ELISA

ABA concentrations in parasite extracts were determined by ELISA using the Phytodetek® ABA Test Kit (Agdia, Elkhart, IN) after subtraction of the background level of ABA in cultured host cells processed in parallel, and expressed as intracellular concentrations²⁹.

Cyclic ADPR

Freshly harvested parasites were snap frozen at -80°C , extracted, and cADPR levels determined as described previously⁸.

In vitro differentiation

Cyst formation was monitored following treatment with FLU under normal culture conditions as detected using FITC-labeled *Dolichos biflorus* lectin (DBL) (Sigma) or antibodies to the cyst antigens CST1¹⁶ or BAG1¹⁷. Transmission electron microscopy was performed as described previously¹⁵.

Mouse infections

BALB/c mice were infected with type II strain PTG strain by ip injection and treated with fluridone by daily i.p. injection for 12 days. To monitor tissue burdens, mice were challenged with the luciferase expressing Pru-Luc strain and imaged as described previously³⁰.

Full Methods accompany this paper

Methods

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including: ($\pm$) cis, trans abscisic acid (referred to as ($\pm$) ABA or ABA), ($-$) cis, trans ABA ($-$ ABA), retinoic acid, and β -carotene. BAPTA and BAPTA-AM were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Fluridone was obtained from Chem Service Inc., (West Chester, PA). Chemicals were dissolved in DMSO and stored at -80°C .

Protein secretion

To examine the induction of microneme protein secretion, purified 2F strain parasites, which express β -galactosidase, were treated with compounds for 5–10 min at 18°C, and separated into supernatant and cell pellet by centrifugation. MIC2 secretion was detected by Western blotting with anti-MIC2 rabbit antibody (supernatant). Cell pellets were probed with anti- β -galactosidase monoclonal antibody 40a-1 as a control for loading and inadvertent lysis. Secreted MIC2 in the supernatant migrates slightly faster than cellular MIC2 (cMIC2) due to proteolysis during shedding²⁷. To test the roles of extracellular and intracellular calcium, respectively, purified parasites were treated with 1 mM of BAPTA or 50 μ M of BAPTA-AM for 20 min at 18 °C before treatment with 100 nM of ABA for 5 min, as described previously⁷.

Growth and egress assays

Parasite replication and egress were monitored by microscopic examination of parasites grown in human foreskin fibroblast (HFF) cells cultured on glass coverslips. Following culture for specific time intervals, the number of intracellular parasites per vacuole, and the % of intact vs. disrupted vacuoles (egress), was determined by examining specific antibody-stained cells (i.e. SAG1 surface staining, DAPI nuclear staining) under epifluorescence microscopy, as described previously^{13,15}. Egress was monitored following culture in normal medium for defined intervals followed by stimulation with ABA for 1 hr, and microscopic examination to determine the % of intact vacuoles as above. To test the ability of ABA to overcome inhibition of egress by FLU, cells were cultured in control medium or medium containing FLU for 38 hrs, then treated with ABA for 1 hr, and examined microscopically to determine the % of intact vacuoles as above.

GC-MS analysis of ABA

Parasites from heavily infected cultures at 30–36 hrs post inoculation, were snap frozen in an ethanol-dry ice bath, lyophilized, and stored at –80°C. Tissues were extracted in organic solvents and resolved by HPLC using a C18 reverse phase column as described previously²⁸. Fractions containing ABA were collected, dried under vacuum, methylated using diazomethane, and analyzed by gas chromatography in comparison to standards for methyl-ABA (kindly provided by Jan Zeevaert, Michigan State Univ.), as described previously²⁸. Tandem mass spectrometry analyses were performed on an Agilent Technologies GC-MS (6890N network GC system). Samples (dissolved in 10 μ L) and standards (appropriate dilutions) were solubilized in ethyl acetate, and injected into the GC-MS instrument. The samples were introduced through a DB-5 capillary column (30m $\times$ 0.250 mm i.d., film thickness 0.25 μ m (J&W Scientific)) with helium as the carrier gas (flow-rate 1.3 mL / min). The GC oven was programmed from 60 to 210°C at 25°C min^{–1} and from 210 to 280°C at 12°C min^{–1}. The ion-source temperature was 150°C. The mass analyzer was scanned from m/z 50 to 400 at a data rate of 20 Hz for the full scan analyses, and m/z 190 at a data rate of 20 Hz for single ion monitoring (SIM) analyses. Spectra from parasite extracts were compared to the methyl-ABA standard and previously reported literature values of cis and trans ABA¹².

ABA detection by ELISA

ABA concentrations in parasite extracts were determined by ELISA using the Phytodetek® ABA Test Kit (Agdia, Elkhart, IN) after subtraction of the background level of ABA in cultured host cells processed in parallel. Concentrations of ABA in the parasite were expressed as intracellular concentrations based on previous estimates of the parasite volume²⁹.

Cyclic ADPR

T. gondii parasites were purified from culture and treated with doses of ABA for 10 min. Parasites were snap frozen at -80°C , extracted in 5% v/v trichloroacetic acid at 4°C , and cADPR levels determined using the cyclase reaction, as described previously⁸.

In vitro differentiation

Type II strain ME49 parasites were induced to differentiate by treatment with FLU in normal culture medium (DMEM, 10% FBS, 10 mM HEPES, gentamicin) during culture for 60 hrs at 37°C , 5% CO_2 . Alternatively, induction was performed by culture 72–96 hrs in RPMI (pH 8.1) in atmospheric air at 37°C . Cysts were stained with FITC-labeled *Dolichos biflorus* lectin (DBL) (Sigma), DAPI to label DNA, anti-CST1 mAb73.18 16, and anti-BAG1 17 (kindly provided by Louis Weiss, Albert Einstein College of Medicine) followed by fluorescently conjugated secondary antibodies. For ultrastructural analysis, cultures were induced as described above and processed for transmission electron microscopy, as described previously¹⁵. Cysts were classified based on the presence of a convoluted cyst wall, amylopectin granules, solid-staining rhoptries, and the posterior position of the nucleus¹⁵.

In vivo infections in mice

BALB/c mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and infected with tachyzoites of the type II strain PTG, or the type II PRU strain expressing firefly luciferase (LUC) under the control of the *T. gondii* alpha tubulin promoter (kindly provided by John Boothroyd, Stanford Univ.). Parasites were propagated *in vitro* in monolayers of HFF cells and tachyzoites were injected by intraperitoneal (i.p.) route. An inoculum capable of resulting in death in 90% of untreated animals (LD_{90}) was used for challenges that consisted of 2×10^3 parasites for PRU-LUC and 2×10^5 for PTG strain parasites. Animals were treated for 12 days by daily i.p. injection with 1 mg/kg or 2 mg/kg of fluridone (Chem Services, West Chester, PA) that was dissolved in DMSO and diluted in PBS. Experiments were conducted with 3–5 animals per group and were repeated 3 or more times. Survival was recorded for 30 days.

For bioluminescence experiments, animals were imaged at two day intervals, following mild anesthesia using isofluorene and i.p. injection of 0.15 mg/kg body weight of firefly luciferin D (Biosynth AG, Switzerland). Animals were imaged using a Xenogen IVIS 100 (Caliper LifeSciences, Hopkinton, MA). Data are expressed in relative light units and are shown as mean $\pm$ s.d.. Experiments contained 5 animals per group (except a single animal was used for the non-infected negative control). Animals were followed for 30 days post-infection, after which chronic infection was confirmed by examining homogenates of the brain for tissue cysts, as described previously¹⁵. Briefly, animals were humanely sacrificed, the brains removed and homogenized in 1 ml of sterile PBS. Homogenates were examined by bright field or phase contrast microscopy at 40X and cysts were counted from 4 separate 10 μL aliquotes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jan Zeevaart for conducting the initial purification and analysis of ABA and for supplying standards, John Boothroyd, and Lou Weiss for providing reagents, Wandy Beatty, Silvia Moreno, Boris Striepen, Andy Waters and Liming Xiong for helpful comments, and Julie Nawas and Darcy Gill for expert technical assistance. Supported by the Uehara Medical Foundation (to K.N.), the Mayo Clinic and American Heart Association (to E.N.C.), and a grant from the NIH (to L.D.S.).

References

- Moreno SN, Docampo R. Calcium regulation in protozoan parasites. *Curr. Opin. Microbiol* 2003;6:359–364. [PubMed: 12941405]
- Berridge MJ, Bootman MD, Roderick HL. Calcium signaling: dynamics, homeostasis and remodelling. *Nat. Rev. Molec. Cell Biol* 2003;4:517–529. [PubMed: 12838335]
- Bothwell JHF, Ng CKY. The evolution of Ca^{2+} signaling in photosynthetic eukaryotes. *New Phytologist* 2005;166:21–38. [PubMed: 15760348]
- Xiong L, Zhu JK. Regulation of abscisic acid biosynthesis. *Plant Physiol* 2003;133:29–36. [PubMed: 12970472]
- Schwartz SH, Qin X, Zeevaart JAD. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiol* 2003;131:1591–1601. [PubMed: 12692318]
- Wu Y, et al. Abscisic acid signaling through cyclic ADP ribose in plants. *Science* 1997;278:2126–2130. [PubMed: 9405349]
- Lovett JL, Marchesini N, Moreno SN, Sibley LD. *Toxoplasma gondii* microneme secretion involves intracellular Ca^{2+} release from IP_3 /ryanodine sensitive stores. *J. Biol. Chem* 2002;277:25870–25876. [PubMed: 12011085]
- Chini EN, Nagamune K, Wetzel DM, Sibley LD. Evidence that the cADPR signaling pathway controls calcium-mediated secretion in *Toxoplasma gondii*. *Biochem. J* 2005;389:269–277. [PubMed: 15773818]
- Nagamune K, Sibley LD. Comparative genomic and phylogenetic analyses of calcium ATPases and calcium-regulated proteins in the Apicomplexa. *Molec. Biol. Evol* 2006;23:1613–1627. [PubMed: 16751258]
- Puce S, et al. Abscisic acid signaling through cyclic ADP-ribose in hydroid regeneration. *J. Bio. Chem* 2004;279:39783–39788. [PubMed: 15252012]
- Zocchi E, et al. The temperature-signaling cascade in sponges involves a heat-gated cation channel, abscisic acid, and cyclic ADP ribose. *Proc. Natl. Acad. Sci. USA* 2001;98:14859–14864. [PubMed: 11752433]
- de Almeida JAS, Kascheres C, Pereira MDA. Ethylene and abscisic acid in the control of development of the rhizome of *Koleria eriantha* (Benth.) Hanst. (Gesneriaceae). *Braz. J. Plant Physiol* 2005;17:391–399.
- Moudy R, Manning TJ, Beckers CJ. The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J. Biol. Chem* 2001;276:41492–41501. [PubMed: 11526113]
- Endo T, Sethi KK, Piekarski G. *Toxoplasma gondii*: calcium ionophore A23187-mediated exit of trophozoites from infected murine macrophages. *Exp. Parasitol* 1982;53:179–188. [PubMed: 6800836]
- Fux B, et al. *Toxoplasma gondii* strains defective in oral transmission are also defective in developmental stage differentiation. *Infect. Immun* 2007;75:2580–2590. [PubMed: 17339346]
- Zhang YW, Halonen SK, Ma YF, Wittner M, Weiss LM. Initial characterization of CST1, a *Toxoplasma gondii* cyst wall glycoprotein. *Infect. Immun* 2001;69:501–507. [PubMed: 11119543]
- Zhang YW, et al. Disruption of the *Toxoplasma gondii* bradyzoite-specific gene BAG1 decreases *in vivo* cyst formation. *Molec. Microbiol* 1999;31:691–701. [PubMed: 10027984]
- Leef, JL.; Carlson, PS. USA: Potomax Ltd. Prtn.; 1999. p. 1-8.
- Jomaa H, et al. Inhibitors of the non-mevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* 1999;285:1573–1576. [PubMed: 10477522]
- Ralph SA, et al. Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. *Nature* 2004;2:203–216.
- Waller RF, McFadden GI. The apicoplast: a review of the derived plastid of apicomplexan parasites. *Curr. Issues Mol. Biol* 2005;7:57–79. [PubMed: 15580780]
- Hirsch R, Hartung W, Gimmler H. Abscisic acid content of algae under stress. *Botanica Acta* 102:326–334. 19889.
- Cowan AK, Rose PD. Abscisic acid metabolism in salt stressed cells of *Dunaliella salina*. *Plant Physiol* 1991;97:798–803. [PubMed: 16668469]

24. Kobayashi M, Hirai N, Kurimura Y, Ohigashi H, Tsuji Y. Absciscic acid-dependent algal morphogenesis in the unicellular green alga *Haematococcus pluvialis*. *Plant Growth Reg* 1997;22:79–85.
25. Razem FA, El-Kereamy A, Abrams SR, Hill RD. The RNA-binding protein FCA is an absciscic acid receptor. *Nature* 2006;439:290–294. [PubMed: 16421562]
26. Liu X, et al. A G protein-coupled receptor is a plasma membrane receptor for the plant hormone absciscic acid. *Science* 2007;315:1712–1716. [PubMed: 17347412]
27. Carruthers VB, Sherman GD, Sibley LD. The *Toxoplasma* adhesive protein MIC2 is proteolytically processed at multiple sites by two parasite-derived proteases. *J. Biol. Chem* 2000;275:14346–14353. [PubMed: 10799515]
28. Tian L, DellaPenna D, Zeevaart JAD. Effect of hydroxylated carotenoid deficiency on ABA accumulation in *Arabidopsis*. *Physiol. Plant* 2004;122:314–320.
29. Rodrigues CO, Ruiz FA, Rohloff P, Scott DA, Moreno SNJ. Characterization of isolated acidocalcisomes from *Toxoplasma gondii* tachyzoites reveals a novel pool of hydrolyzable polyphosphate. *J. Biol. Chem* 2002;277:48650–48656. [PubMed: 12379647]
30. Saeij JP, Boyle JP, Grigg ME, Arrizabalaga G, Boothroyd JC. Bioluminescence imaging of *Toxoplasma gondii* infection in living mice reveals dramatic differences between strains. *Infect. Immun* 2005;73:695–702. [PubMed: 15664907]

A Induction of protein secretion by ABA

B

Calcium-dependence of ABA

C

D

Fig. 1.

ABA induced cADPR production and calcium-dependent protein secretion by *T. gondii*. (A) Protein secretion induced by ABA was compared to the positive control ethanol (EtOH) as detected by Western blotting with anti-MIC2 (supernatant) and anti-β-galactosidase antibodies (cell pellet) 7·8. (B) Induction of MIC2 secretion was specific for (±) ABA and was not seen with (-) ABA, β-carotene, or retinoic acid. (C) cADPR production in *T. gondii* was increased by treatment with ABA. Student's *t*-test. *, *p* < 0.02; **, *p* < 0.01 vs. control, Mean ± s.e.m., N = 3 experiments. (D) ABA-induced secretion required intracellular calcium. Treatment with the membrane permeable calcium chelator, BAPTA-AM, blocked ABA-induced MIC2

release, while non-permeant BAPTA had no effect. Cellular MIC2 (cMIC2) migrates slower due to absence of processing.

Fig. 2.

Biochemical evidence for the production of ABA in *T. gondii*. (A) GC traces of ABA standards and *T. gondii* samples. A single peak with similar retention time (8.0 min) to authentic methyl-ABA was detected in extracts of the parasite following chemical extraction and methylation. (B) Mass spectra of methyl-ABA standard and *T. gondii* sample from A, showing characteristic fragment at m/z 190, with additional minor peaks in common. (C) Detection of ABA by ELISA in control parasites (*T. gondii*) or treated with 50 μ M of fluridone (FLU) (*T. gondii* + FLU). Mean $\pm$ s.e.m., N = 3 experiments, Student's t test, $P < 0.005$ vs. control (*). (D) Time course of ABA production by *T. gondii*. Values are intracellular concentration in the parasite. Time 0 refers to newly invaded parasites at the start of infection. Mean $\pm$ s.e.m., N = 3 experiments

Fig. 3.

Fluridone blocked parasite growth by preventing egress. (A) FLU prevented parasite growth as monitored by β -galactosidase activity in the parasite. (B) Continuous culture in FLU did not affect replication but inhibited egress from the vacuole. (C) Treatment with exogenous ABA induced egress of parasites from mature vacuoles. Hours refers to length of culture prior to addition of ABA for 1 hr and scoring of egress. Student's *t* test: *, $p < 0.02$; **, $p < 0.05$. (D) ABA reversed the inhibition of parasite egress caused by FLU. Parasite infected cells were treated with FLU for 38 hrs, then stimulated with ABA for 1 hr, and evaluated for egress. Student's *t* test: *, $p < 0.002$; **, $p < 0.05$; ***, $p < 0.02$. A–D, Data shown are means $\pm$ s.e.m., $N = 3$ experiments.

Fig. 4. Fluridone treatment induced development of tissue cysts and protected mice against toxoplasmosis. (A) FLU treatment enhanced cyst formation, as revealed by *Dolichos biflorus* lectin (DBL). Student's *t* test: *, $p < 0.02$; **, $p < 0.005$. Means $\pm$ s.d., N = 3 experiments. (B) FLU-induced cysts stained positively with DBL and expressed cyst-specific antigens, CST-1 and BAG1, similar to control samples. (Scale bar = 5 μ m) (C) Cysts induced by FLU contained amylopectin granules (A) and posterior nucleus (N) and prominent cyst wall (W) by EM. (Scale bar 200 nm). (D) Survival following acute challenge with *T. gondii* and treatment with FLU. Two experiments combined, N = 10 animals per group. (E) Luciferase imaging revealed decreased burdens in mice treated with FLU ($\dagger$, 4 of 5 control animals died). Mean $\pm$

s.d. from a representative experiment, N= 5 animals each. (F) Reduced cyst counts were observed in the brain of mice treated with 2 mg/ kg FLU. Two experiments combined, N = 8 animals / group, except for control where only a single animal survived